SEQUENCING OF *BACILLUS SUBTILIS* DNA FRAGMENTS

Alexis Harington

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DECLARATION

I declare that this dissertation is my own unaided work and that the technical assistance which I have received is detailed in the Acknowledgements.

No part of this dissertation has been submitted in the past, or is being submitted, or is to be submitted, for a degree at any other university.

A Harington

ABSTRACT

The cloning vector pPL6C3b.1 carries a promoterless chloramphenicol acetyltransferase gene and is designed to facilitate the cloning of sequences that allow transcription of this gene. This vector has been used in the construction of recombinant plasmids bearing genes for α-amylase and protease. It was found that in all of these recombinant plasmids, a fragment (the P2 fragment) had been co-cloned with the α-amylase and protease gene. The P2 fragments of the independently isolated recombinant plasmids pVC102, pSR11 and 12 appear to be similar in size, and in the possession of a unique SstI site. In contrast the P2 fragment of pSR51 (which carries a protease gene) seems to be different. At present it is unclear why the same P2 fragments are independently isolated or why the P2 fragments recovered with the α-amylase genes differ from the one associated with the protease gene in pSR51. The aim of this project was to characterise the P2 fragments of pSR11, 12 and 51 by DNA sequencing in order to elucidate in more detail the nature of any differences or similarities between themselves and with the P2 fragment of pVC102 which has already been sequenced. To this end, the relevant P2 fragments were isolated by restriction digestion and preparative electrophoresis. After extraction and appropriate digestion of the bacteriophage M13 vectors, the fragments were ligated to them. After the cloned fragments had been identified as being correct, the orientation was established where necessary. Subsequently chain termination sequencing of the cloned fragments was done. Partial sequences in one orientation (pSR11 and 12) or both orientations (pSR51) were determined. These results confirmed the identity of the P2 fragments from pVC102, pSR11 and 12, as well as the distinctness of the P2 fragment of pSR51. This work was complicated by the fact that the pSR11 and 12 appeared to be structurally unstable.

The second part of the project was to investigate certain aspects of structural instability in pVC102. First, attempts were made to use direct plasmid DNA sequencing to determine the endpoints of previously

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The second part of the project was to investigate certain aspects of structural instability in pVC102. First, attempts were made to use direct plasmid DNA sequencing to determine the endpoints of previously

isolated deletion mutants of pVC102. Although this sequencing was unsuccessful, associated dot blotting experiments with end-labelled *primers* allowed the endpoints of the deletions to be confirmed or determined more precisely. Second, additional deletion mutants were isolated from prolonged cultures and plasmid screens showed that these deletants fell into three size classes: larger, smaller, or equal to the smallest deletion type previously isolated from fermentations. Preliminary restriction analysis, which was partly successful, was done on three of these deletants. In general the deletions studied here seem to be similar to those previously characterised.

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The logistical assistance of the technical staff of the Laboratory for Molecular and Cellular Biology is gratefully acknowledged.

LIST OF ABBREVIATIONS USED IN THE TEXT AND FIGURES

amy$^{+/-}$	amylase phenotype
APS	ammonium persulphate
ATP	adenosine triphosphate
$Cm^{R/S}$	chloramphenicol resistance/sensitivity
CsCl	caesium chloride
Ct_1	C terminus 1 primer
dH_2O	double distilled sterile water
dNTP	deoxynucleotide triphosphate
ddNTP	dideoxynucleotide triphosphate
DTT	dithiothreitol
EDTA	ethylene diamine tetra acetate
5xH	5xhigh salt buffer
IPTG	isopropyl-β-D-thio-galactopyranoside
LB	Luria Broth
LMCB	*Laboratory for Molecular and Cellular* Biology
LMCB 1	pVC102 deletant, ±2.3kb loss, Nm^RCm^R *amy*$^-$
LMCB 2	pVC102 deletant, ±2.7kb loss, Nm^RCm^R *amy*$^-$
LMCB 3	pVC102 deletant, ±4kb loss, Nm^RCm^S *amy*$^-$
10xM	10xmedium salt buffer
Nt_1R	N terminus 1 reverse primer
$Nm^{R/S}$	neomycin resistance/sensitivity
PEG	polyethylene glycol
RE	Restriction Enzyme
RF	Replicative form of M13
ssDNA	single stranded DNA
SDS	sodium dodecyl sulphate
TBE	Tris-borate-EDTA buffer
TE	Tris-EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine

List of Abbreviations (continued)

TES	Tris-EDTA-salt buffer
Tris	Tris hydroxymethyl aminomethane
X-gal	5-*bromo*-4-*chloro*-3-*indolyl*-β-D-*galactopyranoside*
11A	P2 fragment isolated in this work from pSR11
12A	P2 fragment isolated in this work from pSR12
112	P2 fragment isolated by LMCB from pSR11
122	P2 fragment isolated by LMCB from pSR12
mp10	M13mp10
mp11	M13mp11

RESTRICTION ENZYME ABBREVIATIONS USED IN FIGURES

B	*Bgl*II
E	*Eco*RI
H	*Hind*III
P	*Pst*I
U	Uncut

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GENERAL INTRODUCTION

In order to use genetically-engineered micro-organisms for industrial use, it is necessary to have a detailed knowledge on their molecular genetics. Most work involving recombinant DNA research has been done on Escherichia coli. At the present time it has become necessary to apply these principles and techniques to organisms that are better suited to applied use. The Bacilli display many features that could be used to advantage in industry. These organisms secrete proteins into the medium, function well in fermentations and are historically used in the fermentation industry (Doi 1984, Ostroff and Pène 1984). Amongst them, Bacillus subtilis has been better characterised genetically than other members (Doi 1984) and information is available on the genetic map, transformation systems, cloning vehicles and bacteriophages (Ostroff and Pène 1984). If this organism is to be modified in order to house and allow expression of artificially manipulated plasmids, four factors require attention (see Doi 1984):

- *the transcription, translation and regulation of cloned* genes;
- the utilization of stable recombinant plasmids;
- the study of protein secretion;
- the reduction of protease and nuclease contents of host bacteria.

A fifth factor that will undoubtedly have a significant role to play is the relationship between the bacterium and its environment. Seo and Bailey (1985), for example, have demonstrated a two-fold higher specific activity of cloned gene product, obtained by environmental manipulation as opposed to genetic manipulation. It is unlikely that any of these factors could be manipulated extensively in isolation of one another; it could be argued that a negative feature, that of plasmid instability, acts as "common ground" between different aspects of gene cloning and use.

Instability

The question of instability is important in the context of new developments in fermentation strategy. At the present time efforts are under way to utilize the process of continuous culture. In theory, this method presents many advantages over traditional methods such as batch culture. Continuous cultivation makes it possible to separate and define various parameters independently of each other, such as nutrient supply, effluent removal, growth rate and cell density. Despite the positive features associated with this form of fermentation, it is not often used. The major problem in this regard (and most difficult to correct) is that of strain degeneration (Stafford 1986). This degeneration is manifested as a gradual and irreversible loss of product expression. By means of a growth advantage, hosts with smaller or no plasmids, or with plasmids expressing less of the cloned gene product, soon become the dominant bacterial species.

In broad terms, the most important factors influencing the stability of a (recombinant) plasmid are the following:

- The replication functions of the plasmid (Doi 1984).
- Partition and segregation functions (Meacock and Cohen 1980).
- The size of the cloned insert (Warnes and Stephenson 1986).
- The structural qualities of the plasmid (including the insert), the presence of repeated sequences, sequence similarities and palindromes (Albertini et al 1982, Hagan and Warren 1983, Alonso and Trautner 1985a, Hahn and Dubnau 1985).
- The degree of expression (promoter activity) of the cloned gene (Gentz et al 1981, Stassi and Lacks 1982).
- The host background. This includes the rec phenotype and the influence of other genes such as, for example, the pleiotropic sacU9 mutation in B. subtilis (Uhlén et al 1981, Hahn and Dubnau 1985, Vehmaanperä and Korhola 1986).
- The metabolic environment within which the host proliferates. This includes the role of selective (antibiotic) pressure on the plasmid and the host.

Given the complexity of plasmid instability, an investigation of the phenomenon would have to be done from various specific aspects.

The Host-Plasmid Relationship

Since plasmids are dependent on the host for proliferation, there is a definite link between the phenotype of the plasmid and its success as replicon. The metabolic influence from the exterior (the medium) dictates to an extent the response of the host to the plasmids. The presence of large inserts in recombinant plasmids results in an instability which is caused by the metabolic load the large plasmid places on the cell and which favours cells with less "expensive" or no plasmids. This point has been empirically demonstrated by Godwin and Slater (1979). Accordingly, large inserts often result in segregational instability (Kim and Ryu 1984, Bron and Luxen 1985, Warnes and Stephenson 1986). In some cases, however, exceptions to this rule have been noticed. Hakkaart et al (1985), for example, reported the loss of the smaller plasmid from a population. Such exceptions can often be accounted for in terms of the net load the plasmid places on the host. This load is composed of various subsections including at least the size of the plasmid and the level of expression of the cloned gene. Certain plasmid functions are associated with their preservation, and serve as linkages between the plasmid and host fitness. These functions involve diverse genes, such as segregation (seg) (Alonso and Trautner 1985b), partition functions (par) (Meacock and Cohen 1980, Gruss and Novick 1986), copy number controlling genes (see Seo and Bailey 1985), pleiotropic genes such as sacU9 (Vehmaanperä and Korhola 1986), functions affecting multimerisation and concatemerisation (see Bron and Luxen 1985), membrane binding regions (McKenzie et al 1986) and the replication origin of the plasmid. These interactions appear to be multiple, highly developed (selected) functions. This fact, in conjunction with environmental selective pressure, makes the form of instability represented here (segregational instability) very difficult to prevent or to eliminate.

The structure of the plasmid and the influence of certain host genes are connected with structural instability. The maintenance of a plasmid, including any cloned DNA, is limited by the requirement that certain forms of DNA structure are incompatible with survival. The presence of repeated, similar or inverted sequences, palindromes or "deletion generators" often result in the elimination of certain stretches of DNA or complete loss of the plasmid (Albertini et al 1982, Hagan and Warren 1983, Alonso and Trautner 1985a, Hahn and Dubnau 1985). Such rearrangements can be independent of the general recombination systems of the host, but it does appear that rec and related enzymes in the general sense often play a causal but incidental role. This includes other enzymes such as polymerases or gyrases which are also thought to be involved with the generation of deletions (Albertini et al 1982, Hahn and Dubnau 1985). Due to the primary influence of the sequence on this type of instability, deletion prone areas or "hot spots" are created (see Primrose and Ehrlich 1981). Thus deletions often fall into various defined classes, although this phenomenon is not always observed (Lehmkänpärä and Korhola 1986). The factors causing structural instability are unlikely to have evolved for this purpose and structural instability can be viewed as an escape "mechanism", based on chance, by which unfavourable host-plasmid interactions are modified. This modification is enforced by selection affecting the host-plasmid relationship and is mediated by segregation. The latter phenomenon explains how a population becomes homogenous for a certain structural variant. Although it is possible that the final results of structural instability may vary, it is likely that most variants produced during instability are part of a series leading towards a specific endpoint. The immediate endpoint in a trend of deletion events is determined by the ease with which the physical process of elimination can take place and the metabolic load the plasmid represents. Without selection, the endpoint is almost always elimination of the plasmid.

SECTION

AN INVESTIGATION INTO THE NATURE OF THE P2 FRAGMENTS OF PLASMIDS pSR11, 12 AND 51

1. Introduction

The vector pPL603 (3.1 megadaltons) (see Williams et al 1981) is a recombinant plasmid, constructed so as to facilitate the cloning of DNA fragments which will act as promoters. The plasmid was derived from an earlier construct, pUB110 (which confers neomycin resistance) by the insertion of an EcoRI fragment of Bacillus pumilis DNA which bears a functional chloramphenicol acetyltransferase (CAT) gene. This functional CAT gene was subsequently deprived of its promoter (or whatever sequences were acting to allow expression of the gene). The CAT gene was also placed in reverse orientation with respect to sequences with potential promoter activity located in pUB110. A derivative plasmid of pPL603, pPL603b, was created by inserting an M13 linker containing a BamHI site into the EcoRI site of pPL603 (see Corfield et al 1984). Since pPL603b showed some CAT gene activity in stationary phase, a variant pPL603b.1, chloramphenicol sensitive at all growth stages, was isolated (Corfield et al 1984).

This vector is specifically designed for the detection and cloning of sequences that can allow transcription of the CAT gene. In an analysis of fragments cloned into pPL603, Williams et al (1981) found that some sequences permitted expression in only one and others in both orientations. By virtue of the construction of pPL603, it is not possible to clone in isolation a gene consisting of promoter, structural and terminator regions and have simultaneous CAT gene expression. If the terminator is absent, the gene fragment could be cloned, providing that read through into the CAT gene takes place. The remaining possibility is that the CAT gene be activated by a promoter (or a

sequence with similar activity) unrelated and unlinked to the promoter activating the cloned gene.

The cloning of certain exoenzyme genes with pPL603b.1

This plasmid was used to clone BglII digested host DNA and the resulting recombinant plasmids were screened for α-amylase (or protease) activity. When such genes were cloned in the vector, it was found that a similar fragment with promoter activity had been repeatedly cloned. This fragment, distinct from the promoter of the cloned α-amylase or protease gene is known as the P2 fragment. It is about 0.7 kb in size and is present in the separately isolated plasmids pVC102, 210, 220, 230, 310, 320 and pSR11, 12 and 51 (Corfield 1986a) (see Figure 1). Similarity between all these P2 fragments (except the P2 of pSR51) was emphasized by the fact that all could be characterised by a unique internal SacI site. In pVC102, this enzyme cleaves the fragment in two pieces of roughly 560 and 180 bp. The identity of P2 fragments in α-amylase gene bearing recombinant plasmids was confirmed by hybridization studies. The pVC102 P2 fragment was cloned into the RNA probe vector pSR65 and transcripts were used to probe pVC102, 210, 220, 230, 310, 320, pSR11, 12 and 51. Only pSR51 did not have sequences complementary to the probe (Youngleson 1986).

