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IS2 insertion is a major cause of spontaneous mutagenesis of the bacteriophage P1: non-random distribution of target sites

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Insertion mutations arising spontaneously in the P1 prophage and affecting vegetative phage reproduction were screened for the presence of insertion sequence 2 (IS2). Filter hybridization identified 28 out of 44 independent insertions as IS2. Their target specificity is not random. A region that amounts to <2% of the phage genome had trapped 15 of the 28 IS2 elements. However, precise mapping of nine mutants in this hot spot segment revealed no preferred insertion site. Rather, the nine IS2 are distributed over the whole target segment and IS2 are found in both orientations. Sequence data indicate that at least two sequence variants of IS2 participated in mutagenesis of the phage genome. The detectable transposition of IS2 from the host chromosome to the prophage occurs with a frequency of 3 x 10^{-5} per cell per generation under the particular experimental conditions. It is concluded that IS2, a natural resident of *Escherichia coli* K12 strains, is an important agent for spontaneous mutagenesis and exerts this action non-randomly along the genome.

Key words: bacteriophage P1 mutants/insertion element IS2/IS elements of *E. coli*/spontaneous mutagenesis/transposition target

Introduction

The transposable element IS2 is a normal resident in the chromosome of *Escherichia coli* K12. Its copy number per chromosome was reported to vary from four to twelve for different K12 strains (Saedler and Heiss, 1973; Hu and Deonier, 1981). IS2 is also carried in the fertility plasmid F and in some R plasmids (Hu *et al.*, 1975a, 1975b). By transposition to new sites, IS2 is known to have caused mutations in the *gal* and *argECBH* operons of *E. coli* (Hirsch *et al.*, 1972; Glansdorff *et al.*, 1980) and in the *y* region and genes *int xis* of phage λ (Brachet *et al.*, 1970; Zissler *et al.*, 1977). The detection of these and similar, more recently isolated mutations depended on an alteration in the activity of the particular gene or operon being investigated. For that reason the target for the insertion was limited to a rather small region, and no conclusions could be drawn with regard to the importance of IS2-mediated transposition events.

These limitations are less stringent in an experimental set-up developed in this laboratory (Arber *et al.*, 1978, 1980). We studied spontaneous mutations in the P1 prophage and its close relative P1-15. Surprisingly, a majority of all independently isolated mutants affected in vegetative phage reproduction turned out to be due to transposition of IS elements into the phage genome. Although some segments of the 90 kb long P1 genome are non-essential for phage growth, a considerable part of the P1 DNA is occupied by essential genes, so that the target for detectable IS transposition in these experiments can be considered to be relatively large. Still, the mutant genomes are amenable to an efficient physical mapping by restriction cleavage analysis. This allowed Arber *et al.* (1978, 1980) to show a non-random distribution of IS-mediated mutations in the phage genome.

We show here that more than half of the IS elements thus trapped on the P1 prophage are IS2. The distribution of their locations on the P1 genome is even less random than that of the total IS population, but precise mapping has not yet revealed any single IS2 insertion site that has been used twice. Comparative sequence analysis shows that different IS2 variants participate in the mutagenesis of the P1 genome. It is concluded that the IS2 elements resident in *E. coli* K12 represent an efficient, natural mutagenic power.

Results

Identification of IS2 in insertion-mediated mutations of the phage P1 and P1-15 genomes

Most of the mutants analyzed in this investigation have been described previously and their locations have been determined on the physical map of the phage genome (Arber *et al.*, 1978, 1980). A few additional mutations have now been mapped by restriction cleavage analysis with respect to the standard restriction maps of P1 (Bächi and Arber, 1977) and its close relative P1-15 (Arber *et al.*, 1980; Meyer *et al.*, 1983). All these mutants are listed in Table I. Because of the presence of either *Bgl*II, *Eco*RI or *Pst*I sites in the inserted DNA, 10 mutants are unlikely to contain an IS2 element. With the exception of these, all of the mutant genomes, mostly isolated from lysogenic bacteria as plasmids, but in some instances from phage particles, were hybridized with an IS2 probe (λr32 or pAW14). Some mutants were, in addition, checked for the presence of the internal 0.6-kb *Bgl*I fragment of IS2 (Figure 1), and some insertions were confirmed to be IS2 by sequencing the ends of the IS element (see later section). This study showed that 28 of the 44 insertions are IS2. The insertions carried in most other mutants that did not hybridize with the IS2 probe are not yet characterized, except for a few cases shown in Table I.

