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The phasmid as a tool for plasmid genetics

I. Fine structure of the β-lactamase gene

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SUMMARY

The reversible integration of a plasmid into a phage genome via *int*-mediated recombination permits rapid and facile analysis of plasmid genes. Seventy-six independent mutants in the *β-lactamase* (*bla*) gene have been isolated and genetically mapped. The end points of deletions generated by partial digestion with restriction enzymes have been identified and this, together with the assignment of some nonsense mutations to specific amino acid residues, allows alignment of the genetic map with the DNA sequence.

INTRODUCTION

Small multicopy plasmids are invaluable tools for investigating many biological phenomena. First, they are ideal for the study of replication of supercoiled DNA both *in vivo* (Bazara & Helinsky, 1970; Oka & Inselburg, 1975) and *in vitro* (Sakakibara & Tomizawa, 1974; Tomizawa, Sakakibara & Kakefuda, 1975). Secondly gene expression can be analysed in the absence of a background of chromosomal transcription and translation by segregation of the plasmid into minicells (Levy, 1971; Roozen, Fenwick of Curtis, 1971). Thirdly, the possibility of easily purifying large amounts of supercoiled DNA is useful for biochemical and DNA sequencing experiments.

Using *in vitro* DNA recombination methods it is now possible to join prokaryotic or eukaryotic genes to plasmid replicons (for review see Murray, 1978). This allows the application of plasmid techniques to the study of the expression of cloned genes, and the amplification of their gene products. Unfortunately, the versatility of plasmids for biochemical analysis is not matched by an equivalent ease of genetic manipulation. Small plasmids are usually not easily transferable and their large copy number makes the segregation of homogeneous clones after mutagenesis or mixed infection very time-consuming. It is therefore especially difficult to isolate

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pure clones of plasmid mutants that are recessive or have a slight selective disadvantage with respect to the wild type. Some of these problems have been circumvented by the use of transposons which can alter plasmid functions by insertion and which can be selected for by antibiotic resistance markers (Inselburg, 1977; Dougan & Sherratt, 1977). Mutations can also be produced *in vitro* by genetic manipulations (Backman *et al.* 1978; Collins, Yanofsky & Helinski, 1978) but mutant plasmids need to be introduced into bacteria by transformation. The inefficiency of transformation hinders genetic mapping and complementation analysis because of the difficulty of obtaining bacterial cells heterozygous for two mutants with a controlled number of each plasmid. It has so far not been possible to study plasmid functions by the classical genetic techniques of isolation of point mutants and fine-structure mapping.

We have shown that a plasmid can be reversibly integrated into a phage genome by the *int*-mediated site-specific recombination system (Brenner *et al.* 1982). The resulting 'phasmids' offer the possibility of applying the powerful tools of genetics to the study of plasmid genes. Mutants can be isolated, characterized and mapped in the phasmid structure. Whenever the plasmid structure is more convenient, either to study the physiology of the mutants or to purify the DNA, the mutant plasmid can be released from the phage chromosome.

The paper describes the genetic analysis of the β-lactamase gene by these methods. This gene was chosen because its product is secreted into the periplasmic space, and we were interested in a detailed analysis of mutations that might affect this process. A comparison of the sequence of this gene in pBR322 (Sutcliffe, 1978) with the amino acid sequence of a related ampicillinase (Ambler & Scott, 1978) suggested that the primary translation product would be larger by 23 amino acids at the N terminus. Such a precursor has been identified *in vivo* in bacterial minicells (Achtman *et al.* 1979) and some of the mutants described here have been shown to affect its processing.

MATERIALS AND METHODS

Chemicals, enzymes, media and buffers

Chemicals and enzymes and their sources were as follows: Restriction endonucleases *Eco*RI, *Bam*HI *Hind*III and *Pst*I from Boehringer; *Hin*fI, *Sau*3a, *Mbo*II from Biolab, *Taq*I was a gift of the late I. Harris; T4 DNA ligase from Miles; S1 endonuclease was a gift of Dr. Jonathan Karn. A sample of the chromogenic cephalosporin Nitrocefin was generously provided by Glaxo.

General microbiological techniques

Most of the techniques used are described by J. Miller (1972). Phage crosses were carried out by infecting 0·2 ml of a culture of exponential-phase EQ82 in CY broth (approximately 4×10^8) with both phages at a multiplicity of 5. After 20 min adsorption at room temperature the infected bacteria were diluted tenfold,

incubated for 2 h at 37 °C with shaking and then lysed with chloroform. Combinations of β-lactamase mutants and λ genetic markers were obtained by standard genetic crosses using the phages described in Brenner *et al.* (1982) and appropriate selections.