These observations raised the question as to why the P2 fragments were always the same (with respect to the criteria mentioned above), why the P2 fragment from pSR51 was different, and why the cloned gene was in opposite orientation to the CAT gene.

Two explanations for the P2 fragment cloning phenomenon can be forwarded (Corfield 1986a). Either the P1 and P2 fragments are linked on the chromosome of the host or they are not. In the former case a partial digest of the host DNA at the first cloning step would explain the presence of a BglII site between the two promoters. In the latter, the same P2 fragment would have to be repeatedly and independently cloned. Blotting and hybridization studies have strongly suggested that the two fragments are unlinked on the chromosome (Corfield 1986a).

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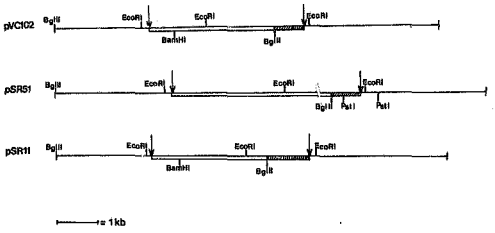


Figure 1

Linearized restriction maps showing P2 fragments of pVC102, pSR51 and pSR11. P2 fragments are indicated by cross hatching. The P1 regions (that is, the rest of the cloned inserts) are indicated by a *double line*. *Arrows show the location of* BamHI - BglII junctions.

These experiments are based on the patterns resulting from the presence or absence of at least one PstI site in the DNA presumed to separate the P fragments in vivo. Because of a deletion event shortly after cloning (see Corfield 1985, 1986a) which may have altered the intervening DNA, it is not rigorously proven that the P2 fragments are separate in the host. Therefore explanations have to be found to account for the repeated co-cloning of the α-amylase genes and the P2 fragments. The phenomenon is complicated by the fact that the P2 fragment of pSR51 is different from the others.

The project attempted here was to determine the nucleotide sequence of the P2 fragments of pSR11, 12 and 51. This would allow comparison with the corresponding fragment in pVC102 which has already been sequenced (Herbst pers. comm.), and reveal any differences between the P2 fragments of pSR11 and 12 and 51. In order to achieve this goal the following strategy was adopted:

- Preparation of CsCl extracted plasmid DNA
- Identification and isolation of P2 fragments by agarose electrophoresis
- Preparation of M13 vectors
- Appropriate digestion of M13 vectors
- Ligation of vectors and fragments
- Transfection of recombinant vectors
- Screening of vectors and identification of cloned inserts
- Preparation of single stranded DNA
- Sequencing

2. Materials, Methods and Results

To avoid unnecessary repetition, in each experiment the techniques used are given, followed by the results obtained in each case. The bacterial strains used in this work are listed in Appendix 1.

2.1 Preparation of plasmid DNA

The preparation of large amounts of pure plasmid DNA was essential to this project. For this to be done, the following procedure was adopted:

(1) The strain was streaked to single colonies on selective medium.

(2) 10ml of culture in selective medium was grown overnight (LB and antibiotics).

(3) 250ml of LB and antibiotics were inoculated with 1ml of overnight culture and allowed to grow for 18-20 hours.

(4) The DNA was then extracted by the following modified protocol of Lovett and Keggins (1978):

   (a) 250ml of culture were centrifuged at 7000 rpm in a JA10 rotor in a Beckman J2-21 centrifuge for 10 minutes.

   (b) The cell pellet was resuspended in 50ml of TES (pH 7.5) and again centrifuged.

   (c) The pellet was resuspended in 4ml of lysozyme buffer and transferred to a 50ml centrifuge tube.

   (d) 0.2ml of 10mg/ml lysozyme in TES was added (final enzyme concentration 500µg/ml) and incubated at 37°C for 30 minutes.

   (e) 16ml of SDS buffer were added and the solution mixed with a rod until clear.

   (f) 5ml of 5M NaCl were added, mixed gently and the mixture stored overnight at 0°C.

   (g) The mixture was centrifuged at 18000 rpm in a J20 rotor for 30 minutes and the supernatant decanted.

   (h) 5ml of 50% PEG 6000 were added, well mixed in, and the resulting mixture stored on ice for several hours or overnight.

   (i) The mixture was centrifuged at 10000 rpm for 10 minutes and the supernatant discarded.

(j) The pellet was redissolved in 3ml of TES, containing 0.2ml of 2mg/ml heat-treated RNase. The solution was then incubated at 65°C for 30 minutes by which time the pellet was completely resuspended.

(k) 4.25g of CsCl was added, dissolved at 37°C and the mixture cleared by centrifugation at 15000 rpm in a J10 rotor. The supernatant was carefully aspirated off and the refractivity adjusted to 1.395.

(l) 0.1ml of ethidium bromide (10mg/ml) was added. If the volume was not sufficient to fill an ultracentrifuge tube, it was brought to volume with TE-CsCl-ethidium bromide of the right refractivity.

(m) After sealing, the tubes were spun at 45-50000 rpm in a *Vti 65 rotor in a Beckman* LB-55 *ultracentrifuge* for 14 hours.

(n) Following the centrifugation the plasmid band was aspirated off by side puncture with a syringe.

(o) Ethidium bromide was extracted with isopropanol saturated with 20xSCC until no red colour could be detected, and then extracted once more.

(p) The volume was dialysed in 3 changes of TE buffer (about 300ml TE per sample).

(q) It was often necessary to phenol extract the DNA (that is, two phenol-chloroform extractions, one chloroform *extraction and two ether extractions*). Subsequently the DNA was sometimes concentrated by ethanol precipitation done as follows: 1/10 the volume of sodium acetate was added, 2 volumes of 96% ethanol added, mixed, stored at -70°C for 20 minutes then centrifuged in a microfuge for 30 minutes, the supernatant poured off, 1ml 70% ethanol added, centrifuged for 5 minutes, and the pellet dried under vacuum and finally resuspended in TE.

(r) The samples were then analysed by agarose gel electrophoresis (see Appendix 3 for details). Figure 2 depicts the result of such a CsCl extraction.

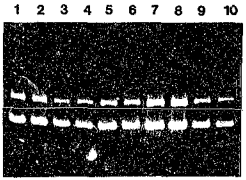


Figure 2

Caesium chloride preparations of plasmid DNA (1.2% agarose).

Lane 1	pSR11A	Lane 6	pSR12A
2	"	7	"
3	"	8	"
4	"	9	pSR61
5	"	10	"

2.2 Identification of P2 fragments and their isolation by gel electrophoresis

According to the restriction maps (Figure 1) the P2 fragments (pSR11 and 12) can be excised by BglII - EcoRI digestion. Excision of the P2 fragment of pSR51 presents problems because it has internal EcoRI sites. This prevents the EcoRI site, close to the uncleavable BamHI - BglII junction, being used to remove the fragment as is done in the cases of pVC102, pSR11 and 12. With this in mind, a *PstI* - *PstI* fragment was cleaved out. This fragment consists of about 800 bp. In this work this fragment is called the P2* fragment of pSR51.

Restriction digests were done in order to confirm that the fragments were released as expected. The P2* fragment of pSR51 appeared as expected (see Figure 3). pSR11A and pSR12A, however, did not give rise to the same fragment pattern as the corresponding LMCB DNA preparations. As can be seen in Figure 4, EcoRI - BglII digestion of pSR12A and 12L give similar patterns. pSR12A has however an additional small fragment (of about 250 bp). The P2 fragments, approximately 900 bp in size, comigrated. The patterns of pSR11A and 11L were not equivalent and seemed to differ more from each other than the above case. In pSR11L, the EcoRI - BglII fragments can be divided into two groups. The larger size group consists of 3 fragments ranging between ±4000 and 1900 bp. pSR11A also yields 3 fragments but their size range is between ±4000 and 3500 bp. The second size group of pSR11L included two fragments, their size between ±850 and 900 bp. The upper of these is the P2 fragment and comigrates with a single fragment (assumed to be P2) from pSR11A which lacks altogether the smaller fragment in this size range. In addition, it also has a small fragment as described for pSR12A. It was subsequently determined (at least in the case of pSR11A) that this small fragment is released on EcoRI digestion. This implies that both of the plasmids have generated an additional EcoRI site, perhaps by duplication. Both 1A297 (pSR11A) and 1A297 (pSR12A) have all three markers associated with the plasmids (that is Nm^R, Cm^R and α-amylase production).

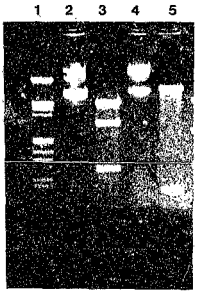


Figure 3

RE digested DNA showing the release of P2* fragments (arrowed) from pVC102 and pSR51.

Lane 1 Lambda III marker
2 pVC102, *uncut*
3 pVC102, BglII - PstI
4 pSR51, uncut
5 pSR51, PstI

The size of the pVC102 P2* fragment is about 1300 bp, the P2* fragment from pSR51 800 bp. About 0.4µg DNA per lane.

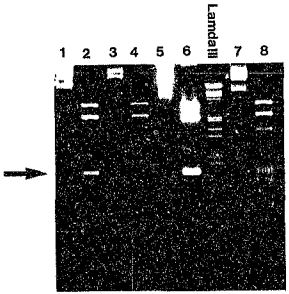


Figure 4

Release of P2 fragments (arrowed) from pSR12A, 12L, 11A and 11L by RE digestion with BglII and EcoRI.

Lane 1 pSR12A, uncut
2 pSR12A, cut
3 pSR12L, uncut
4 pSR12L, cut
5 pSR11A, uncut
6 pSR11A, cut
7 pSR11L, uncut
8 pSR11L, cut

The size of the P2 fragments is roughly 900 bp, that of the small fragment present in pSR11A, 12A about 250 bp.

Preparative gels

For pSR5l, 5 digests each in a total of 50µl were set up. The amount of DNA per tube was about 4µg, thus a total of 20µg DNA was cut. Each tube received the appropriate amount of 10xM and PstI enzyme. The digests were incubated for 2 hours at 37°C. In the case of pSR11A and pSR12A, for the BglII digestion, 5 digests were set up in a volume of 40µl. Each tube contained about 1µg (pSR12A) or 3µg (pSR11A) of DNA. After digestion for 90 minutes the NaCl and Tris concentrations in the tubes were raised from 50 to 100 mM (NaCl) or 10 to 50 mM (Tris), prior to the addition of the high-salt enzyme (EcoRI). After a further 90 minutes, a small aliquot (1µl) was removed and analysed for completeness of digestion. If successful, the full volume was loaded onto a 0.8% agarose gel and electrophoresed for 50-70 minutes at 50V (5V/cm). Subsequently, the relevant bands (see Figure 5) were excised with a scalpel, trimmed close to the DNA and electroeluted as soon as possible.

Electroelution

An electroluter of the IBI type was used to recover the fragments contained in the preparative agarose. The apparatus was filled with TBE buffer and the channels cleared of all bubbles and filled with 100µl of high salt buffer (7.5M ammonium acetate with 0.1% bromophenol blue). The excised pieces were moved as close as possible to the channels and elution started at 100V. It was found that after 20 minutes the fragments were completely eluted. At this stage, two samples of 400µl of fluid from each channel were removed, the bromophenol blue extracted with butanol and the DNA precipitated with ethanol.

2.3 Preparation of M13 vectors

2.3.1 Background

M13 is a filamentous coliphage (see Messing 1983), and has a life cycle that consists of two stages. The infective form of the virus carries

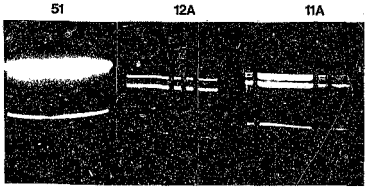


Figure 5

Preparative gels of pSR51, pSR12A and pSR11A. About 20, 5 and 15µg of DNA respectively per gel (0.8% agarose).

a single non-sense (+) strand of DNA. This particle infects E. coli cells by means of their F-pili and these structures are obligatory for infection. This form gives rise, intracellularly, to a sense or - strand. This double stranded replicative form (RF) of M13 represents the active form of the virus and up to 300 copies can be present in a cell (Davies 1982). At a certain stage of growth, the RF forms switch to producing single stranded infectious particles that are released from the cell without cell lysis.

This dual form life cycle makes M13 a useful vector for cloning and for the production of single stranded DNA for sequencing. The wildtype phage has been modified in two main ways to facilitate cloning (see Sanger et al 1980). First, part of the lac operon has been inserted into the phage in the intergenic region between genes IV and II (Messing et al 1977). The segment consists of the operator, promoter and approximately 15% of the amino terminal end of the structural gene coding for β-galactosidase (known as the α segment). This peptide can take part in complementation with the ω peptide (part of the C terminus of the identical subunits of β-galactosidase). If the reading frame coding for the α peptide is disrupted sufficiently, α peptide complementation is abolished.

Second, it was found that the insertion of a multiple cloning site into the coding region itself does not disrupt complementation. This polycloning site has been modified in different M13 vectors so that the restriction enzyme sites are present in reverse (mirror image) orientation. This property permits the vector to accommodate the cloned DNA in reversed polarity, which eventually allows complementary strands of the insert to be sequenced.

The multiple cloning sites of M13mp10 and 11 appear as in Figure 6. For the cloning of the pSR51 P2* fragment a single digestion with PstI is sufficient whereas vectors for the BglII - EcoRI-released fragment require double digestion. It should be noted that following ligation, excision of the fragment would require the use of another enzyme (SalI in this work) because the BamHI - BglII junction is not recognised by either enzyme (Maniatis et al 1982).

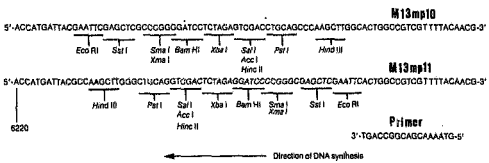


Figure 6

The multiple cloning sites of the single stranded phage vectors M13mp10 and M13mp11. The polycloning site is located within the structural portion of the inserted β-galactosidase gene fragment found in the intergenic region of the phage located between genes IV and II. The universal primer is shown (from Amersham).

Prior to concluding this introductory section it is of interest to note that the host for M13mp10 and 11, JM109, has been extensively modified to facilitate cloning (see Appendix 1). A few of the important changes are the following (see Yanisch-Perron et al 1985):

rec⁻, gyr	- to increase stability of the insert.
Δlac	- to prevent interaction between similar (lac) sequences on the F' plasmid.
Δpro	- to make the host dependent on the F' plasmid which has a functional pro gene. This ensures the presence of F-pili for infection.

