Efficient oligonucleotide-directed construction of mutations in expression vectors by the gapped duplex DNA method using alternating selectable markers

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ABSTRACT

An efficient method for the construction of multiple mutations in a sequential manner is described. It is based on the gapped duplex DNA approach to oligonucleotide-directed mutagenesis (Kramer *et al.* 1984, Nucl. Acids Res. **12**, 9441−9456) and a set of newly constructed phasmid vectors. These are characterized by the following features. Presence of the phage f1 replication origin permits ready conversion to the single stranded DNA form. An amber mutation within, alternatively, the *bla* or *cat* gene provides a means for ready selection of the strand into which the mutagenic oligonucleotide has been incorporated. By means of the alternating antibiotic resistance markers any number of mutations can be constructed in consecutive rounds of mutagenesis. The optional presence of gene expression signals allows the direct overproduction of structurally altered proteins without re-cloning. Both the mutagenesis and expression aspects were tested using the *lacZ* gene as a model.

INTRODUCTION

A widely used strategy for site-directed mutagenesis involves the annealing of synthetic oligodeoxyribonucleotides to single stranded DNA (ssDNA) templates carrying the target sequences (reviewed in 1). Filamentous phages provide a means by which such ssDNA can be easily and rapidly obtained. A number of procedures have been described to increase the yield of mutants and thus to facilitate screening in the absence of a phenotype (1−6). One powerful method relies on the use of gapped duplex (gd) DNA (6) combined with a selection in favor of progeny derived from the gapped strand (i.e. the strand into which the mutagenic oligonucleotide is incorporated *in vitro* by enzymatic reactions). In this strategy, a crucial factor in increasing the percentage of mutants harvested is the use of a DNA mismatch repair deficient *E. coli* strain (i.e. *mutS* or *mutL*). Absence of this cellular repair system has the effect of preserving the linkage between the selectable marker and the newly introduced mutation. Procedures including such refinements allows mutants to be harvested with an efficiency of up to 80%.

Due to their general use as vehicles in expression studies, it is attractive to construct precise mutations directly in plasmids. For this, target sequences cloned in double stranded vectors need to be converted to a single strand. A simple means of achieving this conversion is the use of vectors which, in addition to a plasmid replication origin, carry one derived from a filamentous phage (7,8; hereafter referred to as phasmids). Upon infection of cells harbouring such a phasmid with an appropriate helper phage, induction of the phage origin occurs and one specific DNA strand becomes packaged and secreted as pseudo-viral particles.

In the present paper, we describe a series of new phasmids. These vectors contain genes conferring resistance to the antibiotics ampicillin (Ap) and chloramphenicol (Cm).

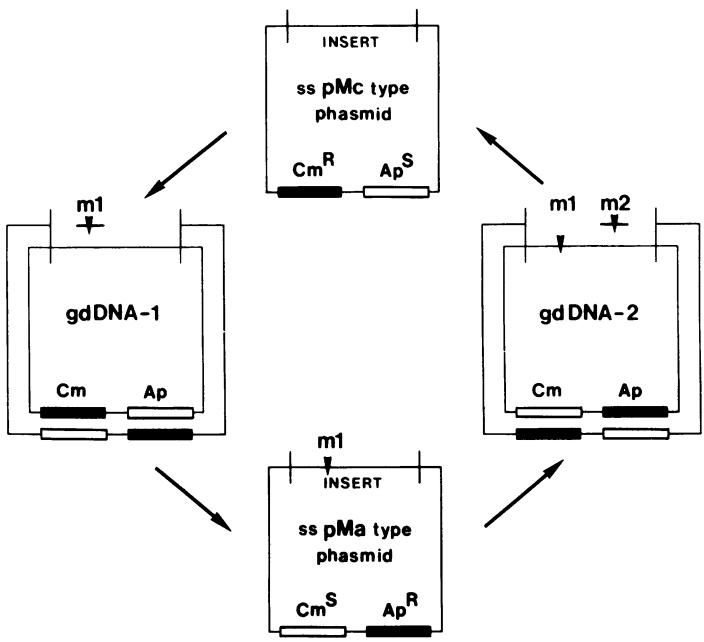

Figure 1: Consecutive introduction of mutations.
Multiple mutations can be introduced into a target sequence ('INSERT') by cycling between pMc type (Cm^RAp^S; contains an amber mutation in the Ap marker) and pMa type (Cm^SAp^R; amber mutation present in the Cm marker) phasmids. The wild type antibiotic resistance genes are shown by filled rectangles, the amber mutant counterparts by open rectangles. Single stranded pMc type phasmid is annealed to a fragment of the complementary pMa phasmid, giving rise to gdDNA-1. Following incorporation of the mutagenic oligonucleotide (the arrowhead represents the desired alteration) into the gapped strand, progeny deriving from this strand are selected on ampicillin containing medium. The resultant pMa type phasmid containing mutation 1 (m1) can, via gdDNA-2, be used to introduce a second mutation (m2) thereby returning to the pMc type configuration.

Introduction of amber mutations into either one or the other gene makes it possible to use the markers alternatively in successive mutagenesis rounds in order to introduce mutations consecutively into a target sequence (Fig. 1). We have also constructed derivatives which contain transcriptional and translational regulatory signals. Such phasmids are of considerable interest for protein engineering studies since they avoid the necessity of shuttling between specialized mutagenesis and expression vectors. The *lacZ* gene was adopted as a model to demonstrate the effectiveness of the vector system.