Replica plating techniques for lifting and release of plasmids

The selective integration of a plasmid into a phage chromosome by 'lifting' crosses has already been described (Brenner *et al.* 1982). This can be accomplished by inserting the λ *att* site into a multicopy plasmid. By crossing appropriate phages in a bacterial strain harbouring the *att* plasmid it is possible to select for recombinant phages which carry an integrated plasmid. The 'lifting phages' contain a short arm balanced by an appropriate long arm to ensure viability. Under normal circumstances the small recombinant carrying the two short arms is not found when such phages are crossed. However, if the host cell contains a plasmid with an *att* site and if appropriate integration functions are provided, then viable recombinants appear in which the length of the phage is extended by the integration of one or more plasmids. We refer to this tri-parental site-specific recombination event as 'lifting' and call the parental phages 'lifting phages'. The phasmids carry multiple *att* sites located at the ends of the plasmids. Recombination at these *att* sites may be exploited to effect 'release' of phasmids, reversing the original integration.

For the present work, replica plating techniques were devised for lifting and releasing large numbers of clones on the same plate. A plate with 50–200 colonies was replica plated on to a second plate spread with 10^8 of each of two appropriate lifting phages. After incubation for 5 h at 37 °C, this plate was replica plated on to a third plate spread with a lawn of the selective strain WY21 (Brenner *et al.* 1982) in 3 ml of top agar and incubated overnight at 37 °C. An area of lysis of the selective strain is only produced by colonies which contained a plasmid with an *att* site. The phasmids were purified on strain Q1 to select against possible contamination by the *h*80 prophage in WY21, and then grown into stocks.

Release of a large number of plasmid clones was achieved by replicating plates containing 100–300 phasmid plaques on to a lawn of the releasing strain EQ84 in 3 ml top agar overlay. Depending on the selection, the plate contains either 100 μg/ml of ampicillin or an excess of colicin E1. After overnight incubation at 37 °C bacterial growth corresponds to plaques of phasmids carrying a releasable genetic determinant for resistance to the reagent used. The DNA of the excised plasmid can be prepared from liquid cultures of these clones without further purification.

Mutagenesis

N-Methyl-*N*-nitro-*N*-nitrosoguanidine (NG), ethyl methane sulphonate (EMS) and ultraviolet light (UV) mutageneses were by the methods of Miller (1972). After

mutagenesis, the culture was diluted 10^{-2}, and 100 fractions dispensed into tubes to generate a set of independent cultures. After 3 h at 37 °C the cultures were lysed with chloroform. Not more than one mutant of a given phenotype was isolated from each culture. The effectiveness of the mutagenic treatment was checked by measuring the enhancement of plating efficiency on a *gro*P strain (Georgopulos & Herskowitz, 1971). For 2-aminopurine (AP) mutagenesis, phages from a single plaque of the phasmid mutant to be reverted were inoculated into 0·2 ml of a fresh saturated culture of EQ82. After 20 min adsorption at room temperature 5 ml of CY broth supplemented with 5 mM-$MgSO_4$ and 0·6 mg/ml of AP were added. The infected culture was shaken at 37 °C until lysis occurred.

Identification of β-lactamase mutants

β-lactamase (*bla*) mutants were identified by one of two methods. In the first method, mutagenized phasmid stocks were plated on a lawn of CA 274 to give about 400 plaques per plate. After overnight incubation at 40 °C the plates were covered with 0·5 ml of Nitrocefin dissolved in 0·1 M phosphate buffer pH 7·4 at a concentration of 0·5 mg/ml. After about 30 s the plates were examined against a white background. Wild type, *bla*$^+$, plaques stain red (O'Callaghan *et al.* 1972) because *β*-lactamase hydrolyzes the chromogenic cephalosporin. Plaques which do not contain the enyzme can be identified by the absence of the colour reaction.

In the second method, phasmids were screened for their ability to confer ampicillin resistance to a host bacterium. This screening method was devised to identify those mutants which are unable to confer ampicillin resistance because of an altered location in the cell of the *β*-lactamase enzyme, but which are still able to hydrolyse the cephalosporin substrate when bacteria are lysed in plaques. About 500 mutagenized phasmids were plated on a lawn of mixed indicator containing 0·1 ml each of fresh saturated cultures of CA274 and the *int*-constitutive releasing strain EQ84. Phasmids grow on CA274 and generate plaques but infection of the *int*-constitutive strain EQ84 leads to plasmid release within that strain. After overnight incubation at 32 °C the turbid plaques are replica plated on to plates with 100 *μ*g/ml of ampicillin. After overnight growth at 40 °C the replicas were screened for bacterial growth. A missing patch corresponded to a plaque with a putative *bla* mutation; such plaques were purified and checked in the same way. *Col*E1-sensitive mutants were isolated using the same release method.

