Genet. Res., Camb. (1982), **40**, *pp.* 233–247
Printed in Great Britain

The phasmid as a tool for plasmid genetics

II. Isolation of point mutations that affect replication of a ColE1-related plasmid

BY GIANNI CESARENI,* LUISA CASTAGNOLI* AND SYDNEY BRENNER

MRC Laboratory of Molecular Biology, University Postgraduate Medical School, Hills Road, Cambridge CB2 2QH, England

(*Received 25 August 1981 and in revised form 22 June 1982*)

SUMMARY

The insertion of a high-copy-number plasmid into a lambdoid phage chromosome which lacks a functional repressor gene confers on the hybrid 'phasmid' the capacity to grow on an immune lysogen. This was found to be due to titration of repressor because of plasmid replication. We have exploited this property in order to isolate mutants that affect plasmid replication. These mutants have been mapped in a region that was previously characterized as necessary for plasmid replication and incompatibility properties. Some of the mutations could revert at frequencies characteristic of single-base-pair change mutations.

INTRODUCTION

The small multicopy plasmid ColE1 and its close relative pMB1 do not specify any proteins required for their own replication, which depends entirely on the host replication machinery (Donoghue & Sharp, 1978; Kahn & Helinski, 1978). Faithful initiation and completion of ColE1 replication has been achieved in bacterial extracts (Tomizawa, Sakakibara & Kakefuda, 1975). Itoh & Tomizawa (1978) have shown that RNA polymerase, DNA polymerase I and RNAse H are sufficient for the correct initiation of replication of this plasmid *in vitro*. DNA synthesis is primed by an RNA fragment transcribed by RNA polymerase and processed by RNAse H (Itoh & Tomizawa, 1981). This processing step is essential for the initiation of DNA synthesis and it is specifically inhibited by a small RNA molecule (RNA I) synthesized in a region approximately 400 nucleotides upstream from the origin of DNA replication (Tomizawa *et al.* 1981). The DNA sequence of the region that codes for the primer precursor (Backman *et al.* 1978; Ohmori & Tomizawa, 1979; Oka *et al.* 1979) contains particular sequences and potential secondary structures

* Present address: European Molecular Biology Laboratory, Postfach 102209, 6900 Heidelberg, Federal Republic of Germany.

that may be relevant to the processing reaction and its regulation. Unfortunately few point mutants are available, to help correlation of functional steps in replication initiation with the DNA sequence (Naito & Uchida, 1980; Muesing *et al.* 1981). In this paper we describe the use of a pMB1-λ hybrid (Brenner, Cesareni & Karn, 1982) in a classical genetic approach to the study of plasmid replication. Seventy-two independent mutations that affect the replication of a pMB1 derivative have been isolated and a fine-structure genetic map of some of them has been obtained.

MATERIALS AND METHODS

Media chemicals and enzymes

The composition of media and source of enzymes and chemicals have been described (Brenner *et al.* 1982; Castagnoli, Cesareni & Brenner, 1982).

Construction of plasmids and phages

Phages, phasmids and plasmids used in this work were constructed by standard *in vivo* or *in vitro* recombinant DNA technology. The construction of pacl29, pacl30 and pacl53 has already been described (Brenner *et al.* 1982). pcl7 was constructed, inserting the *Eco*RI fragment carrying the *b*522 deletion to the right of *att* site (Parkinson & Davis, 1971) into the *Eco*RI site of the plasmid pVH51 (Herschfield *et al.* 1976).

Identification of plasmid replication mutants

General microbiological techniques as well as special phasmid methods are as described previously by Brenner *et al.* (1982) or Castagnoli *et al.* (1982). Phasmids containing plasmids with functional ColE1 replication origins are virulent. A colour test for phasmid virulence was devised for rapid screening of plaques of mutants or recombinants, based on a double-indicator plating technique. One of the two strains, the 'plating strain' (usually CA274) is used simply to generate plaques from every phage, virulent or not. This plating strain must itself carry a mutation in the β-galactosidase *lacZ* gene. The second strain, the 'tester strain', is an immune lysogen and carries a mutation in the *lacY* gene which specifies β-galactoside permease. β-galactosidase synthesis was induced in this strain with isopropyl-β-D-thio-galactopyranoside (IPTG), but hydrolysis of the dye 5-bromo-4-chloro-3-indolyl-β-D-galactoside (BCIG) is very poor, since it cannot penetrate into the bacteria. However, if it is lysed in a plaque, a strong reaction is found. To prevent plaque formation by phages produced by spontaneous induction of the prophage in the lysogen, a plating strain preventing growth of the prophage was used. Generally we used a prophage with a *P* amber mutation and an Su^- plating strain. Wild type (*vir*$^+$) and *vir* phasmids when plated on the double indicator in presence of IPTG and BCIG gave blue and white plaques respectively, after overnight incubation at 37 °C. Phasmids were pre-adsorbed to 0·05 ml of a fresh stationary phase culture of CA274. After 15 min at room temperature 0·05 ml of Q37 + 0·04 ml of IPTG (20 mg/ml in water) and 0·04 ml of BCIG (20 mg/ml in dimethyl formamide) were added, and the mixture plated in 3 ml of top agar. Virulent phasmids make blue plaques while *vir* mutants make white plaques.