MATERIALS AND METHODS

Enzymes and chemicals

Restriction enzymes were purchased from New England Biolabs or Boehringer. T4 DNA ligase, T4 polynucleotide kinase, *E. coli* DNA polymerase I-large fragment (Klenow enzyme), alkaline phosphatase and the deoxynucleotide triphosphates were obtained from Boehringer. Low-melting agarose and ONPG were purchased from Sigma.

Synthetic oligonucleotides

The 28bp double stranded *trpA* transcription terminator was purchased from Pharmacia. All mutagenic oligonucleotides were synthesised by the phosphoramidite method (9) on

Table I: List of *E. coli* K-12 strains used

Strain	Genetic characters	Source/ reference
WK6	*Δ(lac-proAB), galE, strA/F'lacI^q, ZΔM15, proA$^+$B$^+$*	19
WK6mutS	*Δ(lac-proAB), galE, strA, mutS*::Tn10/F'*lacI^q, ZΔM15, proA$^+$B$^+$*	19
K12ΔH1Δtrp	Sm^R, *lacZam, Δ(bio-uvrB), ΔtrpEA2* (λ*Nam7am53cI*857ΔH1)	20
K514	*thr*1, *leu*6, *thi*1, *supE*44, *lacY*1, *tonA*21, r$^-$m$^+$	21
QD5003	Hfr,*supF*	E. Remaut

an Applied Biosystems 380A DNA synthesizer. The oligonucleotides dGTTGAGTAGTCACCAG (underlined residue differs from the wild type sequence) and dCCAGCTAAACGGTC were designed for introduction of an amber nonsense codon in the *bla* and the *cat* gene, respectively. The EcoRI, BalI and NcoI sites present in the *cat* coding region were removed making use of the following oligonucleotides : dCCATACGAAATTCCGG (EcoRI), dCCATATTCGCCACG (BalI) and dGCCCATAGTGAAAACG (NcoI). The oligonucleotides used for construction of an amber mutation in *lacZ* (dGGTTTTCCTAGTCACG) and for reversion of this amber codon to the wild type codon (dGGTTTTCCCAGTCACG) have been described previously (6). The *bla* am-primer was enzymatically phosphorylated using γ-^{32}P-ATP (Amersham) and purified by preparative gel electrophoresis (10). The other oligonucleotides were purified by reversed phase high pressure liquid chromatography while the extent of 5'-phosphorylation was determined by chromatography as described (11).

Bacterial strains and plasmids

The strains used in this study are listed in Table I. These were routinely grown in LB medium (12). Strains WK6 and WK6mutS were, however, regularly streaked on minimal agar plates (12) to select for the *pro* marker present on the F'-episome. The antibiotics Ap and Cm were used at concentrations of 100μg/ml and 25μg/ml, respectively. The plasmid pcI857 has been described by Remaut *et al.* (13). This multicopy plasmid is compatible with the mutagenesis vectors described in this paper, specifies resistance to kanamycin (20μg/ml) and encodes a temperature sensitive repressor of the lambda P_L promoter. A *cI* wild type variant of this plasmid, designated pcIwt, was constructed using pKB280 (14) as a source of the wild type *cI* gene. The origin of replication of the mutagenesis vectors as well as the *bla* gene derive from pPLc28 (15). The plasmids pBR325 (16), pEMBL8(+) (8) and pPLcAT14-βgal (17) were used as a source of the *cat* gene, the f1 origin and the *lacZ* gene, respectively. The multicloning site (EcoRI-SmaI-BamHI-SalI-PstI-HindIII-XbaI) and the fragment containing the two copies of the phage fd transcription terminator in a tandem configuration were taken from pLK54 (18). P_L promoter containing vectors were propagated in the hosts listed in Table I after they were made lysogenic for phage lambda or after introduction of the pcI857 or pcIwt plasmid.

Construction of mutations

The steps involved in a single round of mutagenesis are depicted in Fig. 2. These are: *1. Preparation of single stranded DNA.* Overnight cultures of phasmid containing hosts were diluted 1:50 in fresh medium without antibiotics. They were grown to a density of about 2×10^8 cells/ml at 37°C and infected with phage f1 (strain IR1; 22) or M13KO7 (23) at a m.o.i. of 20. After a 5−16h incubation period, viral and pseudo-viral particles were recovered from the supernatant and the ssDNA was extracted essentially as described