Suppression and reversion

The suppressibility of the 76 *β*-lactamase mutants isolated was tested in two ways. The phasmid mutants ordered on a grid were replica plated on to eight suppressor strains (Table 1). After overnight growth at 40 °C the areas of lysis were assayed by overlaying the plates with a solution of nitrocephin. Semi-quantitative results were obtained this way. Alternatively, the master plate was replica plated

Table 1. *Bacterial strains*

Strain	Genotype	Source
CA274	Hfr C *lac-125 trp*(amber)	Cambridge collection
EQ82	F⁻ r_K^- m_K^+ *supE supF met*	Cambridge collection
WY21	EQ82 (*ϕ80 att80 i81 c*⁺ *Sam* S_7) $\phi 80^r$	Cambridge collection
Q1	*thr leu lac supE* $\phi 80^r$	Cambridge collection
EQ84	HfrH *galT* λV (*int*-FII) (λ*c*⁺ *int*C226)	Enquist LE122
XA101	Δ (*lac-pro*) *nalA metB argE*(amber) *rif thi supD*	Miller *et al.* (1977)
XA102	Δ (*lac-pro*) *nalA metB argE*(amber) *rif thi supE*	Miller *et al.* (1977)
XA103	Δ (*lac-pro*) *nalA metB argE*(amber) *rif thi supF*	Miller *et al.* (1977)
XA105	Δ (*lac-pro*) *nalA metB argE*(amber) *rif thi sup5*	Miller *et al.* (1977)
XA106	Δ (*lac-pro*) *nalA metB argE*(amber) *rif thi sup6*	Miller *et al.* (1977)
XA10B	Δ (*lac-pro*) *nalA metB argE*(amber) *rif thi supB*	Miller *et al.* (1977)
XA10C	Δ (*lac-pro*) *nalA metB argE*(amber) *rif thi supC*	Miller *et al.* (1977)
T9OC	*ara* Δ (*lac-pro*) *mutT thi* *sup*UGA	Miller *et al.* (1977)
CAJ101	F⁻ Δ (*lac-pro*)	Cambridge collection
*GC*40	*CA*274 λ^r	Cambridge collection
Q202	F⁻ $groP_{A15}$ *thr leu thi supE* $\phi 80^r$	Cambridge collection
Q214	Q202 434^r	Cambridge collection

on to a lawn containing a triple indicator composed of 0·1 ml of each of CA274, a streptomycin-resistant derivative of EQ84 and an *int*-constitutive derivative of a suppressor strain. After overnight incubation at 32 °C the plate replica plated on to two further plates containing respectively 100 μg/ml of ampicillin and 100 μg/ml of ampicillin plus 20 μg/ml of streptomycin. Suppressed mutants grew on the first plate but not the second. Plates with different amounts of ampicillin were used to assess the efficiency of suppression. Similar results were obtained with the two methods.

The reversion frequency of a mutant phasmid was measured by singly infecting an EQ84 culture grown in CY + 0·5 mM-$MgSO_4$ to O.D. 0·3 at 600 nm. After 1 h incubation at 32 °C dilutions of the infected bacteria were spread on plates containing ampicillin (300 μg/ml) or colicin E1 and these were incubated overnight at 37 °C. The reversion frequency is the ratio between the concentrations of ampicillin-resistant and colicin-immune bacteria.

Construction of deletions

Deletions generated by *Sau*3a, *Pst*I and *Hind*II restriction enzymes were obtained in the following way. One microgram of pacI29 DNA in 20 μl of *Hind* buffer was incubated at 37 °C with restriction endonuclease. Reaction conditions were chosen to produce an average of one cut per molecule of plasmid. The enzyme was inactivated by heating for 5 min at 70 °C and the cleaved plasmid DNA ligated for 30 min at 37 °C in 100 μl of ligase buffer with 0·1 Weiss units of T4 DNA ligase. Digestions with *Taq*I were at 60 °C and the enzyme was inactivated by phenol

extraction, and after ethanol precipitation the DNA was ligated as above. Endonucleases *Mbo*II and *Hph*I cut DNA some distance from their recognition site and do not leave a constant set of protruding ends. The ends were therefore removed by S1 digestion before ligation. Partially digested plasmid DNA was treated with an excess of S1 for 30 min at 37 °C in 30 μl of S1 buffer. Blunt-end ligation required approximately 10 times more ligase than used for sticky end ligation. The plasmids were recovered by transformation of EQ82 cells made competent according to Lederberg & Cohen (1974). After incubation for 30 min at 37 °C, aliquots were plated on colicin E1 plates, and incubated overnight at 37 °C. Colicin immune clones were checked for the presence of an intact β-lactamase gene by replica-plating on to ampicillin (100 μg/ml) plates. Ampicillin-sensitive clones were checked for their ability to rescue either the marker *bla-1* or *bla-173*. Deletions that could rescue at least one of the two markers were purified and tested for the *att* site by replica plate lifting. Only 3 out of 14 tested were positive in this test. The phasmids containing the three lifted deletions were purified and used for deletion mapping by phage crosses.