Frequency of transposition of IS2 from the host chromosome to the P1-15 genome

The isolation and characterization of some mutants (Table I: mutants with prefix M) allowed us to estimate the transposition frequency of IS2. For obtaining independent mutants, 100 subclones of WA921(P1-15) were grown in tryptone broth at 30°C and diluted 1:100 once a week with fresh medium. At various times between 7 and 12 weeks the subclones were screened for spontaneous defective phage mutants (Arber *et al.*, 1978). In the course of this experiment, 59 defective lysogens were found (Table II). The defective P1-15 genome of 17 independent lysogens was isolated and subjected to restriction cleavage analysis. All mutants analyzed turned out to be due to insertions. Upon hybridization 13 of them were identified as IS2. Assuming that this fraction

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Table I. List of phage P1 and P1-15 insertion mutants analyzed

Mutant	Derived from	Production of phage particles[a]	Insertion hybridizes with IS2	Insertion located in genome region (see Figure 2)
33	C600(P1)	n.d.	+	H
63	W3350(P1)	n.d.	+	H
64	W3350(P1)	n.d.	−	U
65	W3350(P1)	−	−	R[e]
66	C600(P1)	−	−[d]	P
69	C600(P1)	n.d.	+	M
72	C600(P1)	n.d.	−[d]	V
73	C600(P1)	n.d.	+	H
202A	WA921(P1)	n.d.	+	O
209B	WA921(P1)	+	−[c]	H
220A	WA921(P1)	n.d.	+	H
302B	AB1157(P1)	n.d.	−[d]	W
302E	AB1157(P1)	n.d.	−[d]	W
504A	C600(P1)	n.d.	+	M
508B	C600(P1)	n.d.	+	M
520B	C600(P1)	+	−[c]	H
702A	DW103(P1)	n.d.	+	H
705A	DW103(P1)	n.d.	−[d]	W
711A	DW103(P1)	n.d.	−[d]	Q
713B	DW103(P1)	n.d.	+	M
718A	DW103(P1)	+	−[c]	H
M6	WA921(P1-15)	−	+	T
M8	WA921(P1-15)	+	+	H
M9	WA921(P1-15)	−	−	W
M10	WA921(P1-15)	n.d.	+	S
M28	WA921(P1-15)	−	+	H
M34	WA921(P1-15)	+	+	H
M36	WA921(P1-15)	+	+	N
M41	WA921(P1-15)	+	+	H
M47	WA921(P1-15)	+	+	H
M48	WA921(P1-15)	+	+	H
M55	WA921(P1-15)	n.d.	−[d]	P
M58	WA921(P1-15)	+	+	H
M66	WA921(P1-15)	−	+	T
M76	WA921(P1-15)	n.d.	−[d]	O
M80	WA921(P1-15)	+	+	H
M86	WA921(P1-15)	−	+	U
M96	WA921(P1-15)	−	−[b,d]	M
R31	WA921(P1-15)	n.d.	+	H
30R014	WA921(P1-15)	+	+	H
30R025	WA921(P1-15)	+	+	M
RRT033	WA921(P1-15)	+	+	M
RRT076	WA921(P1-15)	−	+	L
RRT093	WA921(P1-15)	+	−[d]	V

[a]Production of phage particles upon thermoinduction was shown with the resulting lysates by the presence of a phage band in a CsCl gradient, as described in Materials and methods. + particles are produced; − no particles are produced; n.d. not determined.
[b]Insertion is identified as IS1 (Arber *et al.*, 1980).
[c]Insertion is identified as IS30 (P. Caspers and C. Sengstag, unpublished data).
[d]Hybridization with IS2 not tested, but unlikely because of restriction patterns incompatible with IS2 structure.
[e]Electron microscopical heteroduplex analysis localizes mutation 65 at a distance of 0.3 kb from the left end of segment R (J. Meyer, personal communication).

(13/17) for the occurrence of IS2 holds for the remaining 42 mutants that were not analyzed in more detail, we calculate the transposition frequency to equal:

$$\frac{13}{17} \times \frac{M}{N} \times \frac{1}{G}$$

where M is the number of defective mutants detected at a particular time point, N the total of colonies tested and G the number of generations of growth before screening was done.

An average transposition frequency of 3×10^{-5} per cell per generation can be calculated for IS2 with the P1-15 prophage as target in these particular growth conditions (Table II). It has to be noted that for most of the experiment the cells were in the stationary phase and therefore the expression 'per generation' must not be conceived strictly. Indeed, a preliminary experiment, in which the P1-15 lysogenic bacteria grew without interruption for 200 generations in the exponential phase of growth, yielded considerably fewer spontaneous prophage mutants than we found under the conditions defined above.