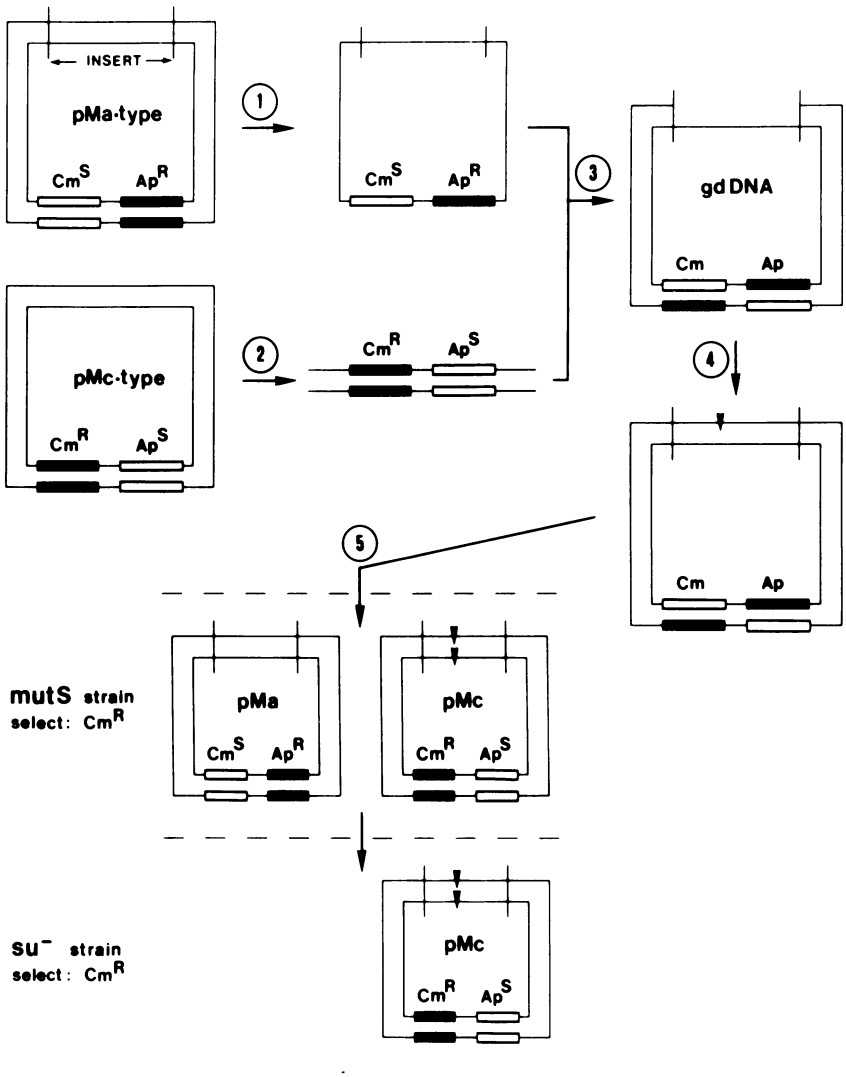

Figure 2: Schematic representation of a single mutagenesis round. For explanation of symbols see legend to Fig. 1. The various steps (1 to 5) are discussed in the text.

(6). The yield was determined by UV-spectroscopy ($\epsilon_{260nm}=2.86\times10^{-2}$ cm$^2/\mu$g). As judged by analysis of the ssDNA on agarose gels the ratio of ss phasmid over ss phage DNA is typically about 1 when using IR1 as helper. This ratio was significantly improved in cases where the helper was M13KO7. To convert P_L containing phasmids to the single stranded form strain WK6, lysogenic for phage λ or containing pcIwt, was used.

2. Preparation of DNA fragments. If necessary (see discussion), restriction fragments were recovered from low-melting agarose (24). The yield of fragment was quantitated on an ethidium bromide stained agarose gel by comparison of the band intensity with known amounts of DNA.

Table II: Efficiency of mutation construction utilising the alternative selectable markers.

Single stranded template	Double stranded fragment	Expected phenotype	Background (%)	Efficiency (%)
pMc5-βg14	pMa5-βg14 (BalI-MstII)	Lac^-	3	60
	pMa5-14 BamHI-linearised	Lac^-	15	67
	pMa5-14 BamHI/phosphatase	Lac^-	2	72
pMa5-βg14	pMc5-14 BamHI/phosphatase	Lac^-	3	75
pMc5-βg14am16	pMa5-βg14 (StyI-MstII)	Lac^+	0	65

The experiments were carried out as described in Materials and Methods. The background level was determined in experiments in which the mutagenic oligonucleotide was omitted. The efficiency is expressed as the percentage of colonies displaying the expected colour phenotype. For details on the fragment used in each individual experiment see text and Fig. 4. pMc5-βg14am16 designates a clone bearing the amber mutation in the *lacZ* coding region.

3. Construction of gdDNA. This step was carried out as described (6) or according to the following protocol. A 36μl aqueous mixture (containing less than 2mM salt!) of fragment (0.1pmol) and ssDNA (0.5pmol) was incubated at 70°C for 5min; then 4μl 10× hybridisation buffer (1.5M KCl/100mM Tris-HCl pH 7.5), also brought to 70°C, was added, after which the mixture was allowed to cool to room temperature. The latter procedure may offer an advantage in that it does not require heating of the DNA solution to 100°C, a condition which may cause hydrolytic deamination of 2'-deoxycytidine residues (25). Formation of gdDNA was monitored by electrophoresis of an aliquot of the mixture on an agarose gel. The mobility of gdDNA molecules of small gap size is practically indistinguishable from that of relaxed fully double stranded plasmid DNA.