Deletion mapping

Two replica plate techniques were developed to allow rapid screening of ampicillin-resistant mutants. Bacteria containing the deletions were ordered on a grid and replica plated on to a plate on which 10^8 plaque-forming units (p.f.u.) of a given phasmid mutant had previously been spread. After 5 h at 37 °C the plates containing the lysed colonies were replicated on to ampicillin plates (300 μg/ml) overlaid with 3 ml of top agar containing 0·2 ml of a stationary-phase culture of EQ84. After overnight incubation at 37 °C an area of bacterial growth appeared only in the position of those clones containing deletions that did not overlap the mutation in the phasmid β-lactamase gene.

The second technique allowed screening of mutants that were rescued by a given deletion. Fifty to one hundred β-lactamase mutants were streaked on a plate overlaid with a lawn of bacteria harbouring a plasmid with a given deletion. After overnight growth at 37 °C, the plates were replicated on to ampicillin plates (300 μg/ml) overlaid with EQ84 in 3 ml of top agar. Mutations not overlapped by the deletion gave an area of bacterial growth on the ampicillin plate.

Restriction endonuclease mapping of deletion mutants

The 14 restriction enzyme-generated deletions were characterized by restriction enzyme mapping. The DNA was purified from 100 ml of chloramphenicol-amplified cultrues as described by Clewell & Helinski (1969). The approximate lengths of the deleted plasmids were obtained by measuring the mobilities of the DNAs in 1% agarose gels after linearizing by digestion with the restriction enzyme *Eco*RI. None of the plasmids had lost the *Eco*RI site. Further characterization was carried out by digesting with other restriction enzymes and analysis by 15% acrylamide–bisacrylamide gel electrophoresis.

Table 2. *Mutation frequencies*

Mutagen	Growth on *groP*	Phenotype *bla*⁻	Phenotype *col*⁻
NG	2×10^{-4}	9×10^{-4}	3×10^{-4}
number of mutants		69(10)	6 (1?)
UV	1×10^{-5}	3×10^{-4}	10^{-4}
number of mutants		7(0)	4(0)

Mutation frequencies are expressed as the total number of mutants divided by the number of plaque-forming units. In parentheses are the number of mutants suppressible by one of the suppressor strains listed in Table 1.

RESULTS

Isolation of β-lactamase mutants

Mutations in plasmid genes are readily isolated in phasmids, given methods to identify phenotypes in plaques. Table 2 shows the frequency of *bla* mutants in phasmid $\phi 1$ (*hλ b*189 < pacI29 < Δ[*int-c*III] *c*I857 *chi*3) after mutagenesis with nitrosoguanidine or by ultraviolet irradiation. For comparison the frequency of mutants in the colicin E1 immunity gene is also shown. Most of the *bla* mutants were detected using the cephalosporin dye test on plaques, but 23 were isolated by their failure to confer ampicillin resistance on a host bacterium. All of these were also negative in the plaque dye test, suggesting that the latter assay does not miss a significant fraction of mutants.

Ten out of the seventy-six mutants were found to be suppressible by at least one of the eight suppressors listed in Table 1. Six of the mutants were suppressed both by amber and ochre suppressors and are therefore UAG mutants. *bla-144* and *bla-136* were suppressed only by UGA supressors and *bla-35* and *bla-525* only by UAA suppressors (Table 3).

Reversion analysis

All the mutants tested reverted spontaneously at frequencies of the order of 10^{-8}, which corresponds to the reversion rate expected for single base substitutions (data not shown). Reversion was strongly enhanced by 2-aminopurine mutagenesis. EMS induction of reversion was found for only a few mutants. This is as expected if most of the forward mutations were GC–AT transitions.

Mapping bla *mutants by four-point crosses*

The relative positions of the *bla* mutations were determined by crossing phasmids carrying *bla* mutations and appropriate outside markers. The strategy used is illutrated in Fig. 1. Recombinants between the host range and immunity markers were selected and the frequency of recombinant phasmids carrying a

Table 3. *Suppression of nonsense mutants of β-lactamase*

Mutant	*supD* Amber Ser	*supE* Amber Gln	*supF* Amber Tyr	*su5* Ochre Lys	*su6* Amber Leu	*supB* Ochre Gln	*supC* Ochre Tyr	UGA Trp	*sup*$^+$
pacl29 (wild type)	+++	+++	+++	+++	+++	+++	+++	+++	+++
bla-1	++	++	++	+	++	+	+	−	−
bla-3	++	+	++	+	++	+	+	−	−
bla-322	−	−	+	−	−	−	−	−	−
bla-35	−	−	−	+	−	+	+	−	−
bla-525	−	−	−	+	−	+	+	−	−
bla-136	−	−	−	−	−	−	−	+	−
bla-144	−	−	−	−	−	−	−	+	−
bla-21	−	−	+	−	−	−	+	−	−
bla-76	++	++	++	+	++	+	+	−	−
bla-72	++	++	++	+	++	+	+	−	−