2.3.2 Preparation of replicative forms of M13mp10 and mp11

In order to extract RF DNA, phage supernatant was taken and about 10μl added to 3 tubes containing 2ml each of a 1:100 dilution of JM109 in 2xTY medium. This pilot culture was grown for 3-5 hours after which it was added to 500ml of 2xTY medium. After 20 hours of shaking at 37°C, the cells were extracted. The protocol used was the large-scale alkaline-lysis method described by Maniatis et al (1982). The following modifications were introduced:

(1) The cells were pelleted at 7000 rpm for 10 minutes, then resuspended in 5ml of solution 1 and subsequently mixed with 5ml of solution 1 containing 10mg/ml of lysozyme. This ensured even and complete lysis.

(2) The alkaline lysis, and potassium acetate and isopropanol precipitation steps were done as described in the Maniatis protocol.

(3) The pellets were dried in a vacuum oven at room temperature, and resuspended in 4ml in TE.

(4) 3.8-3.9g of CsCl and 0.4ml of ethidium bromide (10mg/ml) were added and the total mixture sealed in an ultracentrifuge tube and spun at 45-50000 rpm in a Vti 65 rotor in a Beckman L8-55 Ultracentrifuge for 12 hours. The viral band was

removed and added to fresh TE-CsCl-ethidium bromide mixture and recentrifuged.

(5) After the second gradient purification the DNA-CsCl was extracted as described on page 9, step (o), for CsCl plasmid extraction.

(6) Instead of dialysing the DNA it was concentrated by precipitation as follows (Mizrahi pers. comm.): After extraction of ethidium bromide, 2 volumes of TE were added to the CsCl DNA solution, mixed with 2.5 volumes of absolute ethanol and precipitated as described earlier (page 9, step (q)). After resuspension in an appropriate volume, the DNA was phenol extracted as detailed on page 9, step (q).
Using the alkaline method, about 250µl of M13mp11 at a concentration of 235mg/ml were produced. Similarly, 250µl of M13mp10 at 710mg/ml were purified.

RF DNA can also be successfully produced using the PEG method described on page 8 (Herbst pers. comm.).

2.3.3 Digestion of M13mp11 with PstI

The following digestion was used to produce PstI cleaved M13mp11:

DNA	10µl	(2.35µg)
10xM	2.5µl	
dH_2O	10µl	
PstI	2.5µl	(9U/µl)
	25µl	

After the volume of the digest was raised to 50µl with TE the digest was extracted once with an equal volume of phenol-chloroform, once with chloroform and twice with ether; finally 1/10 of the volume of 3M sodium acetate (pH 5.5) was added and precipitation done as described earlier

(page 9). The DNA was resuspended in 25µl of TE. As seen in Figure 7, the digest was successful.

2.3.4 Digestion of M13mp11 with EcoRI and BamHI

To produce single cut mp11 vector the following digestion was used:

BamHI			EcoRI		
DNA	11µl	(2.6µg)	DNA	11µl	(2.6µg)
10xM	2.5µl		5xH	5µl	
dH_2O	9µl		dH_2O	6.5µl	
BamHI	2.5µl	(12U/µl)	EcoRI	2.5µl	(5U/µl)
	25µl			25µl	

The DNA was extracted and ethanol precipitated as described in Section 2.3.3. As is apparent from Figure 8, the first digestion was effective.

Of each such digestion about 5µl (0.52µg) were stored to be used in transfections. A comparison between this single digested DNA and that of the double digested vector gives an indication of the efficiency of the second digestion.

2.3.5 Digestion of M13mp10 with EcoRI and BamHI

The digestion performed here was as above except that 7.1µg of DNA was used per digestion. As seen in Figure 8, the digestion was successful.

2.3.6 Second digestion of M13mp11 with BamHI and EcoRI

Because the BamHI and EcoRI sites are close together in the polycloning site of M13 (separated by ±16 nucleotides), the second digestion can be a difficult procedure. Since no change in mobility can be detected on

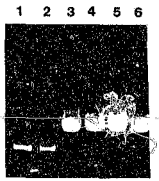


Figure 7

Cleavage of M13mp11 with PstI and BamHI within the polycloning site.

Lane 1 Vector, uncut
2 Vector control (digestion conditions minus enzyme)
3 PstI cut, immediately after addition of the enzyme
4 PstI cut, after 90 minutes
5 BamHI cut, immediately after addition of the enzyme
6 BamHI cut, after 90 minutes

The uncut vector is present at a concentration 1/10 of that of the cut vector (235ng per lane).

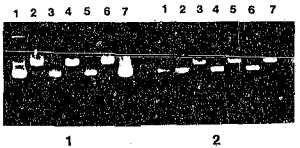


Figure 8

Cleavage of M13mp11 and M13mp10 with BamHI and EcoRI.

Gel 1, mp11

Lane 1 BamHI, uncut
2 BamHI, cut
3 EcoRI, uncut
4 EcoRI, cut
5 BamHI, uncut
6 BamHI, cut
7 mp10, uncut
(About 230ng DNA/lane)

Gel 2, mp10

Lane 1 mp10, uncut
2 BamHI, uncut
3 BamHI, cut
4 BamHI, uncut
5 BamHI, cut
6 EcoRI, uncut
7 EcoRI, cut
(About 100ng DNA/lane)

agarose gels, the efficiency of double digestion has to be assessed in terms of the ligation efficiency. In theory, double digested vector should yield close to zero transformants because of the incompatible nature of the ends, which are very rarely, if ever, joined by ligase.

The second digestion was done as follows:

BamHI cut DNA	20µl	(1.6µg)
5xH	5.3µl	
dH_2O	-	
EcoRI	1.5µl	(5U/µl)
	26.8µl	

A similar digest with 10xM was set up for BamHI digestion. Following 2 hours of digestion, the DNA was phenol extracted and ethanol precipitated as described earlier. It was then resuspended in 20µl of TE to give a final concentration of 80ng/µl. For further calculations, a DNA loss of 10-20% due to phenol extraction was assumed.

In order to test for digestion and double digestion in a ligation assay, about 20µg of vector was taken and ligated and transformed by methods to be described later in this work. Following ligation, the DNA was transformed into competent host cells and plated with a lawn of infectible cells. The plaques resulting from this transformation were scored. The results for this transformation with mp11 were as follows:

EcoRI - BamHI double digestion

First digestion (EcoRI)		
	+ ligase	- ligase
	> 100 plaques	1 plaque
Second digestion (EcoRI - BamHI)		
	+ ligase	- ligase
	100 plaques	0 plaques

In the first digestion, the control without ligase gives an indication of the amount of uncut vector. The ligation assay reveals that this double digestion was partially successful. Although very little uncut vector is present, a significant proportion of the DNA was not cut by the second enzyme, thus explaining the presence of about 100 plaques on ligation of the double digested vector.

When having problems with double digestion it is often useful to digest *with different enzymes in the first or second instance* (Mizrahi pers. comm.). In the following ligation assay a BamHI ~ EcoRI double digestion -- as opposed to the reverse case just described -- was attempted.

BamHI - EcoRI double digestion

First digestion	(BamHI)	
	+ ligase	- ligase
	> 100 plaques	> 100 plaques
Second digestion	(BamHI ~ EcoRI)	
	+ ligase	- ligase
	> 100 plaques	15 plaques

In this case, the first digestion, although successful as viewed on a gel, does not appear so in the ligation assay. In this case the results of the second digestion are obscured. The reduced control without ligase after the second digestion shows that most of the vector had been cut by one or the other enzyme.

At the same time as ligatio were transformed, 1ng of uncleaved vector DNA was used as a transformation control. These results are as follows:

mp10	80 plaques
mp11	160 plaques

These transformation controls are not particularly good since up to 300

plaques can result from 1ng of phage DNA under optimal conditions (Amersham).

2.3.7 Second digestion of M13mp10 with BamHI and EcoRI

The second M13mp10 digestion was as follows:

BamHI cut DNA	20µl	(5µg)
5xH	5.3µl	
dH_2O	-	
EcoRI	1.5µl	(5U/µl)
	26.8µl	

A similar digest was used for the BamHI cut. The digests were allowed to proceed for 2 hours at 37°C and resuspended in 20µl TE (248ng/µl) after phenol extraction and ethanol precipitation.

Ligation assay for double digestion

The results following transformation were as follows:

First digestion (EcoRI)

+ ligase	- ligase
> 100 plaques	7 plaques

Second digestion (EcoRI ~ BamHI)

+ ligase	- ligase
20 plaques	3 plaques

First digestion (BamHI)

+ ligase	- ligase
100 plaques	20 plaques

Second digestion (BamHI - EcoRI)		
	+ ligase	- ligase
	> 100 plaques	20 plaques
Transformation control (mp10)		37 plaques

The results indicate that double digestion was more successful than the previous case (BamHI - EcoRI digestion).

2.4 Ligation of prepared vectors and fragments

2.4.1 Background

Ligation comprises the formation of a phosphodiester bond between a 5' phosphate and a 3' OH group. This reaction is effected by ATP dependent catalysis and is influenced by several factors of which the most critical are: the concentration of compatible ends, the G+C content of the complementary ends, the temperature and the composition of the reaction mixture (Hackett et al 1984). During a ligation experiment, various controls are required to allow interpretation of the results. These controls entail the following (Amersham, Messing 1983):

- cut vector at 10ng/plate which gives an indication of the success of digestion.
- ligation control (cut and religated vector) at 10ng/plate. This allows the efficiency of ligation to be assessed.
- transformation control. Uncut vector at 1ng/plate is used and shows how well the DNA molecules penetrated into the competent cells.

As regards the enzyme itself, T4 DNA ligase is used in cloning experiments because it is a more permissive enzyme than E. coli DNA ligase (Higgins and Cozzarelli 1979). T4 DNA ligase functions best at 37°C but this temperature is too high to allow cohesive ends to

anneal efficiently. For this reason, ligation is done at 12-14°C which increases the possibility of annealing and ligation, provided sufficient time is given (Hackett et al 1984). After ligation, the naked circular molecules originating from ligation and those used for the transformation control must be transfected into competent host cells. Competence is a poorly understood process that is influenced by various factors such as the physical form of the DNA (closed circles or linear molecules), the growth phase of the bacterium and the method of treatment to produce the competent state (see Hayes 1968). When the naked phage DNA enters the competent cell it replicates and forms infective particles, which give rise to a plaque when plated with lawn cells.

In the case of the M13mp series of vectors, these plaques can be clear or blue in colour. In the former case two possibilities exist (Messing 1983):

- a fragment has been cloned into the polycloning site and has disrupted the α peptide so that no complementation takes place.
- self ligation of the vector has occurred after exonuclease or other changes destroyed the α peptide reading frame. This is a false positive.

Lastly, the unchanged vector can recircularise and the α peptide be produced. It then takes part in complementation and produces an active β-galactosidase. In the presence of the inducer IPTG and the indicator X-gal, a blue plaque is produced.

2.4.2 Ligation conditions

In this work, the ligation parameters mentioned above were assessed in terms of already developed protocols. A vector:insert concentration ratio of 1:5-10 was used. The following conditions were employed:

Amersham		Mizrahi (pers. comm.)	
Insert	5µl (100ng)	Insert	10µl (100-200ng)
Vector	2µl (20ng)	Vector	2µl (20ng)
10xligase buffer	1µl	10xligase buffer	4µl
DTT (50mM)	1µl	DTT (0.1M)	4µl
ATP (10mM)	1µl	ATP (20mM)	2µl
T4 DNA ligase	1µl (1U/µl)	T4 DNA ligase	1.5µl (1U/µl)
	11µl	dH_2O	16.5µl
			40.0µl

The reactions were incubated for 15-20 hours at 14°C.

2.4.3 Transformation and plating of ligations

Producing competent JM109 cells is an important step in M13 cloning and the following protocol, a modification of the Amersham procedure, was used:

(1) An overnight culture of JM109 (10ml) was grown in 2xTY from a loop of bacteria off a Minimal Medium plus glucose plate. Simultaneously, a 10ml aliquot of medium was co-incubated to detect any contamination since JM109 has no antibiotic markers.

(2) 40ml of 2xTY medium were inoculated with 400µl JM109 overnight (1:100). (The Amersham protocol involves a 1:20 dilution which does not allow the cells to grow enough when the OD_{550} of 0.5 is reached (Mizrahi pers. comm.).)

(3) The culture was grown to an OD_{550} of 0.5-0.6.

(4) The cells were then centrifuged at 4-5000 rpm in a J20 rotor in a Beckman J2-25 Centrifuge for 5-10 minutes.

(5) The pellet was resuspended in half the volume of ice cold 50mM $CaCl_2$, using a 10ml pipette, and left on ice for 60 minutes.

(6) After centrifugation as before, the cells formed a diffuse ring-like pellet. They were subsequently resuspended in 1/10 the volume of $CaCl_2$, using a wide bore (1ml) automatic pipette.

(7) At this stage the cells could be stored on ice or used immediately.

(8) The ligation or transformation mixture was made up to 100µl with water (Mizrahi pers. comm.). This step is critical otherwise low transformation efficiencies are obtained.

(9) 300µl of competent cells were added to the DNA, the suspension gently but thoroughly mixed, and left on ice for 60 minutes.

(10) After being heat-shocked at 42°C for 2 minutes, the cells were kept at room temperature and plated as soon as possible.

(11) For plating, a log phase culture of JM109 (lawn cells) was required and was inoculated about 1.5 hours prior to use.

(12) 3ml of molten (42°C) 1xTY top agar, 30µl of 2% X-gal (in diethylformamide) and 20µl of IPTG (100mM in dH_2O) were taken and mixed by vortexing.

(13) 200µl of lawn cells were added to the tube of heat-shocked transformed cells and mixed carefully. This mixture was added to the agar, vortexed briefly and poured onto a prewarmed (37°C) 1xTY agar plate. After the agar had set, the plates were incubated overnight at 37°C.