Mutagenesis, isolation of mutants and reversion tests are as described in Castagnoli *et al.* (1982). *vir* mutants were identified using the blue plaque test, and after purification and retesting, seventy-two independent mutants were finally isolated.

Assay of β-lactamase and colicin immunity

The β-lactamase marker was assayed in two ways as previously described (Castagnoli *et al.* 1982). Staining with nitrocephin could be coupled with the blue plaque test only if the plates were not very crowded (⩽ 200 plaques), otherwise diffusion of the red colour confused the results.

The assay for colicin immunity was as previously described (Castagnoli *et al.* 1982). Crude extracts of colicin E1 were prepared from the strain JC411 (col E1) according to Shafferman, Cohen & Flashner (1978), and kept at −20 °C in the presence of 50% glycerol.

Other methods

Plasmid copy number was estimated by measuring the levels of ampicillin resistance of plasmid-containing bacteria (Uhlin & Nordström, 1977). Plasmid mutants were released into EQ84 by infection with the corresponding phasmid according to the method described in Castagnoli *et al.* (1982). Plasmid-containing bacteria were selected using a low concentration of ampicillin (50 μg/ml), and their efficiency of plating at different ampicillin concentrations was subsequently tested.

DNA synthesis of chloramphenicol-treated cells infected with wild-type or mutant phasmids was measured as described by Donoghue & Sharp (1978).

RESULTS

The isolation of plasmid mutants that are defective in replication requires the use of hybrid molecules carrying two different replication origins. In this way even mutants that are completely defective in one of the two replication systems can be identified and propagated.

Our work employed a 'phasmid', in which a pMB1 derivative was contained within a lambda bacteriophage with its own replication functions (Brenner *et al.* 1982; Castagnoli *et al.* 1982). The combination has distinctive properties which allowed the isolation, maintenance and analysis of replication mutants of the plasmid.

Phasmid virulence

Insertion of a ColE1 plasmid into the phage chromosome conferred on the hybrid the capacity for growth on a strain lysogenic for a prophage with the same immunity. Similar properties have recently been found for other phage-plasmid hybrids (Windass & Brammar, 1979) but since our findings are somewhat different we will describe them briefly. As shown in Table 1, this property was found in

Table 1. *Phasmid growth on lysogenic hosts*

Phage host	EQ82	EQ82 (λ)	EQ82 (434)	EQ82 (81)	EQ82 (21)
λ	+	−	+	+	+
Phasmid (λ)	+	±	+	+	+
Phasmid (434)	+	+	±	+	+
Phasmid (81)	+	+	+	+	+
Phasmid (21)	+	+	+	+	+

Growth was tested by spotting $\sim 10^4$ and $\sim 10^2$ plaque-forming units on a lawn of lysogenic bacteria. +, Growth with an efficiency of plating $> 0{\cdot}1$; ±, single plaques were usually not viable but killing was observed when 10^4 phages were spotted.

phasmids with four different immunities. However, the strength of virulence varied; although single plaques with a plating efficiency of approximately one were found for the *i*21 or *i*81 phasmids on corresponding lysogens, the *i*λ or *i*434 phasmids gave a weaker response, and virulence could only be observed by killing the lysogen with many phages. There are two possible explanations of phasmid virulence: either the hybrid overcomes repression because of activation of transcription of phage genes from a plasmid promoter, or the high-copy-number phasmids titrate out repressor, leading to initiation of transcription from non-repressed promoters. The observations described below show that the phasmid overcomes repression by the resident prophage because the plasmid replication functions support phasmid replication even in the presence of phage repressor.

Virulence depends upon the absence of phasmid repressor function

If the repressor titration hypothesis is correct, virulence should not be observed if the incoming phasmid itself synthesizes active repressor. In this situation titration of repressor would not be possible because repressor synthesis is autocatalytic (Reichardt, 1975) and because the genes for repressor and operators are equally amplified during phasmid replication. In fact, when the infecting phasmid carried a gene for a thermosensitive repressor, virulence was observed only at 37 °C, and not at 32 °C when the repressor is active. When the repressor gene was removed by the KH54 deletion (ϕ81, Fig. 1), no difference was observed when the infection was carried out at either temperature.