* The level of suppression of β-lactamase nonsense mutants was tested as described in Methods. The results are expressed in a semi-quantitative way: +++, resistant to > 1 mg/ml of ampicillin; ++, resistant to 200 μg/ml; +, resistant to 50 μg/ml; −, sensitive to 50 μg/ml of ampicillin.

wild-type *bla* gene determined by assay with nitrocephin. The outer markers were chosen so that recombinants in both directions could be selected. The frequency of plaques carrying a wild-type *bla*$^+$ gene depended on the positions of the *bla* mutations and on the direction of selection of the outside markers. The asymmetry was always marked, the frequency of *bla*$^+$ being at least fivefold and usually tenfold greater in one direction than in the other.

Thirteen *bla* mutations were mapped, each with respect to the remainder, using crosses of the type shown in Fig. 1. The frequency of *bla*$^+$ recombinants is shown in Fig. 2A. Some of these frequencies come from averages of different crosses that showed a variation not larger than 20%. Having checked that these crosses gave consistent results, and that the frequencies of recombination were reasonably additive, we mapped most of the other mutations in the eleven intervals defined by these thirteen mutations. These results are shown in the lower panel of Fig. 2A. Mutations that are shown below one of the reference mutations gave less than 10^{-4} *bla*$^+$ recombinants amongst the total recombinants in the interval $h-P$. Some of these mutations, for which no recombination was detected, are known to be different. For example, *bla–21* and *bla–144* are different nonsense mutations suppressed respectively by amber and UGA suppressors (Table 3).

Isolation of deletions in the β-lactamase gene

Further map information was obtained from marker rescue experiments using deletions in the β-lactamase gene. A series of deletions of plasmid pacl29 were obtained by partial digestion with restriction enzymes. Deletions produced in this

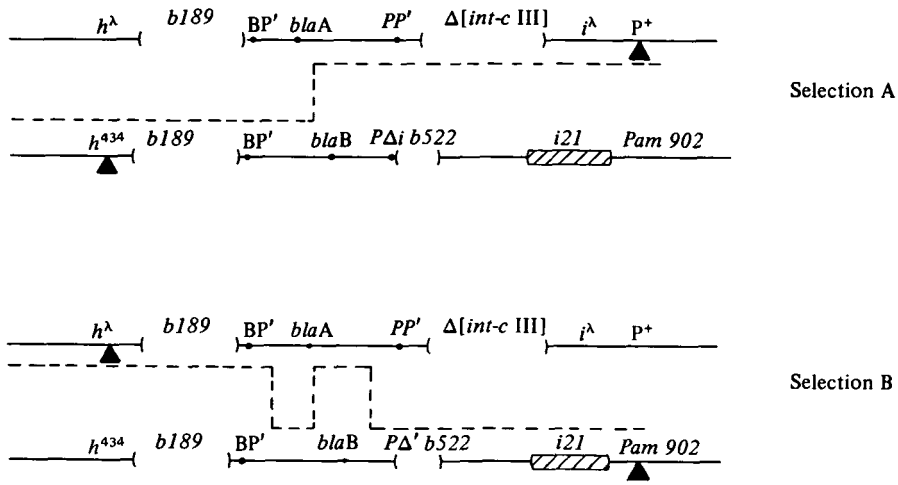

Fig. 1. Strategy used to map *bla* mutations in four point crosses. Selections for external marker recombinants were carried out in both directions by plating either on a ^{r}Su- or a *434*^{r} *groP* strain. In selection A a simple cross-over will generate a *bla*$^{+}$ phasmid, whereas in selection B a triple event is required.

manner could be easily identified from the DNA sequence. Plasmids which were still able to confer colicin immunity but not resistance to ampicillin were tested for their ability to rescue the markers *bla-1* or *bla-173* by means of the replica plating technique described in Methods. Plasmids that gave a positive result with either of the two markers were further tested for their ability to rescue other mutants, and whenever possible were checked by standard phasmid crosses. The results obtained by these series of experiments are plotted in Fig. 2B. The map positions determined by deletion mapping are consistent with those obtained in the four factor crosses, and divide the β-lactamase genetic map in 5 more intervals.