2.4.4 Cloning of the PstI - PstI fragment of pSR51 in M13mp11

A ligation in 11µl total volume (see page 29) was set up. On plating the following results were obtained:

ligation	>100 blue plaques
ligation control	± 70 blue plaques
transformation control	20 blue plaques

Several ligations were done giving this type of result. From one of them a single clear plaque was obtained.

Another 40µl ligation gave the following results:

ligation	>200 blue plaques 6 clear plaques
ligation control	100 blue plaques
ligation without ligase	55 blue plaques
transformation control	5 blue plaques

From these results the following can be concluded:

- ligation was efficient.
- the vector had not been completely digested by PstI.
- transformation was inefficient. (These experiments were done prior to some of the modifications described in the transformation protocol.)
- in view of the small number or clear plaques obtained, it would have been desirable to treat the vector with phosphatase.

After these ligations, 7 potential recombinant M13 vectors had been obtained. The clear plaques were removed with a Pasteur pipette and placed in 1.5ml of a 1:100 dilution of overnight JM109 in 2xTY medium and allowed to grow with agitation for 5 hours at 37°C. The cells were pelleted at the end of the growth period and the supernatant stored at 4°C. In the case of clear plaques picked near blue plaques it was necessary to plaque purify the potential insert bearing phages in order to remove any contaminating wildtypes. This was effected by diluting the supernatant 10^7, 10^8, 10^9, 10^{10} times (using 100µl volumes of 2xTY medium). This full volume at each dilution was plated as described. Clear plaques resulting from the purification were used as a source of infective particles to give pure supernatants.

2.4.5 Cloning of the P2 fragments of pSR11L, pSR12L, pSR11A and pSR12A and M13mp10 and 11

1. 11ul ligations were used to clone the P2 fragments of pSR11A and pSR12A. The following results were obtained on transformation (in all cases of predominantly clear plaques, roughly 5 times less blue plaques were present on the plate):

mp11 + 11A	>100 clear plaques
mp10 + 11A	±100 clear plaques
mp11 + 12A	>100 clear plaques
mp10 + 12A	>100 clear plaques
ligation control (mp11)	>200 blue plaques 2 clear plaques
transformation control (mp11)	7 blue plaques
transformation control (mp10)	9 blue plaques

From each of the first 4 ligations, 4 clear plaques were removed and used to inoculate small cultures to produce stock supernatant.

The ligation control used here was not rigorous because the ends of the vector, being digested with enzymes giving different cohesive ends, hardly ever ligate. The number of plaques is more accurately an indication of single cut (religated) or/and uncut vector. A rigorous ligation control would be a fully single cut vector. The phages present in two clear plaques found on the ligation control plate were almost certainly frameshift mutants.

2. 11ul ligations were set up in order to clone the P2 fragments of pSR11L and pSR12L. These fragments were obtained from the LMCB. On transformation the following results were obtained:

mp11 + 11L	>100 clear plaques
mp10 + 11L	10 clear plaques

mp11 + 12L	>100 clear plaques
mp10 + 12L	>100 clear plaques
ligation control (mp11)	>100 blue plaques 3 clear plaques
ligation control (mp10)	>100 blue plaques 4 clear plaques
transformation control (mp11)	20 blue plaques
transformation control (mp10)	233 blue plaques

The same comments made previously apply to these results. The mp10 transformation control was markedly improved.

2.4.6 Preparation of Replicative Form DNA in order to confirm and establish the nature of the cloned insert

For RF preparation, the Ish-Horowicz alkaline lysis procedure as described by Maniatis et al (1982) was used.

(1) 10µl of supernatant or a plaque were used to inoculate 1.5ml of 1:100 dilution of overnight JM109 in 2xTY medium. The bacteria and phages were allowed to grow for 5 hours.

(2) The cells were extracted as described by Maniatis et al (1982). The procedure consists of 3 steps, the first being the resuspension of the cells in prelysis solution. This was done with a Gilson P200 repeating pipette. Secondly, the cells are lysed with alkaline SDS. This is the critical step in the extraction upon which the success of the method depends. The alkaline SDS was added to the tube which was rotated through 90° 10-20 times, until the solution cleared. Thirdly, potassium acetate precipitation of chromosomal DNA and protein was carried out. At this stage the Eppendorf tube was flicked to ensure complete mixing of the contents, before these were briefly vortexed prior to centrifugation.

(3) The ethanol precipitated DNA was resuspended in 30µl of TE and stored frozen.

2.4.7 Screening of M13mp11 bearing the pSR51 P2* fragment

As can be seen from Figure 9, all clones derived from the clear plaques (except 2 and 3) show a reduced mobility. Clones 2 and 3 appear diffuse because of contamination with wildtype phage DNA as this screen was done prior to plaque purification. Of the 30µl available RF DNA, 8-12µl were used in the following type of digest:

RF DNA	10µl	pSR51 DNA	2µl
10xM	1.5µl	10xM	1µl
dH_2O	2.5µl	dH_2O	6.5µl
PstI	1µl (9U/µl)	PstI	0.5µl (9U/µl)
	15µl		10µl

From Figure 10, it is apparent that clones 1, 3, 4, 5, 8 (and subsequently 9) yielded fragments of the correct size. Clones 6 and 12 were negative controls (wildtype M13) Clone 2, derived from a clear plaque, yielded no fragment. In this case, either the digest failed or no insert is present (the latter suggested by the preliminary screen). The clone is probably a frameshift mutant.

Having identified the presence of an insert of the right size in the above-mentioned clones, the identity of the fragment had to be confirmed. Taking advantage of the fact that the pSR51 P2* fragment has two internal EcoRI sites, clones 1, 4, 5, 8 and 9 were selected for further analysis. The following type of digest was done to establish the identity of the inserts:

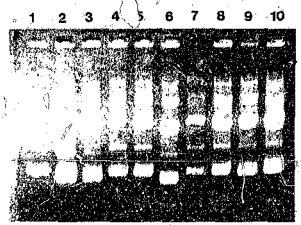


Figure 9

Preliminary screen of clear plaque-derived phages. (M13mp11 - pSR51 P2* ligation.) Alkaline lysis RF preparations of M13mp11.

Lane	Clone	
1	1	Six preparations of six clear plaques
2	2	
3	3	
4	4	
5	5	
6		Vector only, control
7	6	Four preparations derived from the same clear plaque
8	6	
9	6	
10	6	

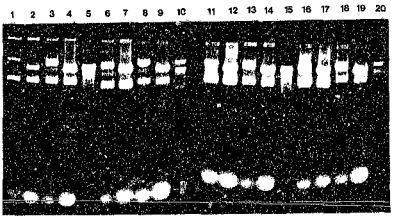


Figure 10

RE digestion of RF preparations on M13mp11 some of which were expected to carry the insert (pSR51 P2* fragment). Numbers represent specific clones. All samples were digested with PstI. Clones 6 and 12 are controls, that is, vector only.

Lane 1	4 uncut	Lane 11	2 uncut
2	4 cut	12	2 cut
3	5 uncut	13	3 uncut
4	5 cut	14	3 cut
5	pSR51 cut	15	pSR51 cut
6	6 uncut	16	12 uncut
7	6 cut	17	12 uncut
8	1 uncut	18	8 uncut
9	1 cut	19	8 cut
10	Lambda III marker	20	Lambda III marker

Clones 1, 3, 4, 5, 8 yielded fragments of the expected size. Clone 2 seems to have been a frameshift mutant because no fragment was released. The slight differences in mobility between the RF and plasmid derived fragments are assumed to be non-significant.

	RF preparations		Plasmid DNA	
	Single Digestion	Double Digestion	Single Digestion	Double Digestion
DNA	10µl	10µl	5µl	5µl
10xM	1.3µl	1.2µl	1µl	1µl
dH_2O	0.7µl	-	3.5µl	3µl
PstI	1µl	0.5µl	0.5µl	0.5µl
EcoRI	-	0.5µl	-	0.5µl
	13µl	12µl	10µl	10µl

In these digestions, the buffer salt concentration was not increr to cater for EcoRI. Advantage was taken of the fact that the enzy functions adequately in the medium salt buffer (Mizrahi pers. comm.). Figure 11 shows that clones 5, 1 and 8 give an identical pattern of single and double digestion fragments to that found in the case of the plasmid and electroeluted fragment. A similar result was obtained for clones 4 and 9.

2.4.8 Screening of M13 vectors suspected of bearing the pSR11L, 11A, 12L, and 12A P2 fragments

RF DNA was isolated as described and gave the results depicted in Figure 12. Of these clones, 4 of each vector-insert type were screened for the presence of an insert of the correct size. As is apparent in Figure 13, all the clones yielded an insert of a size similar to that associated with the plasmids. The following digestion conditions were used:

RF DNA	12µl		plasmid DNA	3µl	
5xH	2.9µl		10xM	1µl	
SalI	0.6µl	(18U/µl)	dH_2O	5.4µl	
EcoRI	0.6µl	(5U/µl)	BglII	0.6µl	(11U/µl)
	16µl			10µl	

Figure 11

RE digestion of recombinant RFs, pSR51 and the isolated P2 fragments in order to confirm the identity of the cloned inserts.*

Lane 1	Clone 5, PstI	Lane 6	pSR51, PstI - EcoRI
2	Clone 5, PstI - EcoRI	7	Eluted P2* fragment, PstI - EcoRI
3	Clone 1, PstI	8	Clone 8, PstI
4	Clone 1, PstI - EcoRI	9	Clone 8, PstI - EcoRI
5	pSR51, PstI	10	Lambda marker

The cloned inserts gave the required pattern and size distribution on single and double digestion.

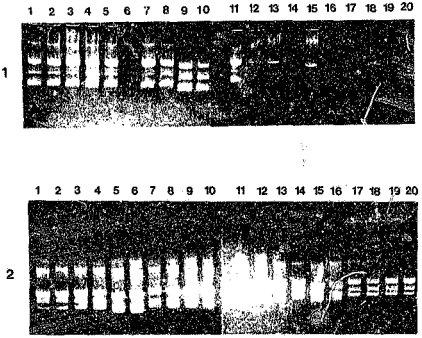


Figure 12

Preliminary screen of clear plaque derived phages. Alkaline lysis RF preparations of M13mp10 and mp11 presumed to carry inserts. All phage DNAs (except the controls) show reduced mobility.

Gel 1

Lane		Lane	
1	mp11+12A	11	mp11+11A
2		12	
3		13	
4		14	
5	mp10+12A	15	mp11 (control)
6		16	mp10 (control)
7		17	mp10+11A
8		18	
9	mp10 (control)	19	
10		20	

Gel 2

Lane		Lane	
1	mp11+11L	11	mp10+11L
2		12	
3		13	
4		14	
5	mp11 (control)	15	mp11 (control)
6	mp10 (control)	16	mp10 (control)
7	mp11+12L	17	mp11+12L
8		18	
9		19	
10		20	

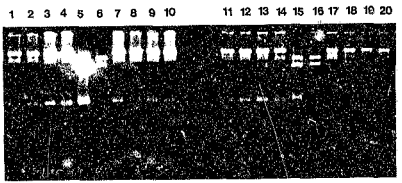


Figure 13

RE digestion of RFs of M13mp10 and 11 suspected of carrying P2 inserts. All samples were digested with EcoRI and BglII.

Lane 1	mp11+11A	Lane 11	mp11+12A
2		12	
3	mp10+11A	13	mp10+12A
4		14	
5	pSR11A	15	pSR12A
6	pSR12L	16	pSR11L
7	mp11+12L	17	mp11+11L
8		18	
9	mp10+12L	19	mp10+11L
10		20	

After 90 minutes of digestion, the plasmid DNA digests had their volumes increased to 20µl and the NaCl and Tris concentrations increased for the EcoRI digestions.

The P2 fragment of pSR11L and 12L has an internal SstI site which can serve as an identification site for the fragment. All the clones previously screened were subjected to the triple digestion (SstI - SalI - EcoRI). Plasmids were digested with SstI - BglII - EcoRI. The following conditions were used:

RF DNA	10µl	plasmid DNA	3µl
Sst core		SstI core	
buffer	1.2µl	buffer	1µl
SstI	1µl (5U/µl)	H_2O	5µl
	12µl	BglII	0.7µl (11U/µl)
		SstI	0.7µl (5U/µl)
			10µl

After 1.5 hours of SstI digestion, the volumes were increased to 20µl and the NaCl and Tris concentrations raised and the EcoRI digestion done. The results of these digestions are shown in Figure 14. All the fragments in the vectors and plasmids show corresponding mobilities except pSR11A. This fragment apparently lacks an SstI site. These results strongly indicate that the P2 fragments have been cloned, with the question of the pSR11A "P2 fragment" unresolved.

Complications arose when the parental plasmids were subsequently digested with the same enzymes, that is, (SstI, BglII, EcoRI). From Figure 15 one can deduce that the SstI digestion was partial. This partial digestion was always observed. The lower of the two fragments was confirmed to be SstI released by BglII - SstI digestion (results not shown). Secondly, the small fragment released by SstI cleavage of the P2 fragments is not visible in Figure 15 and must either comigrate with the small EcoRI fragment (see page 12) (pSR11A and 12A) or be absent (pSR11L and 12L). It should be noted that, in the digestions represented

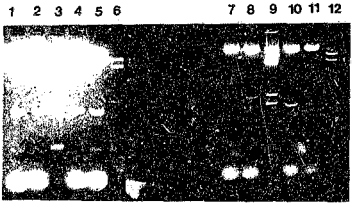


Figure 14

RE digestion of recombinant RFs and plasmids from which fragments were originally obtained. All RFs were digested with EcoRI, SalI and SstI; and all the plasmids with EcoRI, BglII and SstI.

Lane 1	mp11+12A	Lane 7	mp11+11A
2	mp10+12A	8	mp10+11A
3	pSR12A	9	pSR11A
4	mp11+12L	10	mp11+11L
5	mp10+12L	11	mp10+11L
6	pSR12L	12	pSR11L

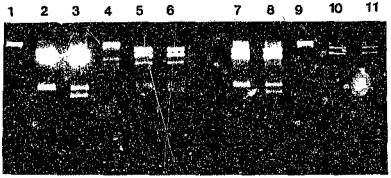


Figure 15

RE digestions of pSR11A, pSR11L, pSR12A and pSR12L.