Furthermore, the expression of phage replication functions did not depend upon the orientation of the plasmid with respect to the chromosome, as would be expected if transcription starting from a plasmid promoter were responsible for virulence. $\phi 1$ and $\phi 2$, which carry the same plasmid in opposite orientations, were both equally virulent.

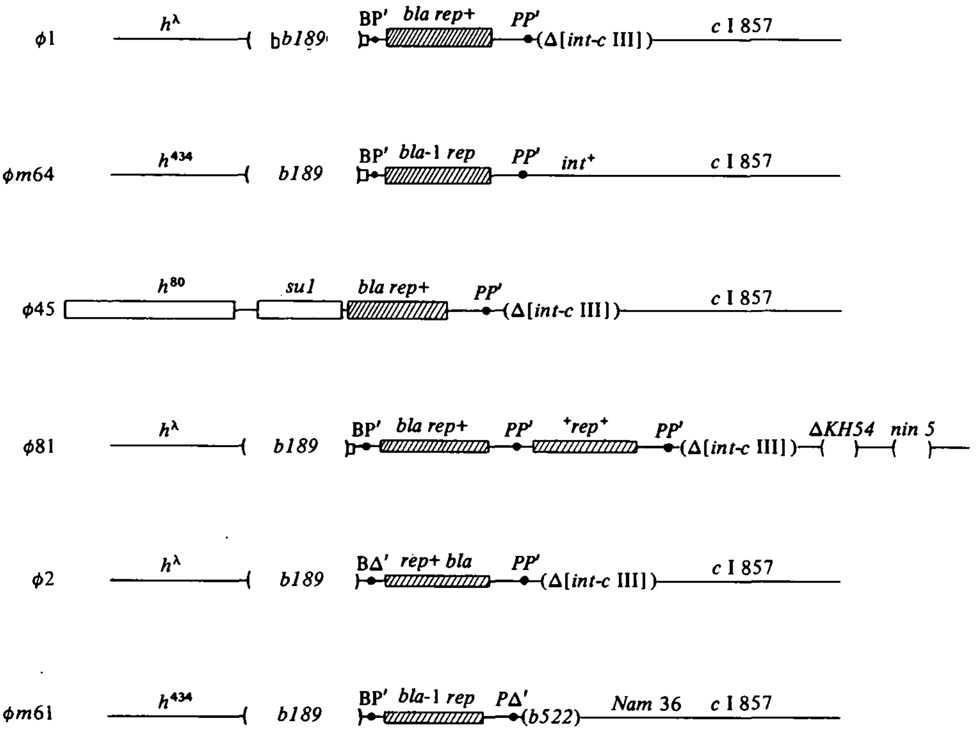

Fig. 1. Genotypes of the phasmids used in this work.

Virulence is linked to the origin of replication of the plasmid

The region of DNA responsible for virulence could be mapped by comparing phasmids carrying different derivatives of the plasmids ColE1 and pMB1. All the phasmids derived from the plasmids pcl7, pacl29, pacl30 and pacl53 (Brenner *et al.* 1982) could grow on homo-immune lysogens. Apart from 250 base pairs on the P′ side of λatt site the only fragment of DNA that they have in common is a region of about 800 base pairs, mostly upstream of the origin of DNA replication. The unlikely possibility that the presence of two λatt sites in the phasmid were responsible for the anomalous behaviour observed was ruled out by constructing phasmids in which the plasmid ColE1 was directly inserted between the two arms by *in vitro* methods. These showed the expected virulence.

Virulence is abolished if the infected lysogen contains a plasmid of the same compatibility group

ColE1 replication is negatively regulated by a *trans*-acting repressor (Shepard, Gelfand & Polinski, 1979). As a consequence, if plasmid-driven replication were responsible for the virulence of the phasmid we expected this to be abolished if the lysogenic bacteria also contained a plasmid of the same compatibility group. Fig. 2 shows that this was correct; phasmids were not produced if a lysogen containing a ColE1 derivative was infected. No difference in burst size was observed after infection of non-lysogenic bacteria with or without the same ColE1 derivative. As expected, the presence in the lysogen of a compatible plasmid such as pSC101 did not affect phasmid growth (not shown).