Identification of the end-points of the deletions in the DNA sequence

The extents of the deletions and their end points in the β-lactamase gene were analysed by digestion with restriction endonucleases and gel electrophoresis (not shown). Most of the deletions, when cleaved with the restriction endonucleases that generated them, showed a pattern similar to wild-type but with some fragments missing. No new fragment appears, confirming that, as expected, the ligation restored a restriction site. However, in the case of deletions 22, 27 or 32, which were obtained by *Mbo*II or *Hph*I partial digestion followed by S1 treatment before ligation, more complicated changes had occurred.

The map positions of the various deletion mutants were assigned with reference to the DNA sequence of the β-lactamase gene (Sutcliffe, 1978). These are plotted in Fig. 2C, which aligns the DNA sequence of the β-lactamase gene with the genetic map.

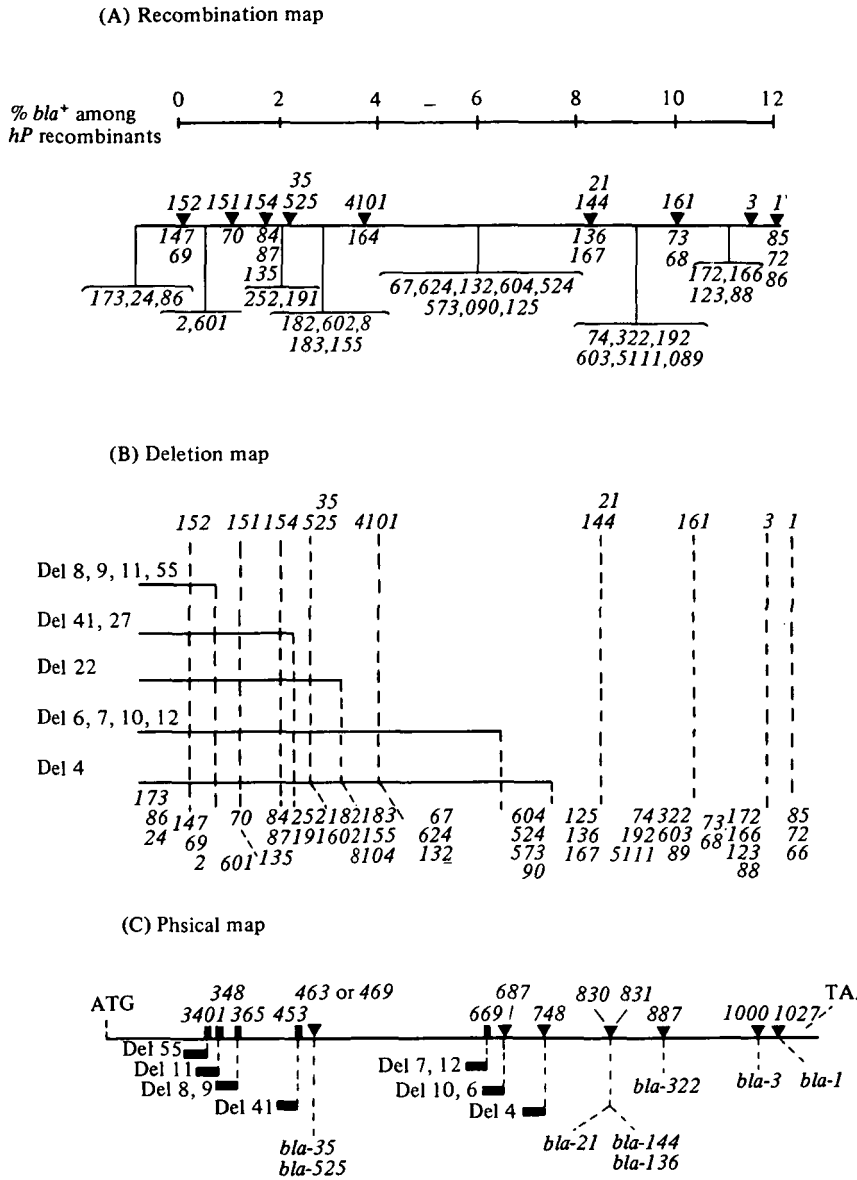

Fig. 2. Genetic and physical map of the β-lactamase gene. (A) Recombination frequency between *bla* mutations. The scale at top represents the percentage of bla^+ among *h P* recombinants in phasmid crosses of the type shown in Fig. 1. The map positions of 13 marker mutations are shown by the triangles. The map positions of 55 additional mutations with respect to the marker mutations are shown below. (B) Deletion mapping of the *bla* mutants. Deletion mutants generated by restriction endonuclease cleavage of pacI29 were ordered by recombination tests between the deletions and point mutants. (C) Physical map of the β-lactamase gene. The numbers represent nucleotides sequence of the gene as determined from the data of Sutcliffe (1978). Bars underneath the scale show the end-points of the deletions obtained *in vitro*. Triangles show the positions of some nonsense codons.