4. Annealing of the mutagenic primer and DNA polymerase/ligase reaction. 5'-phosphorylated oligonucleotide (4 to 10pmol; typically about 2μl) was added to 8μl hybridisation mixture containing the gdDNA. This mixture was heated to 65°C for 5min and then allowed to cool to room temperature. 4μl 10× fill-in buffer (625mM KCl, 275mM Tris-HCl, 150mM $MgCl_2$, 20mM DTT, 0.5mM ATP and 0.25mM of each of the four dNTP's, pH 7.5), water to give a final volume of 40μl, 1 u DNA polymerase I (Klenow fragment) and 5 u T4 DNA ligase were added. The mixture was incubated at room temperature for 45min.

5. Transformation and segregation. The polymerase/ligase reaction mixture was used to transform WK6mutS (or a λ-lysogenic version in the case of P_L-phasmids). An aliquot of the transformation mixture was spread on selective media to determine the efficiency of transformation. In each of the experiments listed in Table II at least 1000 transformants were obtained. The remainder of the transformation mixture was used to inoculate 10ml of LB medium containing the appropriate antibiotic. After overnight growth, plasmid DNA was isolated using a rapid small-scale purification method (26) and used to transform a su^- strain, again selecting for the appropriate drug resistance. In the case of the *lacZ* mutagenesis experiment, transformants of strain K12ΔH1Δtrp or WK6(pcI857) were selected on Eosin Methylene Blue (EMB; Oxoid) indicator plates for determination of the LacZ phenotype. For calculation of the marker yield at least 1000 colonies were counted. Instead of performing a re-transformation, segregation of the mixed phasmid population

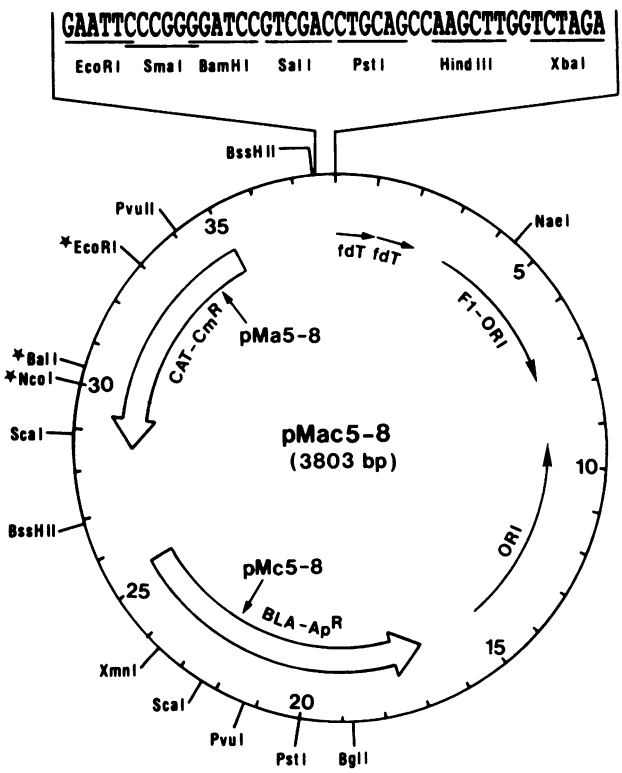

Figure 3: Restriction and genetic map of pMac5-8.
The positions of the most relevant restriction sites are indicated. The EcoRI, BalI and NcoI sites (marked with an asterisk) present in the wild type *cat* gene have been removed by site-directed mutagenesis (see Materials and Methods). The nucleotide sequence of the polylinker is shown above the map. The position of the β-lactamase coding region (BLA-Ap^R), the gene coding for chloramphenicol acetyl transferase (CAT-Cm^R), the origin of replication (ORI), the filamentous phage origin (F1-ORI) and the phage fd transcription termination signal (fdT) is indicated. In all cases the arrow denotes the functional orientation of the genetic element. The positions of the amber mutations present in pMc5-8 (the *bla-am* gene does not contain the ScaI site) and pMa5-8 (*cat-am*; the mutation eliminates the unique PvuII site) are shown.

obtained in the primary transformation may also be achieved by phasduction (see below); in this case, a phage/phasmid stock, prepared by infecting the transformation mixture with helper phage, is used to infect strain WK6 at a low m.o.i. after which antibiotic resistant transductants are selected (K. Friedrich, H. Kolmar and H.-J. Fritz, unpublished).

Induced synthesis of β-galactosidase—Phasduction

The conditions for thermo-induction of the P_L promoter have been described (15). Phasmids can be introduced into a cell not only by transformation but also by a mechanism analogous to phage infection: i.e. upon infection of a host cell with a pseudo-virion, the encapsidated ss phasmid DNA is capable of establishing itself as plasmid. We use the term 'phasduction' to denote such phasmid mediated transduction. We tested whether phasduction of bacteria permissive for expression (e.g. a cI^- strain) by a phasmid carrying a P_L-*lacZ* hybrid gene would result in high level synthesis of β-galactosidase. WK6 cells containing both pcIwt and pMa5-β*g*14 were infected with M13KO7 for production of ss phasmid