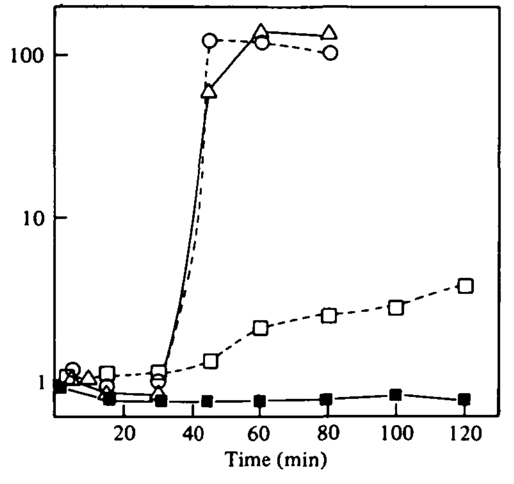

Fig. 2. Single step growth of a phasmid. Exponentially growing cultures of C600, C600 harbouring the plasmid pacl29, C600(*i*81*Sam*7) or C600(*i*81*Sam*7) harbouring pacl29 were grown to O.D. 0·3 (600 nm), harvested and infected at a multiplicity of 0·1 with a phasmid in which the right arm of ϕ1 had been replaced by an *i*81*ts*. The infected cells were diluted 100-fold in CY broth at 37 °C and aliquots were plated on an Su^- indicator at different times.

Virulence is due to titration of repressor

To prove that virulence is due to titration of repressor and not to escape from repression we needed to show that even the resident prophage was eventually derepressed. This was proved by infecting an Su^- lysogen with the phasmid ϕ m61 (Fig. 1) carrying an amber mutation in the *N* gene of lambda. Derepression of the prophage would be required to complement this defect in the phasmid and allow it to grow. This proved to be the case.

Isolation of vir *mutants*

The correlation between phasmid virulence and plasmid replication made it likely that non-virulent mutants of the phasmid would be due to mutations affecting plasmid replication functions. We are able to screen for such mutants by a technique which measures the release of β-galactosidase in plaques. In this system the wild-type virulent phasmid made blue plaques while *vir* mutants made white plaques.

ColE1 replication mutants were first isolated in phasmid $\phi 1$ (Fig. 1). This is a phasmid which shows weak virulence (Table 1). We expected that this would be sensitive even to small defects in plasmid replication and that even a relatively small decrease in plasmid copy number would result in a *vir* phasmid. A total of 72 independent mutants were isolated using either UV (38) or NG (34) mutagenesis. None of these mutations was suppressed either by *supD* or *supF*. This is in accordance with previous evidence that no ColE1-encoded polypeptide is required for ColE1 replication (Donoghue & Sharp, 1978).

Reversion frequency

The replication mutants of ColE1 isolated by Hashimoto-Gotoh & Inselburg (1979) were deletions extending into the origin or replication of ColE1. This suggested that it might be difficult to eliminate ColE1 replication by single base-pair substitutions. With this in mind we tested the reversion frequency of some of our mutants. Most showed a spontaneous reversion frequency in the range 10^{-9} to 5×10^{-8} typical of single base-pair changes. The reversion frequency was increased by mutagens such as 2-aminopurine or EMS. Some of the mutagen-induced revertants proved to carry secondary mutations.

vir *mutants have defects in plasmid replication*

ColE1 replication continues in the absence of protein synthesis in the host bacteria (Clewell, 1972). This allows the study of ColE1 replication *in vivo* in the absence of host DNA synthesis. When exponentially growing bacteria were treated with chloramphenicol, incorporation of ^{3}[H]dTP into bacterial DNA decreased to a level of about 5% of the initial incorporation. As already shown by Donoghue & Sharp (1978), if such bacteria were infected with a phasmid, DNA replication proceeded at levels well above the background for at least 10 h. Most of the ^{3}[H]dTP incorporated under these conditions was found as supercoiled circles of phasmid DNA. Fig. 3 shows that when a *vir* phasmid was used, ^{3}[H]dTP incorporation, though slightly higher than the uninfected control, was clearly below the level of that corresponding to bacteria infected with a wild-type phasmid. This experiment clearly proves that *vir* mutants have defects impairing ColE1-type replication.