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Assignment of nonsense mutations to specific amino-acid residues

The correlation between the genetic map and the DNA sequence was extended by assignment of some of the nonsense mutations to specific amino acid residues. We assumed that NG-generated nonsense mutations result only from GC–AT transitions (Coulondre & Miller, 1977). Tentative assignments were based on the DNA sequence of the β-lactamase gene (Sutcliffe, 1978). Eight out of the ten suppressible mutations isolated were rescued by deletion 4 (Fig. 2) and therefore map in the last third of the protein. Six of these mutants were suppressible by amber suppressors and two by the UGA suppressor. *bla-1*, *bla-72* and *bla-76* had the same phenotype and were not separable by genetic crosses. Therefore they are very likely to be the same mutation. The same is true for *bla-144* and *bla-136*.

In *in vitro* experiments, *bla-3* directed the synthesis of a peptide 20 amino acids shorter than the wild-type β-lactamase protein (data not shown). The mutant was therefore tentatively assigned to position Gln265. In this case it was possible to check the assignment directly because the change of CAG coding for Gln265 to the amber triplet TAG should eliminate a recognition site for the restriction endonuclease *Hinf*I at that position. The DNAs of the mutant and wild-type plasmids were digested with *Hinf*I and analysed on an acrylamide gel. Fig. 3 shows that the two restriction fragments B and G present in the wild-type DNA are joined in one longer fragment in the mutant, confirming the expectation. *bla-1* maps to the right of *bla-3*. The two codons that could generate amber triplets by single base pair transitions are Gln274 and the last amino acid, Trp286. However, *bla-1* directed the synthesis of a peptide about 10 amino acids shorter than the wild-type protein *in vitro*. This suggests that the amber mutation is due to an alteration of the Gln274 codon. *bla-21*, *bla-144*, *bla-136* and *bla-322* mapped between the *Pst*I site (end point of deletion 4) and Gln265. *bla-21* and *bla-322* were suppressed only by *supF* (inserting Tyr) while *bla-144* and *bla-136* were suppressed by the UGA suppressor that inserts the amino acid tryptophan. Only two triplets in this region are candidates for the assignment of the amber mutations: Trp 208 and Trp 227. The same two triplets are also the only candidates for the assignment of the UGA mutations. These considerations together with the mapping data of Fig. 2 lead to the conclusion that *bla-21* and *bla-322* are mutations corresponding respectively to amino acid residues Trp208 and Trp227. The two UGA mutations *bla-144* and *bla-136* probably result from the same TCG→TGA transition at position Trp208, as suggested by the fact that they were not separable, by recombination, from *bla-21*. Finally *bla-35* and *bla-525* were suppressed only by ochre suppressors and mapped between the two deletions 44 and 12. Only two triplets in this region can generate ochre mutations by simple base-pair transitions. These code for the Gln residues at positions 86 and 88. These assignments allow us to extend the correlation between genetical and physical maps as shown in Fig. 2.

These assignments make it possible to examine recombination frequency as a function of physical distance for point mutations. Fig. 4 shows that for the range of distances investigated, the function is approximately linear, apart from a slight