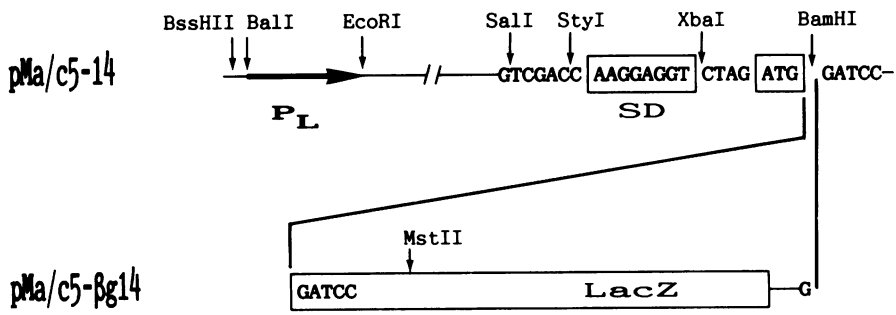

Figure 4: Schematic representation of the structure of pMa/c5-14 (upper panel) and pMa/c5-βg14 (lower panel). The mutagenesis/expression vector pMa/c5-14 (4145 bp) carries the phage lambda P_L promoter and a synthetic translation initiation region. The P_L promoter is shown by a heavy arrow indicating the direction of transcription. The sequence of the synthetic ribosome binding site is shown. The Shine-Dalgarno (SD) element and the ATG initiation codon are boxed. The cleavage sites of the relevant restriction enzymes are indicated. The expression signals are located between the BssHII site upstream of the *cat* gene and the BamHI site contained in the polylinker (see Fig. 3). In pMa/c5-βg14 the *lacZ* coding region is fused in phase with the ATG codon. The vector contains the *lacZ* information as a portable BamHI fragment.

containing particles (i.e. transducing particles). The infection was carried out as noted above. To determine the titer of transducing particles, aliquots of diluted culture supernatant were added to 1ml of a 1:10 diluted fresh overnight culture of WK6(λ). After a 30min incubation at 37°C, the cells were spread on selective plates. The transducing particle titer was scored as the number of colony forming units (Ap^R transductants) per ml. For induction of β-galactosidase synthesis concomitant with phasduction, a ratio of pseudo-virions/recipient WK6 cells of about 10 was used; the cells were infected at a density of about 2×10^8/ml and no antibiotic was added to select the presence of the phasmid. To detect induced β-galactosidase protein, total cellular extracts were fractionated on SDS-10% polyacrylamide gel (27). Proteins were stained with Coomassie Brilliant Blue. Alternatively, β-galactosidase enzymatic activity was assayed by ONPG hydrolysis (17). One unit is defined as the enzymatic activity which produces 1nmol o-nitrophenol/min at 28°C.

Introduction of amber mutations into the bla and cat genes

Codon 103 of the *bla* gene (TAC; tyrosine) and codon 38 (CAG; glutamine) of the *cat* gene were converted to amber codons by oligonucleotide directed mutagenesis essentially as described above. The amber mutations destroy the ScaI and PvuII sites which are present in the *bla* and *cat* gene, respectively. The presence of the intended mutations was confirmed by DNA sequence analysis (28). We have checked the resistance level of the respective amber mutants in *supF* (strain QD5003) and *supE* (K514) backgrounds. A UAG is read as a tyrosine codon by the supF tRNA while the supE amber suppressor inserts a glutamine residue. The *bla-am* gene was found to confer resistance to at least 100μg/ml Ap in a *supF* host and to about 20μg/ml in the *supE* strain. Both the *supE* and *supF* host were resistant to 25μg/ml Cm following transformation with phasmids containing the *cat-am* marker.

Removal of restriction sites within the cat gene

So as to retain maximum flexibility in designing useful polylinker modules, we have removed certain restriction sites from the rest of the vector. Oligonucleotide mediated mutagenesis (see above) was used to destroy the NcoI, BalI and EcoRI sites of the *cat* gene without changing the encoded amino acid sequence. These three mutations were then

combined in a single phasmid in a marker salvage type of experiment (29). Purified restriction fragments containing the respective mutations as well as an additional fragment carrying the wild type Ap marker were annealed to pMc type (Cm^RAp^S) single stranded DNA. After strand segregation of the resultant gdDNA in a *mutS* strain, Ap^RCm^R transformants of a su^- strain were selected. Eight out of ten clones were found to contain the three mutations.

RESULTS

Description of the basic mutagenesis vector

A map of the basic mutagenesis vector, designated pMac5-8, is shown in Fig. 3. The vector contains the following relevant features:

(i) a ColE1 type origin of replication. (ii) the intercistronic region of filamentous phage f1. This region, including the origin of replication and a morphogenetic signal, was cloned as a 456 bp fragment (nucleotides 5488–5943 of the f1 genome). Our data (results not shown) and those of Levinson *et al.* (30) confirm that this fragment contains all the information required for efficient production of pseudo-virions. In pMac5-8 the orientation of the f1 origin is such that the DNA strand complementary to the mRNA of both the *bla* and *cat* genes is encapsidated and secreted into the culture medium. To allow control over which strand becomes packaged, we have also constructed vectors bearing the f1 origin in the opposite direction. (iii) the β-lactamase gene (*bla*) which confers resistance to 100μg/ml ampicillin. (iv) the chloramphenicol acetyl transferase (*cat*) gene. The EcoRI, BalI and NcoI sites present in the wild type *cat* gene have been removed by oligonucleotide-directed mutagenesis. To this end, point mutations which create synonymous codons were introduced. Downstream of the *cat* coding region two copies, in tandem, of a 28bp synthetic transcription terminator (see Materials and Methods) were inserted. Presence of these terminators was found to abolish the spontaneous integration of an IS1 insertion element in this region of the vector which was occasionally observed (to be published in detail elsewhere). The engineered *cat* gene was found to confer resistance to at least 25μg/ml chloramphenicol. (v) a polylinker providing convenient sites for insertion of target sequences. (vi) two copies, in tandem, of the central transcription terminator of phage fd (fdT).