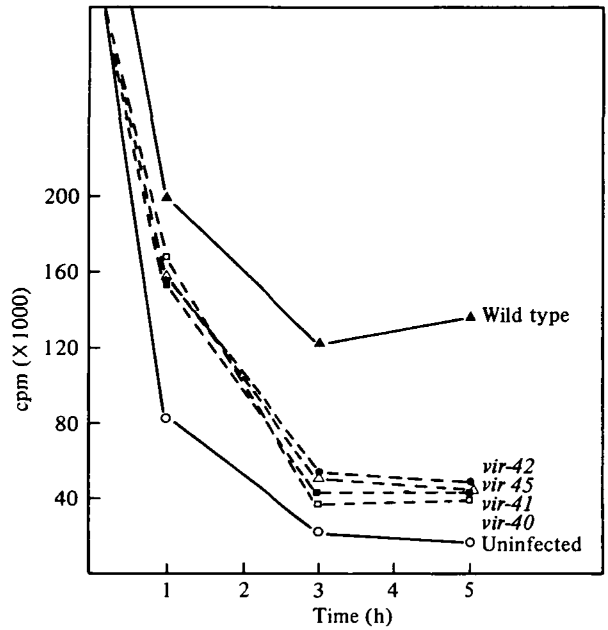

Fig. 3. ^{3}[H] thymidine incorporation in chloramphenicol-treated cells. Plasmid-specific replication was measured as described by Donoghue & Sharp (1978) in chloramphenicol-treated cells. Exponentially growing EQ82 bacteria were infected at a multiplicity of 5 with wild-type of *vir* mutant phasmids. The incorporation of ^{3}[H] thymidine was measured with 10 min pulses at different times from the start of the incubation in the presence of chloramphenicol (time 0).

Table 2. *Transduction of ampicillin-resistance by* vir *mutants*

Mutant	Frequency of transduction
vir$^+$	5×10^{-1}
vir-40	$\sim 10^{-5}$
vir-41	$\sim 3 \times 10^{-3}$
vir-42	$\sim 10^{-3}$
vir-45	$\sim 1{\cdot}5 \times 10^{-4}$

Phasmid lysates were used to infect an exponentially growing culture of the integrase-producing strain EQ84 at MOI $\approx 0{\cdot}1$. After 30 min growth at 32 °C, different dilutions were spread on TYE plates containing 50 μg/ml of ampicillin. The plates were incubated at 37 °C.

vir *phasmids can release plasmids with a low copy number*

The integrated plasmid in the phasmid can be released simply by infection of a bacterial strain containing a prophage that synthesizes the *int* protein constitutively (Brenner *et al.* 1982). Phasmids with *vir* mutations would be expected to release plasmids with defects in replication. In fact, when we excised these plasmids

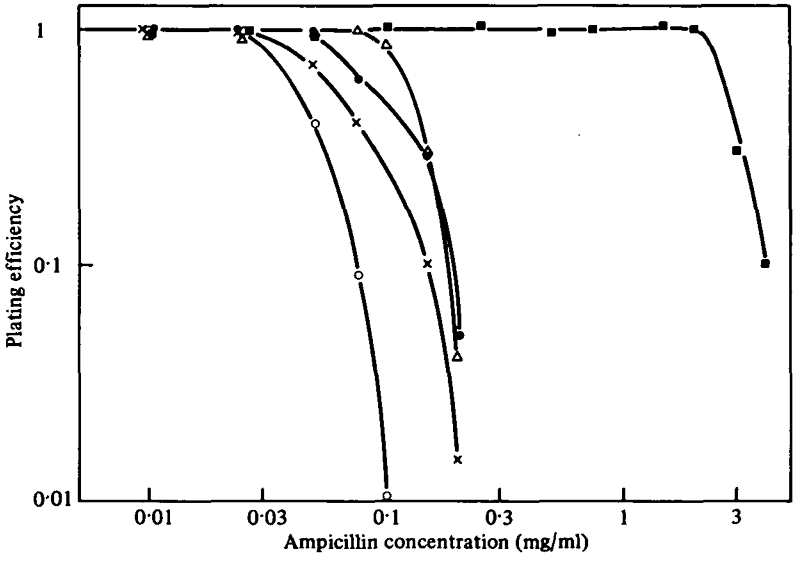

Fig. 4. Relative copy numbers of *vir* plasmids. EQ84 bacteria containing the wild-type plasmid pacl29 (■) or the mutants *vir-40* (○), *vir-41* (●), *vir-42* (△), *vir-45* (×) were grown in 2×TY broth. Aliquots (0·1 ml) of dilutions of the exponentially growing cultures were spread on TYE plates containing different concentrations of ampicillin.

Table 3. *Recombination between* vir *mutants and* bla*

		Percentage of recombinants with:			
vir	*bla*	+	−	−	+
alleles	*vir*	−	−	+	+
vir-40		8·3	4·6	85·5	1·5
vir-364		10·6	6·3	82	1·1
vir-374		7	4·5	88	1·1
vir-41		9·1	5·6	83	1·3
col⁻	*bla*	+	−	−	+
alleles†	*col*	−	−	+	+
col-356		9·2	8·2	81	1·6
col-494		8·1	7·4	82	2·5

* The scheme for the crosses is drawn in Fig. 5A.

† These results were obtained in crosses similar to the one in Fig. 5A in which the *vir*⁻ phasmid was replaced by a *col*⁻ one.

looking for ampicillin transductants in the presence of repressor, very few were obtained, and these gave unusually small colonies. The few large colonies that appeared at a lower frequency were revertants (Table 2).