Lane 1	Lambda III marker	Lane 7	pSR12A (BglII - EcoRI)
2	pSR11A (BglII - EcoRI)	8	pSR12A (BglII - EcoRI - SstI)
3	pSR11A (BglII - EcoRI - SstI)	9	Lambda III marker
4	Lambda III marker	10	pSR12L (BglII - EcoRI)
5	pSR11L (BglII - EcoRI)	11	pSR12L (BglII - EcoRI - SstI)
6	pSR11L (BglII - EcoRI - SstI)		

1. In the pSR11L and 12L triple digests (Lanes 6, 11) no small fragments similar in size to those produced on BglII - EcoRI - SstI of pSR11A and 12A are visible. Such fragments were produced when pSR11L and 12 were similarly digested previously (Figure 14).
2. The small fragment assumed to result from SstI cleavage of the BglII - EcoRI released P2 fragment is either not visible, or is assumed to comigrate with the small EcoRI released fragment (in the cases of pSR11A, 12A).
3. pSR11L showed no change on SstI digestion (Lanes 5, 6).
4. All the digests where an SstI induced change is visible (Lanes 3, 8 and 11) are partial.

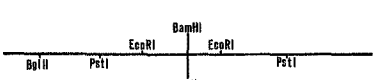
in Figure 14, all the plasmids gave two fragments on SstI digestion presumably corresponding to the cleaved P2 fragments.

The large number of clear plaques obtained, coupled with the fact that the BglII - EcoRI fragment is of the right size and that there is fragment-fragment correlation in Figure 14, suggest that the cloned fragments are the correct ones, with the exception of the problem of the pSR11A fragment.

2.4.9 An attempt to determine the orientation of the pSR51 P2* insert in M13mp11 by restriction analysis

Since the vector has been cleaved at a single site, it was possible for the PstI - PstI insert to be ligated in two orientations. The polarity of such an insert can be determined in at least 3 ways:

- Pairwise hybridization of the single stranded DNA clones. If two clones possess the insert in *opposite orientations* the cloned strands are present in complementary forms and can recognize each other. The viral strands proper are *both identical + strands and thus non-complementary.* Hybridized pairs form dimers and can be detected on agarose gels, allowing the orientation to be determined (Messing 1983).
- The single stranded DNA can be screened by single dideoxy chain termination screening (T or C tracking, for example).
- By means of restriction analysis. As shown in Figure 16 interpretation of single digestions involving internal restriction sites allows the orientation to be deduced. In order to perform this type of analysis on the pSR51 P2* fragment, information about the relative sizes of EcoRI produced fragments is needed. The P2* fragment region of pSR51 has the following expected *appearance:*



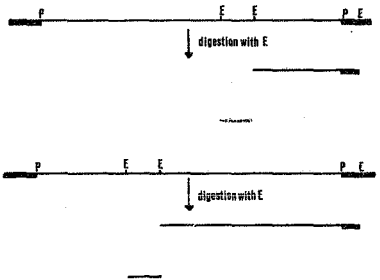


Figure 16

Diagram illustrating the use of restriction analysis to determine the orientation of an insert cloned into a vector. Thick lines indicate vector sequence.

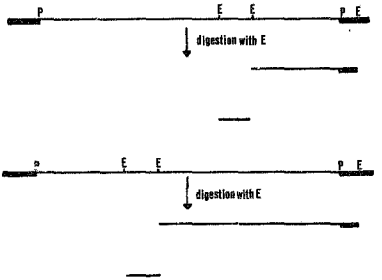


Figure 16

Diagram illustrating the use of restriction analysis to determine the orientation of an insert cloned into a vector. Thick lines indicate vector sequence.

The internal positions of the EcoRI sites have to be determined, and PstI, and BglII - EcoRI digests are needed to make the relevant size determinations. To this end digests in 20µl were done. For double digestions involving EcoRI, the volume was increased to 30µl and the NaCl and Tris concentrations raised. In order to clarify the double and triple digestions, single digestions were done. An LMCB restriction map of pSR51 is presented in Figure 17, and the following results were obtained, some being depicted in Figures 18 and 19.

Table 1. Results of restriction digestions of pSR51.

Enzymes	Expected number of fragments	Results of electrophoresis 1	2	3
PstI	2	-	-	-
BglII - PstI	4	-	-1	-
BglII	2	-	-	-
BglII - EcoRI	6	-1	-1	-
EcoRI	4	+1	-	+2
HindIII - EcoRI	7	-1	-	-2
HindIII	3	-1		
PstI - EcoRI	6	- or -1		-1

- indicates that the expected number was observed.
A -ve number indicates the number of bands missing.
A +ve number indicates the number of additional bands.

Unfortunately the results were too erratic to allow size determinations of the EcoRI - PstI fragments and therefore orientation of the inserts could not be determined by this method.

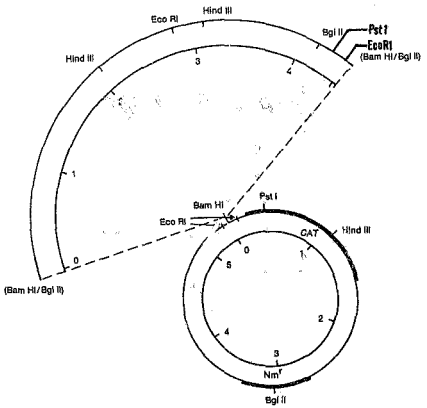


Figure 17

LMCB restriction map of pSR51.

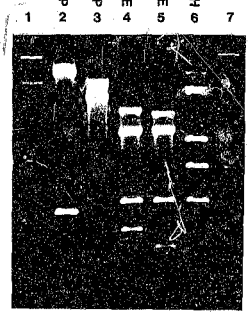


Figure 18

Restriction analysis of pSR51.

Lane 1	Lambda *III marker*	
2	PstI	-
3	BglII - PstI	-2
4	BglII - EcoRI	-1
5	BglII - PstI - EcoRI	-4
6	EcoRI - HindIII	-
7	Lambda III marker	

No numbers - the correct number of bands was observed.
Negative numbers - lacking bands.

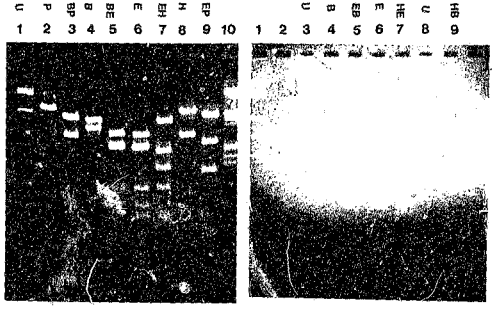


Figure 19

Restriction analysis of pSR51.

Gel 1			Gel 2		
Lane			Lane		
1	uncut		1	Lambda III marker	
2	PstI	-	2		
3	BglII - PstI	-	3	uncut	
4	BglII	-	4	BglII	-
5	BglII - EcoRI	-1	5	EcoRI - BglII	-
6	EcoRI	+1	6	EcoRI	-
7	EcoRI - HindIII	-1	7	HindIII - EcoRI	-
8	HindIII	-1	8	uncut	
9	EcoRI - PstI	-1	9	HindIII - BglII	-1
10	Lambda III marker		10		

No numbers - the correct number of bands was observed.
-ve numbers - lacking bands
+ve numbers - extra bands

2.4.10 Preparation of single stranded DNA

In order to perform chain termination sequence analysis, single stranded template DNA is required. This can be extracted from the infectious M13 viral particles which are released to the exterior from the infected cells. Single stranded DNA extraction was performed by the following method, a modification of the generally available protocol (Mizrahi pers. comm.).

(1) A plaque was removed, or alternatively, 1-10µl of phage supernatant was used and added to 1.5µl of 1:100 dilution of overnight JM109 in 2xTY medium. It was then incubated at 37°C with shaking for 4-5 hours.

(2) The cells were pelleted in a microfuge for 5-8 minutes. About 1.4ml of the supernatant was carefully pipetted off into a tube containing 200µl of 20% PEG/2.5M NaCl. After thorough mixing the mixture was left for 10-15 minutes at room temperature.

(3) After centrifugation for 10 minutes in a microfuge, the supernatant was poured off, leaving a viral pellet of about 1mm diameter.

(4) In order to collect residual PEG/NaCl the tube was recentrifuged for 30 seconds after removal of the supernatant. This residual PEG/NaCl was removed with a pipette.

(5) The pellet was resuspended by vortexing in 100µl TE, and 10µl 3M sodium acetate (pH 5.5) was added.

(6) The TE-virus solution was centrifuged for 10 minutes to remove any contaminating cells and the supernatant transferred to another tube. If a large-scale preparation was being undertaken, the contents of 8 tubes were pooled at this stage.

(7) An equal volume of phenol-chloroform was added and the tube vortexed for 15 seconds.

The mixture was centrifuged in microfuge for 5-10 minutes and the upper phase removed carefully, avoiding the PEG

precipitate at the interface. This extraction was done twice more followed by a chloroform extraction (using half the volume of chloroform) and two ether extractions.

(8) The DNA was precipitated with twice the volume of 96% ethanol, resuspended in 15-30µl of TE and stored frozen.

Figure 20 shows the result of a successful large-scale single stranded DNA extraction. Recovery of ssDNA can fail if the aqueous phase is contaminated with PEG or if the phages do not grow properly or grow for too long a period.

2.5 Sequencing of certain clones by the chain termination method

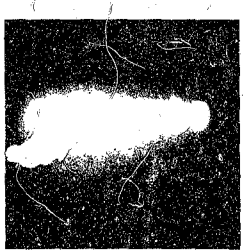
2.5.1 Determination of the orientation of the pSR51 P2* insert in M13mp11 by C tracking

By examination of the pattern of terminations resulting from the presence of a single dideoxynucleotide triphosphate in a polymerisation reaction, the orientation of the "tracked" insert can be established. The protocol used for C tracking was as given in the Amersham M13 Cloning and Sequencing Handbook, except that dideoxy CTP was used instead of dideoxy TTP.

For the preparation of the sequencing gels, the following protocol was adopted:

(1) The glass plate bearing the buffer trough was cleaned with 1N HCl. The plate was subsequently treated with Repel-Silane (dimethyldichlorosilane) according to the LKB instructions. The other glass plate was treated with Bind-Silane (γ-methacryloxypropyltrimethoxysilane) according to the LKB instructions.

(2) The plates were kept apart by thin spacers. At the bottom of the gel, the spacers were doubled in thickness. This results in a field strength gradient which preserves a uniform spacing of oligonucleotides at the bottom of the gel (Olsson et al 1984).



Figure

Single stranded DNA used for sequencing. Each lane represents 15% of the yield from 6ml of phage-containing supernatant. The amount present in one lane above was used in a sequencing reaction.

(3) 50ml of a 6% working acrylamide solution were mixed with 100µl of APS (0.0625g/250µl H_2O) and with 100µl of TEMED, and then poured. The flat end of a BRL Sharkstooth comb was inserted between the plates and the gel allowed to set for 1.5-2 hours. After this the comb was reinserted so that the teeth slightly penetrated the gel. The wells were flushed to remove urea that had leached out of the gel and alternate wells were loaded with 2µl of formamide dye mixture to check for leakage. The cells were pre-electrophoresed at 1400V for at least 30 minutes.

(4) When the gel was hot enough, the wells were cleared of urea, and 2µl of sequencing reaction mixtures which had just been boiled, were loaded. Electrophoresis was carried out until the bromophenol blue dye had reached the bottom of the gel. For longer runs, electrophoresis was continued for 4-5 hours.

(5) At the end of electrophoresis, the plates were separated and the one bearing the acrylamide fixed in 2 litres of 10% acetic acid/10% methanol for 15 minutes with agitation. This fixing (also including the removal of urea from the gel) was repeated with fresh fixer and the gel dried in an oven at 70°C for 2-5 hours. The plate was exposed to X-ray film at room temperature for 24-96 hours.

As can be seen from Figure 21, the cloned inserts were present in two orientations.

2.5.2 Sequencing of P2 fragments from pSR11L, 12L and 5I

For sequencing the plasmid fragments, single stranded template was prepared as described earlier. The general experimental strategy used for sequencing was that developed by Sanger et al (1977), together with the use of ^{35}S label (Biggin et al 1983) and thin acrylamide gels (Sanger and Coulson 1978). The sequencing reactions were performed exactly as outlined in the Amersham guide with the following modifications introduced:

Figure 21

C tracking of the P2* fragment of pSR5I in M13mp11.

Lane 1, 2, 3: orientation 1
Lane 4, 5: orientation 2
Lane 6: control (mp11)
Lane 7: orientation 2
Lane 8, 9: orientation 1
Lane 10: control (mp11)

- the template/primer/enzyme mixture was manually mixed with the nucleotide solution rather than by centrifuging a drop of the former into the latter. The latter practice can result in poor polymerization (Mizrahi pers. comm.).
- According to the Amersham guide, the dNTP/ddNTP mixes are usually made by adding equal volumes of the N° and ddNTP together. In the LMCB, the N°/ddNTP ratios have been adjusted so that the frequency of termination allows the most sequence to be read. The N° and ddNTPs were mixed as follows:

  The N° mixtures are made up exactly as detailed in the Amersham sequencing guide. The ddNTP solutions differ as follows:

	Amersham	LMCB
ddATP	10 mM	0.1 mM
ddCTP	10 mM	0.02 mM
ddGTP	10 mM	0.2 mM
ddTTP	10 mM	0.5 mM

  According to the Amersham guide equal volumes of the N° and ddNTP solutions are combined to give the final nucleotide mix. The volumes mixed in the LMCB which are fine-tuned for the specific DNA in question were as follows:

N°/ddNTP volume ratio
A mix : 1:1
C mix : 1:0.75
G mix : 1:0.75
T mix : 1:1

As regards the results, Figure 22 shows some of the autoradiographs obtained. The pSR51 insert has been sequenced in both directions but not far enough to encounter a degree of overlap. Similarly the P2 fragments of pSR11L and pSR14L have been partially sequenced in one direction.

Figure 22

Autoradiograph of a sequencing gel.

Sequence 1: pSR5I P2* fragment, orientation 2.
2: pSR11L P2 fragment.