The nucleotide sequences across the junctions between the various segments were determined. By combining these data with published sequences, the exact size (3803 bp) and the complete nucleotide sequence of the vector were deduced. To allow selection in each separate mutagenesis round, single amber mutations have been introduced into both the *bla* and *cat* genes. The resultant phasmids have been designated pMc5-8 and pMa5-8. The vector pMc5-8 confers resistance to chloramphenicol and contains an amber mutation in the *bla* gene. The complementary phasmid, pMa5-8, confers ampicillin resistance and harbours a *cat-am* gene. pMac5-8 refers to the vector wild type for both genes. The phasmids also differ at the restriction level; the amber mutation in the Ap^R gene eliminates one of the two ScaI sites present on pMac5-8; similarly, the *cat-am* mutation destroys the unique PvuII site (see Fig. 3 and Materials and Methods).

Introduction of expression signals and synthesis of β-galactosidase

Phasmids pMa5-8 and pMc5-8 represent the two complementary vectors of the basic mutagenesis system. Both vectors contain a multilinker region which provides a ready means for insertion of target sequences for mutagenesis. By inserting an expression module

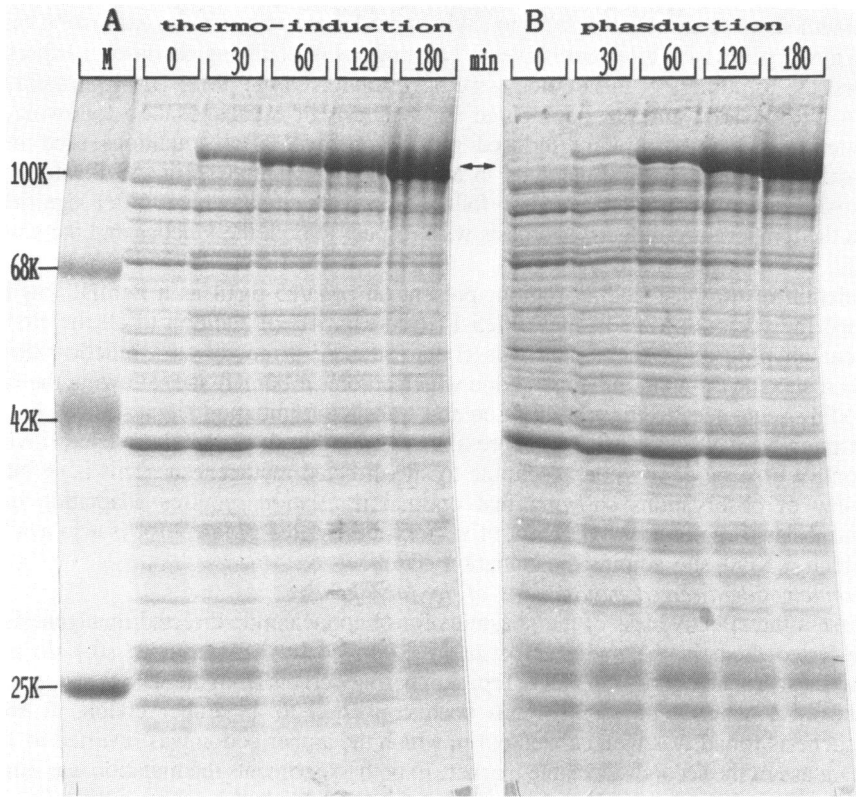

Figure 5: pMa5-βg14 directed β-galactosidase synthesis following temperature induction (A) *versus* phasduction (B). In both experiments samples were collected at various times (indicated above the lanes) after induction and boiled in SDS before electrophoresis. Each lane contains the equivalent of 2×10^8 cells. The thermo-induced synthesis was monitored in WK6 cells containing pcI857. In the phasduction experiment, WK6 was used as the recipient host. As molecular weight standards (M), we used a set of prestained proteins (Bethesda Research Laboratories). An arrow indicates the position of β-galactosidase.

into these phasmids, the functions of mutagenesis and gene expression (protein overproduction) are combined in a single replicon.

To demonstrate the usefulness of the vectors for expression, we have transferred the P_L-*lacZ* hybrid gene of pPLcAT14-βgal (17) onto pMa/c5-8. Upon induction, the resultant recombinants, pMc5-βg14 and pMa5-βg14 (Fig. 4), were found to direct efficient synthesis of β-galactosidase, as shown by the appearance of a new protein band of the expected molecular weight on an SDS-polyacrylamide gel (Fig. 5A). A concurrent dramatic increase in β-galactosidase enzymatic activity was observed. Extracts of cultures containing either pMc5-βg14 or pMa5-βg14 were found to contain similar amounts of enzyme. After a 2h induction period the activities ranged from 3810 to 4050 u/ml/OD_{650} unit. An uninduced control culture contains about 30 u/ml/OD_{650} unit. From this result we conclude that pMc and pMa type phasmids are equally useful for expression purposes.