It has been shown that the production of β-lactamase and the consequent level of resistance to ampicillin is proportional to the number of β-lactamase genes

Table 4. *Recombination between* vir *mutants and* col-*356*

		Percentage of h434 P^+ recombinants			
		Cross A		Cross B	
	col	−	+	−	+
Allele tested	*vir*	−	+	−	+
vir-40		1·1	3·4	6·4	1·2
vir-364		2	4·1	7	1·5

These results refer to crosses of the type shown in Fig. 5B and C.

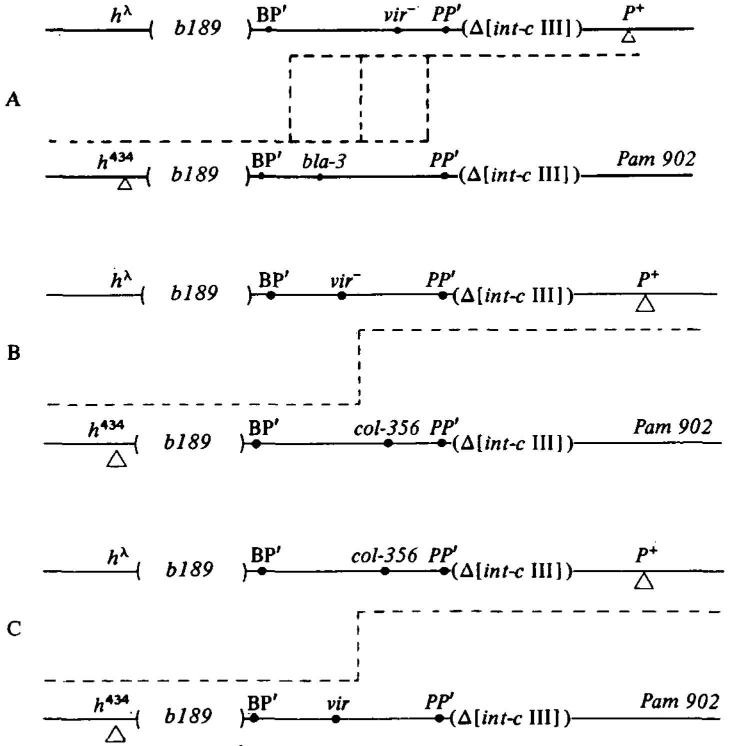

Fig. 5. Mapping *vir* mutants with respect to *bla-3* and *col-356*. (A) The cross used to map *vir* mutants with respect to *bla-3*. Phasmid recombinants carrying the *h*434 P^+ markers were selected on an Su^- host resistant to phage λ. The unselected markers *bla-3* and *vir* were tested among the recombinants as described in Methods. A similar scheme was used to map *vir* mutants with respect to *col-356*. (B and C) The crosses used to map *vir* mutants with respect to *col-356*. Selection in both crosses was carried out on an Su^- host resistant to λ. The percentage of *vir col* or vir^+ col^+ amongst *h P* recombinants was obtained by testing the phasmids recombinant for the two unselected markers as described in Methods.

present inside a cell (Uhlin & Nordström, 1977). Thus a measure of the relative copy numbers of plasmids containing a β-lactamase gene can be obtained by measuring the level of ampicillin resistance. Fig. 4 shows that the wild-type plasmid conferred a level of resistance at least 40 times higher than *vir-40* and approximately 20 times higher than the three other *vir* mutants tested. Although the exact copy number of plasmid pacl29 has not been measured it is likely to be about 50, judging from the copy number of similar ColE1 derivatives. The experiment in Fig. 4 suggests that the average copy number of *vir-40* is very close to one. We were therefore surprised to find that bacteria carrying the *vir-40* plasmid mutant did not segregate the plasmid. This stability seems to be due to integration of the non-replicating plasmid into the bacterial chromosome via *int*-mediated recombination. About 50% of the bacteria can be cured of plasmid functions by infection with a phage that provides the products of the *int* and *xis* genes.

Mapping of the vir *mutations*

A stretch of DNA of about 580 nucleotides upstream of the origin of replication has been shown to be sufficient for ColE1 replication (Backman *et al.* 1978). This segment of DNA is located between the β-lactamase gene and the colicin immunity gene in the plasmid pacl29. To test if *vir* mutations map in this region, a series of four point crosses between phasmids carrying various plasmid and phage markers were performed (Figs. 5 and 6). The results shown in Table 4 are consistent with a position for the *vir* mutations between *bla-3*, an amber mutant in the carboxyterminal part of the β-lactamase gene, and *col-356*, a mutation that affects the colicin immunity gene of pacl29. The data that map the mutations *vir-40* and *vir-42* to the left of *col-356* were not conclusive because of strong negative interference.