The sequences are as follows:

pSR5l P2* fragment

Because of the fact that the quality of some autoradiographs was not optimal, this sequence cannot be regarded as being 100% accurate

Orientation 1:

5'-TCTGTATATGTACTCGGTTATTGTTA
CCTTTACTCAATCACTTACTTACAGTATTATCTT
ACTTATGGAGTCGCCTCTTTTGTATATATCGTGA
TGTTTTTAAGTGCAACCCGCTTCTACTGAATGC-3'

Orientation 2:

5'-GCGAAGGGCAACAAGAGACGACAGA
GTTCGACATACGAGTCGGGCCAAACCCAGACCA
ATAACTATAAAAGCTACAGTTGAGGCCTGTCGG
GCCAAGAACTCCAACGGTTAAGACGTCCAGCTG
AGATCTCTA-3'

pSR7lL P2 fragment in M13mp11

5'-CGAGACTGCCGCATAGAAAAAGGGC
AAGGCAAGACTGGGTGACTTTTTTCTACTGTCG
GTTTATACAAGGCATTTGGCGGAGAACTTACTA
AAAGTAGTCGTGACGGGGTTCGACGAGAGAAGG
TGTTCCTCCTCCTTCGCACAATTTGTAGAGGCC
AGGCCTATTTCCC-3'

pSR12L P2 fragment in M13mp11

5'-GGCGGAGAACTTACTAAAAGTAGTC
GTGACGGGGTTCGACGAGAGAAGGTGTTCCTCC
TCCTTCGCACAATTTGTAGAGGCCAGGCCTATT
TCCC-3'

2.5.3 Conclusion

Two points deserve to be considered in this Section, namely, the instability of the pSR11 and 12 plasmids, and the fact that, according to these results, the P2 fragments of pSR11L and 12L are the same as that of pVC102 and distinct from that of the pSR51 P2* fragment. As described previously, the plasmids used in this work were not the same as the "standard" pattern associated with these plasmids. These differences have been described on page 12 and pages 41-44. These alterations may be similar to the instability associated with the pVC series of plasmids. If strains of the original plasmids were available it would be possible to determine whether this instability is more common and to what extent it takes place. The apparent stability of the pSR51 plasmid may be correlated with the different cloned gene or P2* fragment borne on the vector. This could be tested by joining the α-amylase gene containing fragments of pSR11, 12 or pVC102 with the vector bearing the pSR51 P2 fragment. It would be useful to determine whether the different P2 fragments [illegible] the same level of expression of the CAT gene. It remains unc[illegible] the pSR51 P2* fragment differs from those of pVC102, pSR11 and 12. It may or may not suggest a causal relationship between the cloning of the α-amylase gene and a particular P2 fragment.

SECTION B

SOME ASPECTS OF STRUCTURAL INSTABILITY DISPLAYED BY pVC1D2

1. Introduction

In order to produce hybrid plasmids carrying an α-amylase gene, the vector pPL603b.1 was ligated to BglII digested donor DNA (Bacillus amyloliquefaciens NCPI in the case of the pVC series of plasmids and a different B. amyloliquefaciens for pSR11 and pSR12) (Corfield et al 1984, Reid pers. comm.). Those recombinants were screened on starch and chloramphenicol containing medium. Selection was therefore dual, requiring both the activation of the CAT gene as well as the expression of a cloned α-amylase gene. The most studied of these recombinants, pVC102, consists of an α-amylase gene and its stationary phase promoter, cloned in reverse orientation with respect to a second sequence, the P2 fragment which has promoter activity and allows the CAT gene to be active. The pVC series of plasmids isolated in this way has been characterised by structural and segregational instability (Corfield 1985). Very soon after the creation of the parent plasmid, deletions took place producing a range of smaller plasmids. Amongst these strains, some were selected that were reasonably stable structurally and which simultaneously produced high levels of α-amylase. Thus pVC102, 270, 220, 230 seemed to be the result of a 200 bp deletion which conferred some stability. Since these deletion mutants were screened on both neomycin and chloramphenicol, only losses not affecting these phenotypes were recovered, that is, Nm^R Cm^R amy$^-$ or Nm^R Cm^R amy$^+$. The cause of this instability is unknown but is speculated to be due to the presence of a strong promoter, repeated sequences and/or a deletion generator (Corfield et al 1986c).

When fermentations were performed under different selection conditions, further types of deletions were recovered. In the presence of

chloramphenicol only, the CAT gene, its promoter and the ori region of the plasmid are the primary requirements for survival. This category of deletions is very similar to those resulting from chloramphenicol-neomycin selection. The deletions isolated by the LMCB by chloramphenicol selection fell into two closely related size groups, that is, a loss of 2.3 and 2.7 kb affecting primarily the BamHI - BglIII region of the insert (see Figure 23). When selection is done on neomycin only, a third type of deletion is recovered with the phenotype Nm^R Cm^S amy$^-$ (Watson et al 1986). These plasmids had lost approximately 4 kb, including the better part of the CAT gene.

It is important that two phenomena be studied in order to gain a better understanding of structural instability in this plasmid. An investigation into the different size classes of deletions and the nucleotide sequences at the boundaries of the excisions would be a first step towards determining what factors are responsible for generating and positioning of such deletions. In the Section that follows, two different approaches to this problem were attempted. First, the technique of direct plasmid DNA sequencing was attempted in order to sequence the endpoints of the LMCB deletions. If successful, this method avoids the complication of subcloning into M13 or other vectors that can produce single stranded DNA. This would be useful so that endpoints of other deletions could be easily determined. This modified approach to sequencing is not expected to give results of the same quality as standard sequencing. However, the nucleotide sequence of the α-amylase gene and P2 fragment of pVC102 is known (Herbst pers. comm.), as is that of a large part of the vector. This would provide sufficient background information to make "double stranded" sequencing useful. Second, one can attempt to produce more deletions and determine what size classes (if any) are produced. The physical structure of representatives of the size classes could provide an indication of the extent and position of deletions.

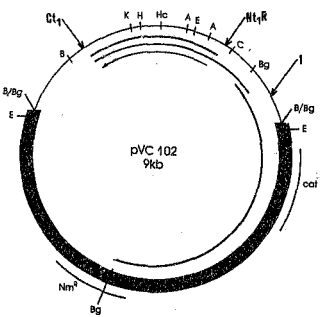


Figure 23

Map showing the location and size of three deletion mutants of pVC102. Also indicated are the approximate positions where primers Ct_1, Nt_1R and 1 are expected to anneal.

2. Methods, Materials and Results

2.1 Direct plasmid DNA sequencing

2.1.1 Background

Although restriction analysis can provide fairly detailed information on the segments of DNA affected by an excision, sequencing is required to provide data on the precise position of a deletion. In addition, the nucleotide sequence will reveal sequences that may be involved in causing, or influencing the position of, the deletion.

The chain termination method of DNA sequencing has an absolute requirement for single stranded DNA which must serve as a template upon which a complementary strand is synthesized. This single stranded template can be produced in a variety of ways, such as by Exonuclease III digestion, the use of specialized phage λ or plasmid vectors which lend themselves to strand separation, or by cloning fragments into filamentous phage vectors such as M13, which produce single stranded DNA as part of their life cycle (see Smith 1980). Certain plasmid vectors with an M13 ori region can yield single stranded DNA with the aid of a helper phage (Russel et al 1986). One possible way to circumvent this problem is by producing single strands by denaturation of native double stranded DNA. This method would allow sequences to be directly determined from a recombinant plasmid (Heinrich 1986). In this regard, the pUC series of vectors would be particularly useful because of the existence of a universal primer and a polycloning site. In the case of pVC102, direct sequencing would be facilitated by the availability of primers annealing to various positions within the cloned region.

Finally, it should be noted that direct plasmid DNA sequencing may be used to sequence fragments that are difficult to clone (promoters, terminators or repetitive sequences) (Chen and Seeburg 1985).

2.1.2 The basic methodology of direct plasmid DNA sequencing

In order to expose single stranded DNA, the plasmid is irreversibly

denatured in the form of a "network" DNA structure (Chen and Seeburg 1985). After alkaline denaturation the sample is neutralized and ethanol precipitated. The source of this DNA can be from caesium chloride preparations or small-scale isolations, such as alkaline-SDS extractions (the Birnboim and Doly technique) (Chen and Seeburg 1985). Following the preparation of the DNA it is subjected to normal annealing and sequencing reactions.

Two different polymerases can be used for direct plasmid DNA sequencing. The Klenow fragment of DNA polymerase 1 can be used (Heinrich 1986), in which case the standard dNTP/dd NTPs are required. If reverse transcriptase is used, lower concentrations of ddNTPs are required as the enzyme accepts these nucleotides more easily than the Klenow fragment. Reverse transcriptase is reputed to give better quality sequences than Klenow under the conditions at issue here (Zagursky et al 1985). This characteristic of the enzyme is perhaps related to the fact that it may be better equipped to deal with polynucleotides which can assume strong secondary structure (such as RNA).

In the sequencing attempted in the course of the project both CsCl and mini-preparation (Birnboim) DNA were used. The CsCl extraction was done as described earlier (page 9) and the Birnboim method used was that given by Maniatis et al (1982). Figure 24 shows such CsCl and Birnboim preparations of plasmid DNA.

The DNA (about 1µg) was precipitated with 7.5M ammonium acetate (1 volume DNA solution, 0.5 volume salt solution and 2 volumes of 96% ethanol, after denaturation with 2M NaOH as described by Zagursky et al (1985).

For Klenow fragment sequencing the protocol of Heinrich (1986) was followed except that deoxycytidine 5'-[α-^{35}S] thiotriphosphate was used as label (1.5µl of [α-^{35}S]dCTPαS 600 Ci/mmol). The nucleotide mixes used were as described on page 55.

When reverse transcriptase was used as polymerase, the sequencing protocol of Zagursky et al (1985) was employed. It was modified to

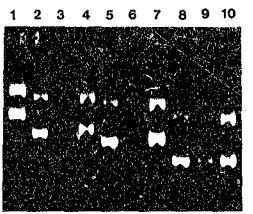


Figure 24

Birnboim and caesium chloride DNA preparations of deletion mutants of pVC102 for direct plasmid DNA sequencing.

Lane 1	pVC102	(CsCl)
2	LMCB 1	(CsCl)
3	LMCB 1	(Birnboim)
4	LMCB 1	(CsCl)
5	LMCB 2	(CsCl)
6	LMCB 2	(Birnboim)
7	LMCB 2	(CsCl)
8	LMCB 3	(CsCl)
9	LMCB 3	(CsCl)
10	LMCB 3	(Birnboim)

accept [α-^{35}S]dCTPαS as label. The dNTP/ddNTP solutions used were those described in the protocol of Biorad (1985).

As regards results, the techniques attempted as described above did not yield readable sequences and in some cases no sequence at all. These problems were perhaps related to a poor DNA template or to other less well-defined factors.

2.1.3 Determining whether primers Nt_1R, Ct_1 and 1 anneal to the deletion mutant DNA

Since the direct plasmid DNA sequencing technique was to be used to determine the borders of deletion mutants LMCB 1, 2 and 3, it was desirable to determine by hybridization whether the primers annealed to the deleted plasmids. The three primers used in this experiment were expected to anneal to the general positions indicated in Figure 24 (on page 64). The three primers had the following sequences:

Ct_1 5' G G A G A C G A C C C 3'
Nt_1R 5' G C T G T C C A G A C T G T C C G C 3'
1 5' G T G C C G T A A T T C A G C T C A G 3'

Since these primers lacked a 5' phosphate (as a result of their artificial synthesis), it was possible to endlabel them with γ-^{32}P and use them as probes in hybridization experiments. This would establish whether the primers could be used for directing the synthesis of complementary strands in a sequencing reaction.

2.1.3.1 End-labelling

For end-labelling, the forward reaction of T4 polynucleotide kinase was used (Maniatis et al 1982).

$$5'\text{-OH-oligonucleotide} \xrightarrow[[\gamma\text{-}^{32}P]ATP]{Mg^{2+}} 5'\text{-}^{32}P\text{-oligonucleotide} + ADP$$

The protocol used for the labelling was as follows (Mizrahi pers. comm.):

10μM oligomer	1μl
10 x kinase buffer	1μl
T4 polynucleotide kinase	1μl (1U/μl)
[γ-^{32}P]ATP	3μl (3000 Ci/mmol)
dH_2O	4μl
	10μl

(1) Incubation was done at 37°C for 1 hour after which the reaction was stopped by heating the mixture to 65°C for 10 minutes.

(2) The kinase buffer was made up as described by Maniatis et al (1982).

(3) The molarity of the oligomers was calculated by using concentrations derived from spectrophotometer readings of 1:100 and 1:50 dilutions (in water) of concentrated primers. It was assumed that an A^{cm}_{260nm} value of 1 corresponded to 35μg/ml. The molecular weight of one dNTP was assumed to be 330 daltons.

(4) The protocol above employs a molar excess of oligomer (5'-OH ends) over γ ^{32}P-ATP. If the reaction is successful, no unincorporated label should remain. If an excess of label is added the labelled oligonucleotides are separated from free label in a spun column (Maniatis et al 1985). For this purpose, the 10μl reaction was made up to 100μl with TE.

(5) To monitor whether end-labelling has taken place, thin layer chromatography on polyethylene-imine cellulose was done. In this procedure, 1cm wide columns were scored on the PEI-cellulose sheet with a sharp pencil. The buffer used as carrier was 0.3M potassium phosphate buffer pH 7 prepared by titrating 0.3M KH_2PO_4 with 5M KOH. After

spotting 0.5µl of 1:100 dilution of label and 0.5µl of the labelled oligonucleotide solution (in 100µl) on to the sheet, the spots were allowed to dry and immersed in a thin layer chromatography tank containing about 0.5cm deep volume of buffer. After 1-2 hours of migration the sheet was allowed to air dry and was then exposed to an X-ray film in a casette with intensifying screens at -70°C for 2-5 hours. As can be seen from Figure 25, the labelling had proceeded satisfactorily, apart from primer Ct_1 which had a low specific activity.

The total radioactivity in the 100µl volumes of each of the primers was as follows:

Ct_1, 0.3×10^7 cpm; Nt_1R, 0.6×10^7 cpm; 1, 2×10^7 cpm, as determined from 1µl placed in 10ml of scintillation cocktail and read in a Packard Minaxi TriCarb Scintillation Counter.