Phasmids can be moved into another genetic background by phasduction (see Materials

and Methods). A logical extension is to exploit phasduction to convey an expression cassette from a non-permissive to a permissive host as a means of obtaining conditional expression. To test the usefulness of this strategy, we have tranferred the pMa5-βg14 phasmid from cI^+ to cI^- bacteria and have compared the synthesis of β-galactosidase following such transfer with the temperature induced production level. The conditions used in this phasduction experiment are desribed in Materials and Methods. Fig. 5B illustrates that the overproduction of β-galactosidase following phasduction does not differ significantly from the temperature induced synthesis with respect to both the kinetics and the absolute levels.

Deletion of the *lacZ* coding region, present on pMa/c5-βg14 as a BamHI fragment, yielded the vectors pMa5-14 and pMc5-14 (see Fig. 4). In addition to all the elements present on pMa/c5-8, these phasmids contain the P_L promoter, a synthetic ribosome binding site and an ATG initiation codon which adjoins a BamHI site, allowing the fusion of coding sequences to the transcription and translation initiation signals. The phasmids have the advantage over existing expression vectors that they make sequences involved in control of gene expression accessible to site-directed mutagenesis. This is of interest in view of observations showing that optimal translation requires adaptation of the 5'-untranslated region (17,31). A set of vectors, designated pMa/c5-10, is also available in which a KpnI site adjoins the initiation codon.

Oligonucleotide-directed mutagenesis of the lacZ gene

To assess the effectiveness of the phasmids for oligonucleotide-directed mutagenesis, we have tested them in the model experiment described by Kramer *et al.* (6). In a first mutagenesis round codon 16 (TGG, Trp: numbering derives from the wild type *lacZ* gene) of the *lacZ* gene of pMc5-βg14 has been converted to an amber codon. A second mutagenesis round was then carried out in which the amber codon was reverted to TGG, making use of the second selectable marker. In both experiments the mutation was directed by a synthetic hexadecanucleotide (see Materials and Methods). The easily discernable $LacZ^+$ and $LacZ^-$ phenotypes provide a means of determining the mutagenesis efficiency in each round.

Both pMa and pMc type phasmids were tested in construction of the am16 mutation in order to evaluate the equivalence of the two selectable markers in recovery of mutations. Restoration of the wild type sequence from the am16 mutant was used to demonstrate that the vectors permit sequential mutagenesis rounds to be undertaken. The two complementary mutagenesis experiments cannot be considered equivalent since the desired phenotype ($LacZ^-$) in the first experiment may also derive from factors other than controlled mutation construction. Unwanted mutations outside the target region which eliminate β-galactosidase activity may occasionally arise as a result of the infidelity of DNA polymerase. During the Klenow polymerase/T4 ligase treatment, circularisation of double stranded fragment remaining after gdDNA formation may occur. Such molecules, since they encode resistance to the antibiotic used for selection of transformants, will also give rise to progeny displaying the $LacZ^-$ phenotype. We therefore measured the background level of white and red colonies in the first and second round respectively in experiments in which no oligonucleotide primer was included.

Three different fragments were used for preparation of gdDNA. Using the large BalI-MstII fragment of pMa5-βg14 results in a gap of about 500 nucleotides encompassing the P_L promoter, the 5'-untranslated region and the first 73 codons of the *lacZ* gene. Use of a StyI-MstII frament gives a gap starting 13 nucleotides upstream of the *lacZ* coding

region. Using pMa5-14 or pMc5-14 cleaved with BamHI creates a gap which spans the entire *lacZ* coding region (see Fig. 4). The BamHI fragment has been used both as such and after dephosphorylation. The results of the various experiments are shown in Table II.

DISCUSSION

We present a series of phasmid vectors for the efficient construction of oligonucleotide-directed mutations. The recovery of mutations relies on the strategy of marker linked mutagenesis, as described by Kramer *et al.* (6). The twin vector system consists of the following phasmids: one conferring Ap resistance and harbouring an amber mutation in the *cat* gene (pMa type; $Cm^S Ap^R$), the complementary version conferring Cm resistance and harbouring an amber mutation in the *bla* gene (pMc type; $Cm^R Ap^S$). This allows gapped duplex DNA consisting of a gapped strand containing a wild type antibiotic resistance gene and a template strand containing the amber mutant version of that same gene to be constructed. By transformation of a non-suppressing host strain to antibiotic resistance, selection for progeny deriving from the gapped strand, to which the mutagenic oligonucleotide is enzymatically linked, is imposed. To preserve the physical linkage between the desired mutation and the vector borne markers, the enzymatically sealed heteroduplex derived from the gdDNA/oligonucleotide structure is first introduced in a mismatch repair deficient (*mutS*) host, giving rise to a mixed phasmid population. An important feature incorporated in the phasmids is the presence of two alternative selectable markers in such a way that an amber marker is retained after each mutational step. Thus, multiple rounds of mutagenesis, involving alternate selection for resistance to Ap and Cm, can be undertaken.