A genetic map of the β-lactamase, *ori V*, and colicin immunity region aligned with a physical map of pacl29 is shown in Fig. 6. We have shown previously that the frequency of recombination between two markers in the β-lactamase gene is approximately proportional to the physical distance between the two markers up to 500 nucleotides (Castagnoli *et al.* 1982). If we assume that this is true also in the neighbouring region containing the origin of replication and the colicin immunity gene we can try to locate the genetic markers more precisely on the physical map. Using as a reference the physical distance between *h* and *bla-3* it is therefore possible to assign the *vir* and *col* mutations to the regions shown in Fig. 6. These regions correspond approximately to the DNA fragment containing the origin of replication and to the sequence that has been identified as the colicin immunity gene (Oka *et al.* 1979).

Fine-structure genetic mapping

A fine-structure genetic map of the mutants affecting replication was determined by phasmid crosses as an aid to subsequent analysis of the mutants by DNA sequencing. Phasmid crosses of the type shown in Fig. 7 allow the relative position of the two alleles *virA* and *virB* to be distinguished. Recombinants for the external markers *h* and *P* were selected on a double-indicator lawn and the vir^+ recombinants were screened for segregation of an unselected marker, *bla-3*. Depending on the relative positions of the *vir* alleles it is possible to predict that in one configuration

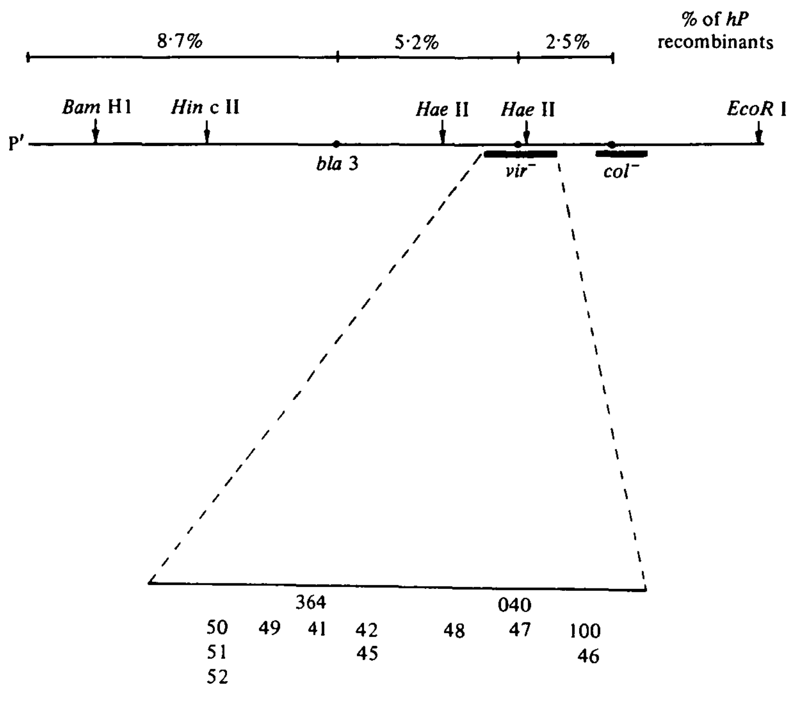

Fig. 6. Alignment of the fine structure map of *vir* mutants with the physical map of the *ori col* immunity region in plasmid pacl29. The physical positions of *vir* and *col* mutants were assigned using the data in Table 3, assuming that the frequency of recombination between two genetic markers is proportional to their physical distance as demonstrated in the case of the β-lactamase gene (Castagnoli *et al.* 1982). The distance between *h* and *bla-3* was used as a standard. The frequency of recombination between *h* and the end point of the deletion *b*189 was negligible. The order of the *vir* mutants was inferred from the results shown in Table 5.

most of the vir^+ recombinants should be bla^-, while in the other configuration a higher fraction of bla^+ should be recovered. In the latter case, another recombination event in the β-lactamase region is necessary to obtain the selected *h*434 marker. In this way thirteen *vir* mutants were mapped with respect to *vir-40* and *vir-364*. The percentage of ampicillin-resistant among vir^+ recombinants (Table 5) divided the mutants into two classes: those with a frequency of ampicillin-resistance of 10% or less and those with a frequency of 30% or more. The first class was considered to be to the right and the second to the left of the *virA* allele, on the