2.1.3.2 Probing with the labelled oligonucleotides

In order to probe the plasmid DNA, it was transferred to a solid support, nitrocellulose paper in this case. Since double stranded DNA binds very inefficiently to this medium it was necessary to linearize the DNA so that it readily denatures. This can be effected by digestion or heat treatment prior to alkaline treatment.

Linearization of DNA by digestion

In this approach, pVC102, and several deleted plasmids were digested with EcoRI (2.5µg DNA in a total volume of 15µl). After separation of the fragments on an agarose gel, an attempt was made to transfer the DNA to nitrocellulose by the Southern procedure, as described by Maniatis et al (1982). The transfer was not successful and could not be improved either by reducing the agarose concentration from 1% to 0.6%, or by increasing the weight on the blot, or by increasing the time of transfer from 24 to 60 hours.

Figure 25

Thin layer chromatography of end-labelled primers.

Track 1 Label
2 Nt_1R
3 Ct_1
4 1

The end-labelling proceeded satisfactorily except for Ct_1 which has a low specific activity. The X-ray film was exposed for 2 hours.

Linearization by heat treatment

Given the problems described above, dot blotting was attempted since this could give a positive or negative answer as to whether the primers were able to anneal to the DNA. In order to linearize the plasmid DNA it was heat treated as described by Anderson and Young (1985). To this end, 2µg of plasmid DNA in 10µl of TE was boiled for 15 minutes. In order to confirm that the concentrations of the DNA (which had been spectrophotometrically determined) were similar, and to examine the effects of the heat treatment, about 1µg was run on a 0.8% agarose gel (Figure 26). The concentration of the DNA appeared similar with evidence of extensive breakdown.

Application to nitrocellulose

The protocol that was used for dotting is given by Anderson and Young (1985).

Hybridizations

The method followed here is a combination of that given by Mason and Williams (1985) and Kunkel et al (1986).

Prehybridization:

This was done in 6xSSC, 10xDenharts and 50µg/ml denatured salmon sperm DNA at the hybridization temperature for 1 hour. The nitrocellulose squares were incubated in 10ml of prehybridization medium in a Petri dish.

Hybridization:

(1) Hybridization was done in prehybridization solution with 0.1µm of oligonucleotide/ml. The blocking DNA was denatured prior to hybridization, a procedure not required for the oligomers.

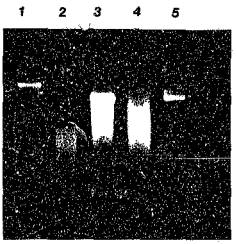


Figure 26

Heat linearized plasmids prepared for dot blotting. Extensive DNA breakdown is apparent.

Lane 1 pVC102 (+ control)
2 LMCB 1
3 LMCB 3
4 LMCB 2
5 pPL603b.1 (- control)

(2) Hybridization was done at a temperature (Td) 5°C lower than the melting temperature of the oligonucleotide. The melting temperatures were calculated according to the formula, Tm = 4(G+C) + 2(A+T) (see Meinkoth and Wahl 1984), and were found to be as follows:

	Tm	Td
Ct_1	42°C	37°C
Nt_1R	70°C	65°C
1	58°C	53°C

(3) Hybridization was done for at least 2 hours after which the blots were washed 2-3 times in 6xSCC, 0.1% SDS at Td for 30 minutes. Finally the blots were washed at Tm for 2 minutes.

(4) The blots were sealed in plastic bags and exposed to X-ray films for 5-24 hours depending on the amount of radioactivity bound.

The results of the dot blotting are depicted in Figure 27, and are tabulated below. (Figure 23, which shows where the primers may anneal, appears on page 61.)

Table 2. Results of dot blotting using deleted plasmids and end-labelled primers.

Primer		Plasmid				
		pVC102 (+ control)	LMCB 1	2	3	pPL603b.1 (- control)
1	expected	+	+	+	-	-
	observed	+	+	+	-	-
Nt_1R	expected	+	?	-	+	-
	observed	+	-	-	-	-
Ct_1	expected	+	?	?	+	-
	observed	+	-	-	+	-

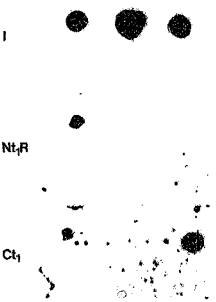


Figure 27

Dot blots of plasmid deletion mutants after hybridization to end-labelled primers.

1 Primer 1
2 Primer Nt_1R
3 Primer Ct_1

The arrangement of dotted plasmid DNA in all 3 cases is as follows:

pVC102 (+ve control)	LMCB 2	LMCB 1	LMCB 3

pPL603b.1
(-ve control)

The location of the sequences on pVC102 complementary to the Ct_1 primer is about 400 bp from the BamHI site in the direction of the KpnI site (see Figure 23). This primer did not anneal to LMCB 1 and 2 and it suggests that the left endpoints of the deletions are closer than 400 bp to the *BamHI site*.

The position of sequences complementary to Nt_1R is approximately 70 bp away from the ClaI site, towards the body of the amylase gene. This primer did not anneal to either LMCB 1, 2 or 3. In order to explain these results it is necessary that the right endpoint of LMCB 1 be closer than 70 bp, assuming that this (ClaI) site remains present. Similarly the endpoint of LMCB 3 would have to be extended considerably further, that is beyond the ClaI site. This would also explain the fact that LMCB 3 is amy$^-$.

The results suggest that neither Ct_1 nor Nt_1R could be used to sequence the LMCB 1 and 2 deletion endpoints but that primer 1 may be more useful in this respect. However it is possible that this primer anneals too far from deletion LMCB 1 and 2 endpoints to give appropriate DNA sequence. In conclusion, it seems that subcloning of the BamHI - EcoRI fragment of deletions 1 and 2 into M13 for standard sequencing may be the simplest method to determine the structure and position of the deletion endpoints.

2.2 Isolation of deletion mutants of pVC102

2.2.1 Generation of deletion mutants

As described earlier (page 60), the LMCB deletions isolated from industrial fermentations fell into distinct size classes and the production of additional deletion mutants was attempted. The following procedure was adopted:

(1) An overnight culture of pVC102 (grown with antibiotics) was taken and diluted 10^6 times in water. 100µl of this were added to 250ml of 2xLB with either neomycin or chloramphenicol. At this stage an aliquot of the diluted

overnight was plated on a neomycin, chloramphenicol, *starch* plate to ensure that the inoculum had only amy$^+$ cells. If any amy$^-$ colonies were found the culture was discarded.

(2) The inoculated culture was grown for 7 days after which an aliquot was plated on to agar containing the same antibiotics as they were cultured in. Dilutions were made to produce single colonies.

(3) At least 96 of these colonies per fermentation were patched on to plates as follows: chloramphenicol, neomycin, starch plus the antibiotic which was present in the culture, and LA only.

(The latter is a control plate to monitor whether viable cells had been patched.) On all such screening plates a positive (1A297 (pVC102)) and a negative control (1A297), derived from freshly streaked single colonies, were included.

(4) Potential deletion mutants were detected by the loss of a marker (amylase phenotype or $Cm^{R/S}$ in the cases studied here). The possible mutants isolated are listed in Table 3 (page 75).

Table 3. Phenotypes of possible deletion mutants isolated from 7 day cultures.

No.	Nm^R	Cm^R	amy^+	No.	Nm^R	Cm^R	amy^+	
1	+	-	-	(18)	+	+	-	LMCB 1
2	+	-	-	(19)	+	+	-	LMCB 2
3	+	-	-	(20)	+	-	-	LMCB 3
4	+	-	-	21	+	+	+	
5	+	-	-	22	+	-	-	
6	+	-	-	23	+	-	-	
7	+	-	-	24	+	-	-	
8	+	+	+	25	+	-	-	
9	+	+	+	26	+	+	-	
10	+	-	-	27	+	-	-	
11	+	-	-	28	+	-	-	
12	+	-	-	29	+	-	-	
13	+	-	-	30	+	-	-	
14	+	-	-	31	+	+	+	
15	+	+	+	32	+	+	+	
16	+	+	-	33	+	+	+	
17	+	+	-					

1. +/- denotes resistance/sensitivity to an antibiotic or presence or not of α-amylase as detected on starch plates.
2. All the isolates referred to in the Table were derived from neomycin only fermentations, except Nos 31-33 (off chloramphenicol) and 18-20 which were already in existence.
3. Some of the isolates are probably wildtype pVC102 and were screened in order to detect any deletions that had not affected the 3 scored phenotypes.

2.2.2 Screening of deletion mutants

In order to screen the possible mutants for the sizes of their plasmids the Quick lysis plasmid screen was done. This technique is a modified version of the procedure developed by Marrero and Lovett (1980).

(1) 2ml of cells were grown overnight in LB with antibiotic(s) in test tubes.

(2) 1ml was transferred to an Eppendorf tube and centrifuged for 1 minute.

(3) The supernatant was discarded and excess fluid removed with tissue paper.

(4) The pellet was resuspended in 15µl of lysozyme buffer by pipetting the compacted cells up and down.

(5) 15µl of 4mg/ml lysozyme solution (made up in lysozyme buffer) was mixed in briefly by pipetting, and the tubes incubated at 37°C for 15 minutes.

(6) 50µl of SDS buffer were added and the tube flicked until the solution cleared.

(7) 20µl of 5M NaCl were added and the tubes stored on ice for 30 minutes, and then centrifuged in a microfuge for 25 minutes.

(8) The pellet was then removed with a pipette and 10µl of the supernatant loaded on to a 0.8% agarose gel.

(9) It was found that the samples could be stored overnight on ice but that they deteriorated rapidly and are therefore best used immediately.

The screened deletion mutants fell into 3 size classes (see Figure 28). Table 4 (page 78) summarizes the result of the gel electrophoresis.

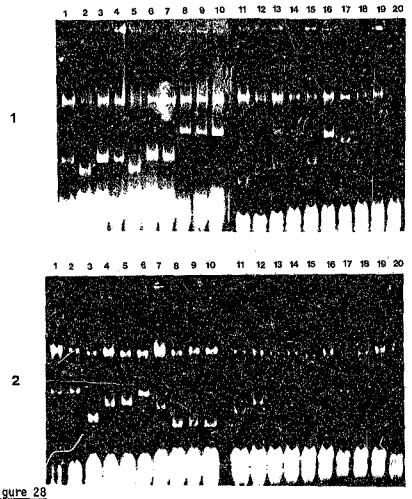


Figure 28

Quick lysis plasmid screen of potential deletion mutants isolated from extended cultures.

Gel 1				Gel 2			
Lane 1	1	11	10	Lane 1	pVC102	11	"pVC102"
2	2	12	11	2	9	12	26
3	3	13	12	3	14	13	23
4	4	14	13	4	16	14	28
5	5	15	14	5	19 (=LMCB 1)	15	24
6	6	16	15	6	21	16	30
7	7	17	17	7	18 (=LMCB 2)	17	31
8	8	18	20 (=LMCB 3)	8	23	18	32
9	8	19	18 (=LMCB 2)	9	24	19	33 (=pVC102)
10	pVC102	20	pVC102	10	25	20	34

The "pVC102" (gel 2 lane 11) apparently suffered a deletion during culture.

Table 4. Size classes of plasmid deletion mutants.

Size	Isolate number
Equal to pVC102	8, 9, 15, 21, 31, 32, 33, 34
Between pVC102 and LMCB 3 (includes LMCB 1 and 2)	16, 17, 18, 19, 26
Equal to LMCB 3	1, 3, 4, 6, 7, 10, 11, 12, 13, 14, 20, 23, 24, 25, 28, 29
Smaller than LMCB 3	2, 5, 10, 13, 30

The results indicate 3 size classes:

- A size class between pVC102 and LMCB 3. All these strains have the phenotype $Nm^R Cm^R \underline{amy}^-$.
- A class equal in size to LMCB 3 ($Nm^R Cm^R \underline{amy}^-$).
- A class smaller than LMCB 3 ($Nm^R Cm^R \underline{amy}^-$).
- All plasmids with all three markers ($Nm^R Cm^R \underline{amy}^+$) have the same size as pVC102 and are probably identical to it.

The Ish-Horowicz procedure (see Maniatis *et al* 1982) was also tried as a plasmid screen. A lysozyme step was included because B. subtilis does not lyse easily with alkali unless it is in log phase (Dabbs pers. comm.). This method was not used extensively because it produced more complex plasmid profiles, presumably corresponding to various forms of the plasmid (see Figure 29). This needlessly complicated interpretation of the results.

2.2.3 Restriction endonuclease digestion of certain deleted plasmids

In an attempt to determine whether structural changes that had affected some of the plasmids were similar or not to the previously mapped

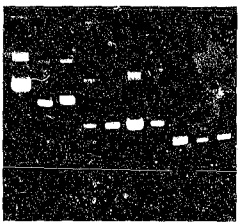


Figure 29

Ish-Horowicz screen of plasmid deletion mutants. More plasmid forms are visible here than in the quick lysis plasmid screen.

Lane 1 pVC102
2 LMCB 2
3 LMCB 1
4 LMCB 3
5 11
6 4
7 7
8 6
9 5
10 2

deletions (LMCB 1, 2 and 3), various digestions were performed. The following strains were used:

plasmid	phenotype	size class
pVC102	$Nm^R Cm^R$ amy$^+$	pVC102
5	$Nm^R Cm^R$ amy$^-$	<LMCB 3
16	$Nm^R Cm^R$ amy$^-$	>LMCB 3 <pVC102
17	$Nm^R Cm^R$ amy$^-$	>LMCB 3 <pVC102

CsCl preparations were made and the DNA digested with *Bam*HI, *Bgl*II, *Eco*RI and combinations of these. The digestions were done in 20µl volumes for single digests, and 30µl for double digests involving enzymes requiring the same buffer (*Bam*HI - *Bgl*II). For sequential digestion the medium salt buffer enzyme was used in a volume of 15µl which was increased to 30µl with a concomitant increase in NaCl and Tris concentrations. At all times a final maximum glycerol concentration of 5% was present. Some of the results are indicated in *Figures 30-1*. It was not possible to draw any detailed conclusions from the results as the digestion patterns were not always reproducible. The results obtained are summarized in the following table:

Table 5. Number of sites for different restriction enzymes and their combinations in deleted plasmids.