Plasmids are the vectors that are commonly used for the synthesis of quantities of foreign proteins in *E. coli*. The most obvious advantage of an efficient phasmid mutagenesis technique is that it allows the modification of coding sequences within their respective expression vectors without further manipulations. The amounts of β-galactosidase directed by *lacZ* bearing phasmids indicate that none of the features of the present vector system prohibits high level expression. The various strains used in mutagenesis experiments have been adapted for use with P_L containing phasmids. Presence of a lambda prophage or of the auxiliary plasmids pcIwt or pcI857 make it possible to exert control over the activity of P_L. Phasmids are maintained in cI^+ host strains during mutagenesis so as to keep the lambda promoter repressed. Maximal induction of P_L, leading to overproduction of the wild type or mutated gene product, is obtained at 42°C in strains which provide *in trans* thermolabile repressor (*cI*857). Note that the $lacI^q$ strains WK6 and WK6mutS can also be used in combination with phasmids containing IPTG-inducible promoters. Our results demonstrate that introduction of phasmids into a permissive host by phasduction may be used as an alternative method to induce overexpression of a cloned gene. In the case of the P_L promoter, this approach allows expression to be achieved at 37°C (or even lower temperatures) which may in particular cases offer an advantage over incubation at 42°C (32). More importantly, a phasduction is accompanied by a change in genotype and may therefore offer opportunities for the design of alternative conditional expression systems. These may provide a much more rigorous control over expression by comparison with systems in which conditional synthesis results from the induction of a phenotypic change during growth of the host cells. For example, we have used phasduction from su^- to su^+ cells for the expression of an amber mutant version of an RNase gene; an expression cassette containing the wild type gene could not be assembled, probably because even under

repressed conditions the level of enzymatically active RNase is toxic to *E. coli* (unpublished data).

As a test for the effectiveness of the phasmids in mutation construction, we have converted *lac* codon 16 to an amber. This model experiment was chosen because a LacZ$^-$ phenotype is readily visualised on indicator plates, allowing accurate determination of the yield of mutant. The results listed in Table II demonstrate that we were successful in harvesting the *lac* am16 mutation with a high efficiency. They also show that the two alternative selectable markers are equally useful, i.e. there is no significant difference in the yield with a pMa or pMc type 'selection strand'. Furthermore, it would appear that single stranded DNA preparations can be used as such without interference from the helper viral genome; thus, no purification of individual DNA species is required at any stage of the *in vitro* manipulations. Given the high efficiency with which mutations can be constructed, direct screening of the candidate mutants by sequence determination becomes realistic.

The experiment in which pMa5-14, opened with BamHI enzyme, was used as fragment reveals one possible source of unwanted background. All of the 24 'background clones' that have been characterised by restriction analysis were found to contain the pMa5-14 phasmid (data not shown). The greater part of this background must, therefore, result from efficient recircularisation of remaining fragment during the gap filling and sealing reaction. We expect that this phenomenon will, to a variable extent, always arise when fragments with cohesive ends are used. The data presented in Table II show that in problematic cases dephosphorylation is an effective countermeasure. The background is also rendered insignificant when fragments with two different ends are used (Table II and data not presented here). Presumably, this is because religation of such fragments, which requires that sticky ends are converted to blunt ends by the Klenow polymerase, is not efficient under the conditions used. The two different cleavage sites used for preparation of the fragment may be located on the insert. This option offers the advantage that small gaps can be generated but requires that both a pMa and pMc type recombinant be constructed. The second route consists of cleaving the parental vector with the restriction enzymes used for cloning the target DNA and eliminates the need to purify the double stranded fragment.

The am16 mutation was, in a second round of mutagenesis, reverted to the wild type codon. Analysis of the mutants obtained after one round revealed that not all of them had retained the *bla-am* (or alternatively, *cat-am*) marker, a prerequisite for consecutive rounds of mutagenesis to be undertaken. We found that a small number of such double resistant clones are actually double transformants (i.e. both a pMa and pMc type phasmid is present); the majority, however, contain a single Ap^{R}Cm^R (pMac) phasmid. This phenomenon of marker dislinkage can in part be accounted for by the presence of nicks in the fragment used for construction of gdDNA; single stranded breaks in the minus strand of the fragment result in the formation of gapped strands which are (much) smaller than expected, including ones that lack the *bla-am* or *cat-am* marker. Marker scrambling may also be attributed to MutS-independent repair and homologous recombination (33). Practically, loss of the amber mutation is not a serious concern, since it is easy to verify the antibiotic resistance phenotype of any one mutant.

In addition to the model experiment described in this paper, the present mutagenesis vectors have been used for introduction of several hundred mutations in a variety of target sequences. These experiments constitute a sample from which statistically more significant mutant frequencies can be calculated. In 65% of the experiments mutants were recovered

with an efficiency of ≥ 30%, in 30% of the experiments mutations were obtained at frequencies of between 30% and 5% while in a few cases the yield dropped below 5%. The average efficiency was on the order of 40 to 45%.

Recently, some further developments of the gdDNA approach to oligonucleotide mutagenesis have been reported (25,34,35). The procedures put forward there can also be applied to the pMac family of vectors.

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ABBREVIATIONS

Ap: Ampicillin, Cm: Chloramphenicol, bp: base pairs, ss: single stranded, gdDNA: gapped duplex DNA, am: amber, m.o.i.: multiplicity of infection, SDS: sodium dodecylsulphate, ONPG: o-nitrophenyl-β-D-galactopyranoside.

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