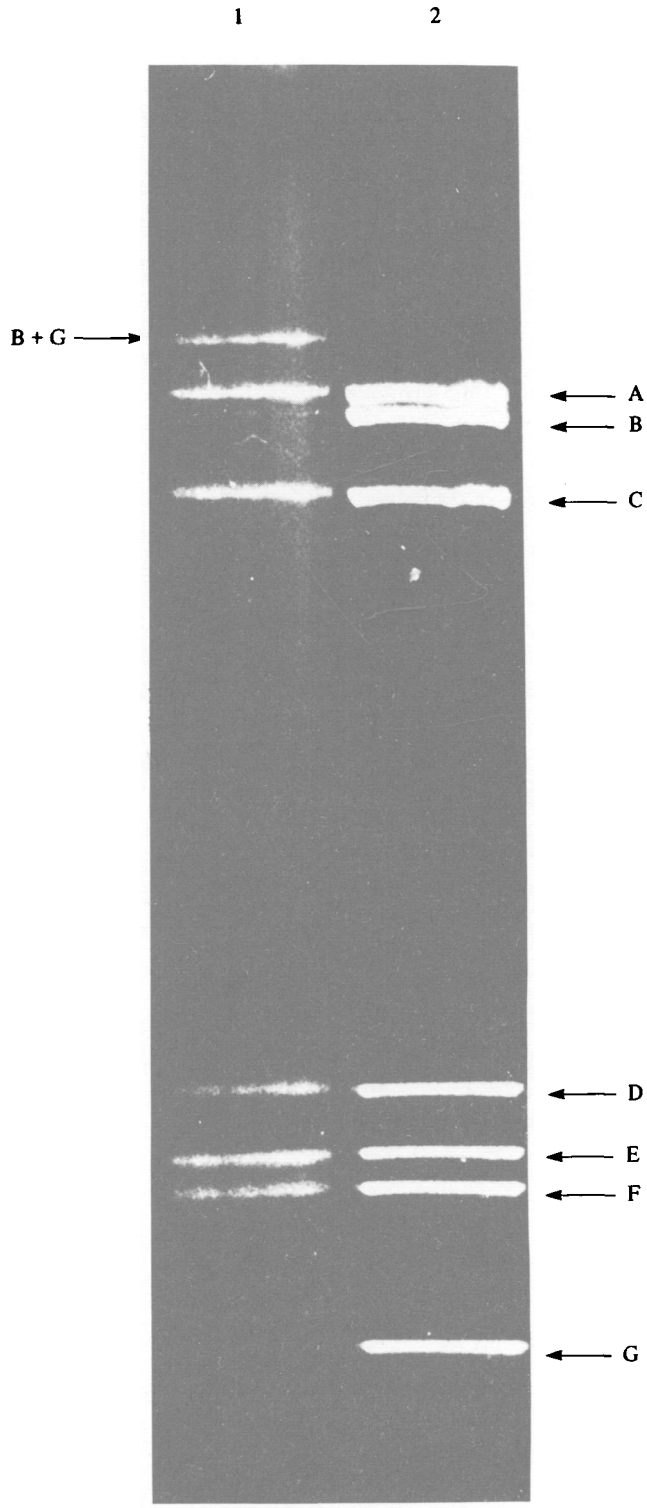

Fig. 3. Mutation *bla-3* causes loss of a *Hinf*I site. *bla-3* (1) and wild-type DNA (2) were digested with *Hinf*I and the fragments separated on a 10% acrylamide gel.

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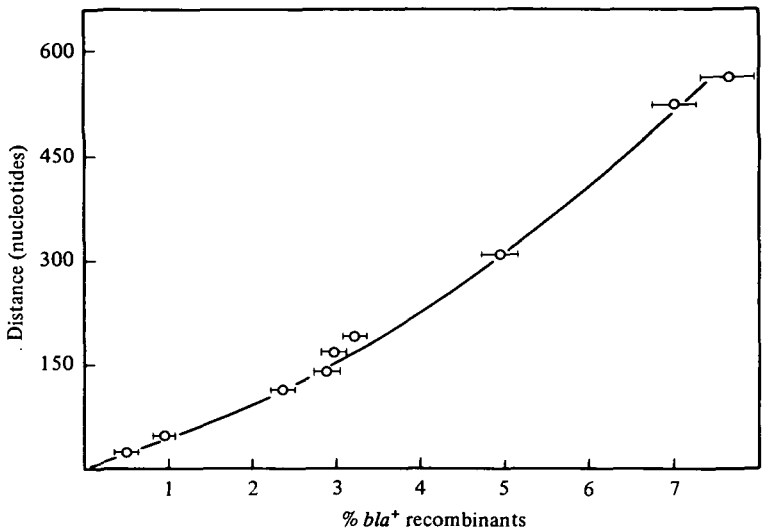

Fig. 4. Recombination frequency as a function of nucleotide distance. The frequency of bla^+ plaques among *h P* recombinants in crosses of the type shown in Fig. 2 is plotted as a function of nucleotide distance.

decrease in average recombination frequency per nucleotide at distances larger than 300 nucleotides.

DISCUSSION

The experiments described in this paper demonstrate how phasmids may be used to study the genetics of plasmid encoded functions. Simple replica plating techniques were devised which allowed the simultaneous lifting and release of large numbers of plasmids. In this way pure clones of plasmid mutants or recombinants were obtained by incorporation of the plasmid into a phage genome followed by subsequent release of the plasmid in an *int*-constitutive strain. Cells heterozygous for two alleles of a plasmid gene with a defined number of copies can be constructed by infection at a known multiplicity with two phasmids.

In the work described in this paper, mutants in the β-lactamase structural gene were isolated and mapped. We correlated the genetic map with the physical map as defined by end points of restriction enzyme-generated deletions and by the assignment of some nonsense mutations to specific amino acid residues.

Since β-lactamase is a periplasmic protein, some ampicillin-sensitive mutants might be defective in maturation or secretion of the enzyme. Mutations of the periplasmic *malE* enzyme and of the outer membrane *lamB* protein which result in cytoplasmic accumulation of precursors, have recently been isolated and characterized (Bassford & Beckwith, 1978; Emr, Schwartz & S'ilhavy, 1978). The selection procedure used probably restricted the mutants to those affected in the

early steps of the secretion process. In contrast, our screen methods for mutants unable to confer ampicillin-resistance are more general and will detect mutants in all steps of the secretory process. Such mutants are being characterized and will be described in detail elsewhere.

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