Plasmid	Enzyme					
	*Bam*HI	*Bgl*II	*Eco*RI	*Bam*HI/ *Bgl*II	*Bam*HI/ *Eco*RI	*Bgl*II/ *Eco*RI
pVC102 (expected)	1	2	3	3	4	5
pVC102 (seen)	1	2	3	3	4-5	3
5	1	1	2-3	-	4-5	3-4
16	1	1	2	2	4	3
17	1	1	2	2	4-5	2-3

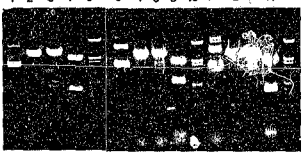


Figure 30

Single digestion of pVC102 deletion mutants.
16,17 -$Nm^{R}Cm^{R}$ amy^{-}, >LMCB 3<pVC102
5 -$Nm^{R}Cm^{S}$ amy^{-}, <LMCB 3

Lane 1	uncut		Lane 10	Lambda III	
2	BamHI	17	11	uncut	
3	BglII		12	BamHI	
4	EcoRI		13	BglII	5
5	Lambda III		14	EcoRI	
6	uncut		15	Lambda III	
7	BamHI	16			
8	BglII				
9	EcoRI				

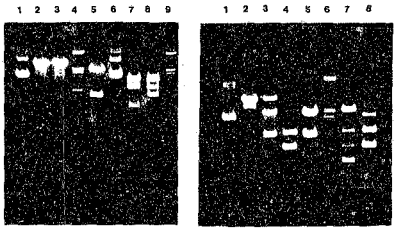


Figure 31

Double digestion of pVC102 deletion mutants (1.8% agarose).
16,17 -$Nm^{R}Cm^{R}$ amy^{-}, >LMCB 3 <pVC102

Lane	16	Lane	17
1	uncut	1	uncut
2	BamHI	2	BamHI
3	BglII	3	BglII
4	Lambda III	4	EcoRI
5	EcoRI	5	BamHI - BglII
6	BamHI - BglII	6	Lambda III
7	BamHI - EcoRI	7	BamHI - EcoRI
8	BglII - EcoRI	8	BglII - EcoRI
9	Lambda III		

Although the digestion patterns were not always reproducible, the following can be concluded from them:

Deletants 16 and 17 ($Nm^R Cm^R$ amy$^-$, >LMCB 3 <pVC102)

These plasmids have single sites for BamHI and BglII. Two sites for EcoRI remain. On BglII - EcoRI digestion 3 fragments are released. These results suggest that the deletions may be similar to LMCB 2. There seem to be differences between 16 and 17 but these could not be rigorously determined.

Deletant 5 ($Nm^R Cm^S$ amy$^-$, <LMCB 3)

This plasmid carries single sites for BamHI and BglII. As in the above deletions, two EcoRI sites are present. It is possible that the EcoRI site within the amylase region is still intact, and that this deletion represents a larger version of LMCB 3. It is possible that the smaller deletions affecting the amylase region (LMCB 1, 2, Nos 16 and 17) are different in their origin from the larger variety (LMCB 3 and No. 5). This would suggest that the larger and smaller deletants do not form part of a series. This would contrast with the results of Vehmaanperä and Korhola (1986). The deletions studied by them all appear to affect primarily the general region of the cloned amylase gene.

2.2.4 Conclusion

In the preceding section, the technique of direct plasmid DNA sequencing was described. No meaningful results were obtained. It is probable that these problems could be eliminated, and with that in mind, three primers were used as probes to detect possible complementary sequences in deletions LMCB 1, 2 and 3. After end-labelling and hybridization to dot blotted fragmented plasmid DNA, it appeared that Nt_1R and Ct_1 primers annealed only to LMCB 3. Primer 1 (whose complementary sequence is found within the P2 fragment of pVC102) annealed only to plasmid DNA from LMCB 1 and 2. One anomalous result, namely the failure of primer Nt_1R to anneal to LMCB, could be accounted for by assuming that this deletion

was more extensive than previously thought. Deletion mutants created by fermentations were screened and fell into 3 main size classes. One of these groups (>LMCB 3 <pVC102) seems to be more heterogenous than the other classes (i.e. <LMCB 3, = LMCB 3). This heterogenous group of deleted plasmids is produced under chloramphenicol selection. Although specific data are not available it would seem that the two largest deletions (i.e. = and <LMCB 3) are more frequent and may represent a more stable "end point" of deletion. Thus, chloramphenicol selection may be necessary to "fix" smaller deletions such as LMCB 1 and 2.

Although the mapping of the deletions has not proceeded very far, it appears, in the cases studied, that BglII - EcoRI sites near the α-amylase gene have been affected. It should be noted that in smaller plasmids it may be difficult to determine which of the two BglII sites of pVC102 has been lost. In future mapping, the distribution of restriction sites in the vicinity of the BglII site associated with the neomycin resistance gene will have to be determined.

APPENDIX 1

BACTERIAL STRAINS USED

Strain	Relevant characteristics	Source
B. subtilis		
1A297	*trp* C2 *asp* T1 *amy*	BGSL
1A297 (*pVC102*)	Nm^RCm^R *amy*$^+$	LMCB
1A297 (pSR11L)	Nm^RCm^R *amy*$^+$	LMCB
1A297 (pSR12L)	Nm^RCm^R *amy*$^+$	LMCB
1A297 (pSR11A)	Nm^RCm^R *amy*$^+$	this work
1A297 (pSR12A)	Nm^RCm^R *amy*$^+$	this work
1A46	*trp*C2 *thr*5 *Sec*E4	BGSL
1A46 (pSR51)	Nm^RCm^R *npr*$^+$	LMCB
E. coli		
JM109	*rec*A1, *end*A1, *gyr*A96, *thi*, *hsd*R17, *sup*E44, *rel*A1, λ^-, Δ(*lac-pro*AB), [F', *tra*D36, *pro*AB, *lacI*^{q}*Z*ΔM15]	LMCB

APPENDIX 2

SOURCES OF REAGENTS AND CHEMICALS

Acetic acid	Merck
Agar (Technical No. 1)	Oxoid
Agar	Merck
Agarose	BioRad
Amberlite MB - 1	BDH
Ammonium acetate	Merck
Ammonium persulphate	BDH
Antibiotics	Sigma
ATP	Sigma
Bisacrylamide	BioRad
Boric acid	Merck
Bovine Serum Albumin	Sigma
Bromophenol Blue	Merck
n-Butanol	BDH
Calcium chloride	Merck
Caesium chloride	Boehringer Mannheim
Chloroform	Saarchem
Diethyl ether	PAL Chemicals
Dimethyldichlorosilane	BDH
Di-potassium hydrogen orthophosphate	Merck
Dithiothreitol	Sigma
DNA molecular weight markers	Boehringer Mannheim
DNA (salmon sperm)	Sigma
Ethanol	CSIR Stores
Ethidium bromide	Sigma
Ethylene diamine tetra-acetic acid (disodium salt)	Kodak, Merck

Ficoll 400	Sigma
Formamide	Merck
Glucose	BDH
Hydrochloric acid	Merck
Instagel scintillation cocktail	Packard
IPTG	Sigma
Isopropanol	Saarchem
Lysozyme	Boehringer Mannheim
Magnesium chloride	Merck
γ-methacryloxypropyltrimethoxysilane	Sigma
Methanol	Merck
NaCl	Merck
Nitrocellulose	Millipore
Nucleotides	Amersham
PEG 6000	Merck
PEI-Cellulose	Merck
Phenol	Merck
Potassium acetate	Merck
Potassium dihydrogen orthophosphate	Merck
Potassium hydroxide	Merck
Radioactive label	Amersham
Restriction Enzymes	Boehringer Mannheim
Reverse transcriptase	Amersham
Ribonuclease A	Sigma
Sephadex G50	Pharmacia
Sodium acetate	Merck
Sodium chloride	Merck

tri-Sodium citrate	Merck
Sodium dodecyl sulphate	Saarchem
Sodium hydroxide	BDH
Spermidine	Sigma
SstI enzyme	BRL
Starch	Saarchem
Sucrose	Merck
Thiamine-HCl	Sigma
T4 DNA ligase	Boehringer Mannheim
T4 polynucleotide kinase	Boehringer Mannheim
Tris base	Sigma, BDH
Tryptone	Oxoid
Urea	Merck
X-gal	Boehringer Mannheim
X-ray film (XAR-5)	Fuji
Xylene cyanol	Hopkins
Yeast extract	Merck

APPENDIX 3

MEDIA, GENERAL BUFFERS, ENZYME BUFFERS, REAGENTS FOR DNA EXTRACTION, REAGENTS FOR SEQUENCING GELS, AND OTHER REAGENTS

1. Media

Luria Broth	Tryptone	10g
	Yeast extract	5g
	NaCl	5g
	dH_2O	2 litres
Luria agar	As above, with	
	Agar	12g
Luria agar with 1% starch	As above, with	
	Starch	10g

When starch is included, the powdered components are first mixed completely, water added, and mixed. The solution is then boiled until the starch goes into solution, followed by autoclaving.

Minimal medium plus glucose

This is made up exactly as detailed in the Amersham M13 Cloning and Sequencing Handbook. Note, however, that the 1M thiamine-HCl was filter sterilized. Merck Agar was used for minimal media because it is purer than other brands (Dabbs pers. comm.).

2xTY	Tryptone	16g
	Yeast extract	10g
	NaCl	5g
	dH_2O	1 litre

1xTY agar	Tryptone	8g
	Yeast extract	5g
	NaCl	5g
	Agar	15g
	dH_2O	1 litre
TY top agar	As above, with half the amount of agar.	

2. General buffers

Tris-Borate-EDTA electrophoresis buffer (pH 8.3)
10x stock

Tris base	108g
Boric acid	55g
EDTA	9.3g
dH_2O to	1 litre

Tris-Acetate electrophoresis buffer
50x stock

Tris base	242g
Acetic acid	57.1ml

For agarose electrophoresis, ethidium bromide to a concentration of 2µg/ml was added to these buffers.

Tris-EDTA (TE) buffer (pH 8)

Tris	10mM
EDTA	1mM

The solution was brought up to pH with HCl

Tris-EDTA-salt (TES) buffer (pH 8)

Tris	30mM
EDTA	5mM
NaCl	50mM
pH with HCl	

Buffer for thin layer chromatography (pH 7)

Potassium phosphate	0.3M

Prepared by titrating KH_2PO_4 0.3M with KOH 5M.

3. Buffers and reaction mixes for enzymes

Klenow reaction buffer	Tris-HCl (pH 8)	100mM
	$MgCl_2$	50mM
Restriction buffer-medium salt	Tris-HCl (pH 7.5)	10mM
	$MgCl_2$	10mM
	NaCl	50mM
Restriction buffer-high salt	Tris-HCl (pH 7.5)	50mM
	$MgCl_2$	10mM
	NaCl	100mM

SstI buffer

This buffer, provided by the supplier, has the following composition:

Tris-HCl (pH 7)	50mM
$MgCl_2$	10mM
NaCl	50mM

Reverse transcriptase reaction buffer	Tris-HCl (pH 8.3)	0.3M
	$MgCl_2$	37.5mM
	Dithiothreitol	2.5mM
	NaCl	0.375M
RNase buffer		
T4 polynucleotide kinase buffer	Tris-HCl (pH 7.6)	0.5M
	$MgCl_2$	0.1M
	Dithiothreitol	50mM
	Spermidine	1mM
	EDTA	1mM

4. Reagents for DNA extraction

Caesium chloride - Quick lysis plasmid screen

Lysozyme buffer	Tris-HCl (pH 8)	25mM
	NaCl	50mM
	EDTA	50mM
	Sucrose	25%
SDS buffer	10% SDS	20ml
	EDTA 0.5M	20ml
	TE buffer (pH 8)	120ml

Ish-Horowicz solutions for small and large-scale alkaline lysis procedures

Prelysis solution (Solution 1)	Tris-HCl (pH 8)	25mM
	EDTA	10mM
	Glucose	50 mM

Alkaline-SDS (Solution 2)	NaOH	0.2M
	SDS	1%
Solution 3	Potassium acetate, pH 4, consisting of 3M K and 3M acetate.	

To avoid precipitation during the preparation of Solution 3, the procedure followed by Maniatis et al (1985) must be followed.

5. Reagents for DNA sequencing gels

40% Acrylamide stock	Acrylamide	38%
	Bis-acrylamide	2%

Deionise with amberlite (20g) and store at 4°C in the dark after filtration.

Acrylamide	6% working solution	
	40% Acrylamide stock	150ml
	10xTBE	100ml
	Urea	460g
	dH_2O to	1 litre
Ammonium persulphate	0.125g/500µl dH_2O	
100µl is used per 50ml acrylamide working solution.		
Bind silane	γ-methacryloxypropyl-trimethoxysilane	75µl
	Add ethanol (96%)	25ml
	Mix completely	
	Add acetic acid (10%)	750µl

Fixer for acrylamide gels	Methanol	10%
	Acetic acid	10%

Two litres are used per wash.

Formamide dye mix	Amberlite deionised formamide	100ml
	Bromophenol Blue	0.03g
	Xylene cyanol	0.03g
	EDTA	0.75g

6. Other solutions

Denaturation solution (for hybridizations)	NaCl	1M
	NaOH	0.5M
Denharts solution (20x)	Ficoll	2%
	Bovine serum albumin	2%

The components are best dissolved separately.

Isopropanol saturated with 20xSCC
Mix the organic reagent with an equal volume of 20xSCC.

Neutralization solutions (for hybridizations)	1. Tris-HCl (pH 7.4)	0.5M
	2. Tris-HCl (pH 7)	0.5M
	NaCl	3M

Agarose

For small gels, agarose was mixed with buffer to give the required percentage (almost always Tris-acetate buffer for small gels and Tris-borate buffer for large gels). Additional distilled water was added

and the solution boiled until clear. After making the solution up to volume, ethidium bromide to a concentration of 2μg/ml was added and the solution poured.

Dialysis tubing was prepared as described by Maniatis et al (1982).

IPTG	2% in dH_2O, stored frozen
X-gal	2% in diethylformamide, stored in the dark at -20°C.

ATP 10mM
The solution must be brought to pH 7 with triethylamine.

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