Table 5. *Recombination frequencies in four point crosses*

virB ...	*vir-40*		*vir-364*	
virA	% *vir*⁺ among *h–P* recombinants	% *bla*⁺ among *vir*⁺	% *vir*⁺ among *h–P* recombinants	% *bla*⁺ among *vir*⁺
vir-40	$< 2 \times 10^{-4}$		3×10^{-2}	36
vir-41	0·4	9	$< 4 \times 10^{-4}$	
vir-42	0·6	3	10^{-4}	54
vir-47	$< 2 \times 10^{-4}$		0·05	34
vir-48	0·2	9	4×10^{-2}	36
vir-49	0·35	3	2×10^{-4}	9
vir-50	0·5	12	0·4	7
vir-51	0·5	8	0·1	8
vir-52	0·7	9	0·2	9
vir-100	0·05	40	0·3	40
vir-45	1·0	4	2×10^{-4}	33
vir-46	$6{\cdot}6 \times 10^{-3}$	36	4×10^{-3}	28
vir-364	0·3	3	$< 2 \times 10^{-4}$	

*h*434 P^+ recombinants carrying a *vir*⁺ phasmid were selected on double-indicator plates from crosses of the type shown in Fig. 7. Dilutions of the cross were preadsorbed to CA274 λ^r. The tester strain Q37 λ^r was subsequently added together with IPTG and BCIG. Blue plaques were purified on CA274 λ^r and screened for the presence of *bla-3* marker with nitrocephin.

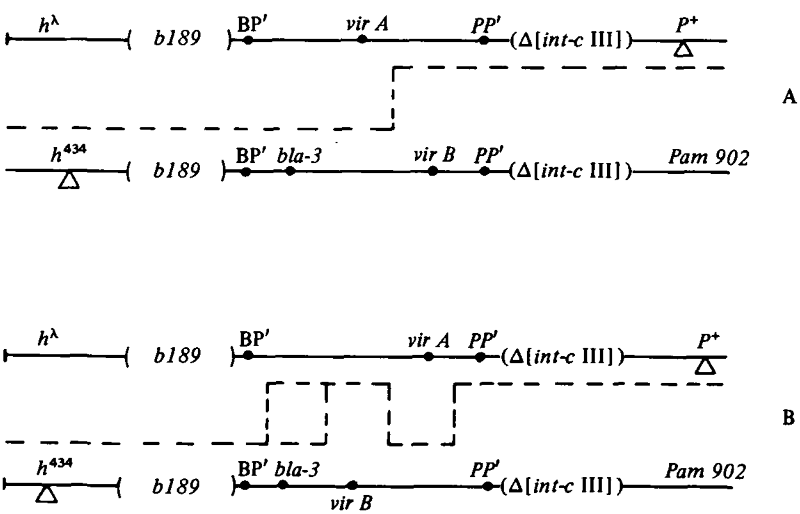

Fig. 7. Fine-structure mapping of *vir* mutants. The rationale of the mapping procedure is described in the text.

basis of internal consistency, allowing the genetic map in Fig. 6 to be drawn. *vir-41* and *vir-47* are shown beneath *vir-364* and *vir-40* since no recombination was observed between these alleles. *vir-49* was drawn in a different position from the group including *vir-50*, *vir-51* and *vir-52*, despite the fact that no direct mapping data was obtained, because of the clear difference in the recombination frequency in the crosses with *vir-364*. The same is true for the position of *vir-42*, *vir-45* and *vir-48*.

CONCLUSIONS

In this paper we have described the isolation, characterization and genetic mapping of mutants in the replication functions of the plasmid pMB1. This was made possible by the observation that phasmids containing integrated plasmids can grow on a lysogen containing a prophage of the same immunity provided the plasmid replication origin is functional.

We have shown that the function that confers this behaviour maps in the region of the origin of replication of the integrated plasmid and that it is inhibited by a resident plasmid of the same in compatibility group. Contrary to findings for similar hybrids (Mukai *et al.* 1978), in our phasmids the plasmid replication functions complemented phage *O* and *P* mutants very poorly, if at all. This allowed us to use *P* amber mutants as selective markers in mapping crosses. We proved that phasmid virulence was not due to a general insensitivity to repression, but rather to titration of the repressor present by the increased copy number of the incoming phasmid due to plasmid replication. This phenotype, together with a convenient colour test, can be used to select *vir* mutants which are defective in plasmid replication functions as shown by infection of chloramphenicol-treated cells. Furthermore, the mutant plasmids excised from the phage chromosome were very poorly propagated; most are probably not self-sustaining.

All the *vir* mutants mapped between the β-lactamase and colicin immunity genes of the plasmid pacl29, which had already been characterized as the region necessary for ColE1 replication. Many of the mutations appear to be single base changes, and a fine-structure genetic map shows that they are at different sites. The genetic studies are a first step and will permit further studies of the relationship between DNA sequence and replication phenotype.

Phasmids have proved to be versatile for the study of ColE1 replication. In addition to the *vir* mutants reported here, high-copy-number mutants and mutants in incompatibility properties have been selected and will be described in detail elsewhere.

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