Distribution of insertions in the P1 and P1-15 genomes

The map positions of 44 independently isolated and randomly chosen insertion mutations are shown in Figure 2. The insertions are scattered over the whole phage genome, but it is obvious that a preferred region exists. This hot spot for insertion is localized between a *Bgl*II site and a *Bam*HI site that define a DNA segment of 1.7 kb (Figure 2, black area). This segment H amounts to only 1.9% of the total phage genome,

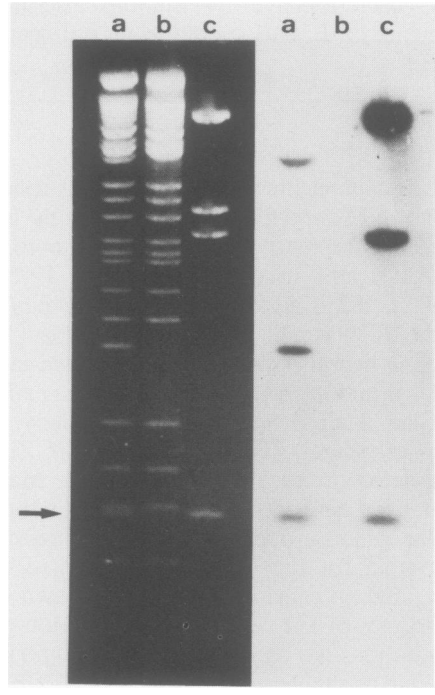

Fig. 1. Identification of IS2 by specific hybridization of restriction fragments. **Left**: electrophoresis patterns on a 1.2% agarose gel of *Bgl*I digests of **a**: P1 mutant prophage 504A, **b**: P1 DNA as control, **c**: pAW14. The arrow indicates the characteristic 0.6-kb internal *Bgl*I fragment of IS2 (Ghosal *et al.*, 1979). **Right**: the gel was blotted to nitrocellulose and hybridized with nick-translated λr32 DNA serving as IS2 probe. The 0.6-kb fragment hybridizes as expected. The other two radioactive bands in tract **a** represent the two terminal *Bgl*I fragments of IS2. The strong hybridization to two bands with larger DNA fragments in track **c** is due to the presence of λ sequences in plasmid pAW14.

but it carries 15 of the 28 independently transposed IS2 elements.

To investigate the reason why the hot spot fragment H preferentially served for IS2 insertion, we asked if the IS2 elements all map at a single target site within this region. For this purpose, the 1.7-kb *Bam*HI/*Bgl*II fragment of P1 was isolated from gel after electrophoresis and cloned into the *Bam*HI site of pBR322 (Bolivar *et al.*, 1977). The resulting plasmid is pAW30. A restriction map for the restriction enzymes *Hae*II, *Hae*III, *Hin*cII, *Hpa*II, *Pvu*I and *Taq*I was determined by application of two methods: firstly single digests with each enzyme showed the fragment sizes; secondly application of the Smith/Birnstiel procedure (see Materials and methods) gave the order of the fragments (Figure 3). The order of some neighbouring restriction sites was in addition checked by double digests.

To localize precisely nine of the 15 IS2 insertions carried in segment H and to determine their orientation, we isolated the hot spot fragments, enlarged to 3 kb due to the IS2 insertion, of each of these mutants and cloned them into the *Bam*HI site of pBR322. By restriction cleavage analysis of the recombinant plasmids the insertions could all be mapped with a precision of ~50 bp (Figure 3). The nine mutations do not map at a single position and no target site was used more than once. Rather, they are distributed over the whole 1.7-kb fragment. The IS2 elements are found in both orientations.

DNA segment M located adjacent to the hot spot fragment H carries six IS2 elements, which is also higher than average. Possibly, the hot region of insertion extends into segment M. The locations of two of the six IS2 elements in segment M are known more precisely: they are 0.6 kb and 1.2 kb, respectively, from the border between segments M and H (Iida *et al.*,

Table II. Estimation of the transposition frequency of IS2

Days of incubation at 30°C	52	62	68	90
'Generations' of growth[a]	77	83	89	110
Number of colonies tested[b]	3375	3250	5600	5000
Number of mutants detected	6	13	16	24
Frequency of IS2 transposition per cell 'per generation'[c]	1.7×10^{-5}	3.6×10^{-5}	2.4×10^{-5}	3.2×10^{-5}

[a]Calculated from the factor of dilution with fresh tryptone broth.
[b]Colonies were replicated onto LA plates seeded with WA921 indicator bacteria. After overnight incubation at 42°C the plates were inspected for colonies not giving rise to lysis of the indicator.
[c]Calculated as described in the text.

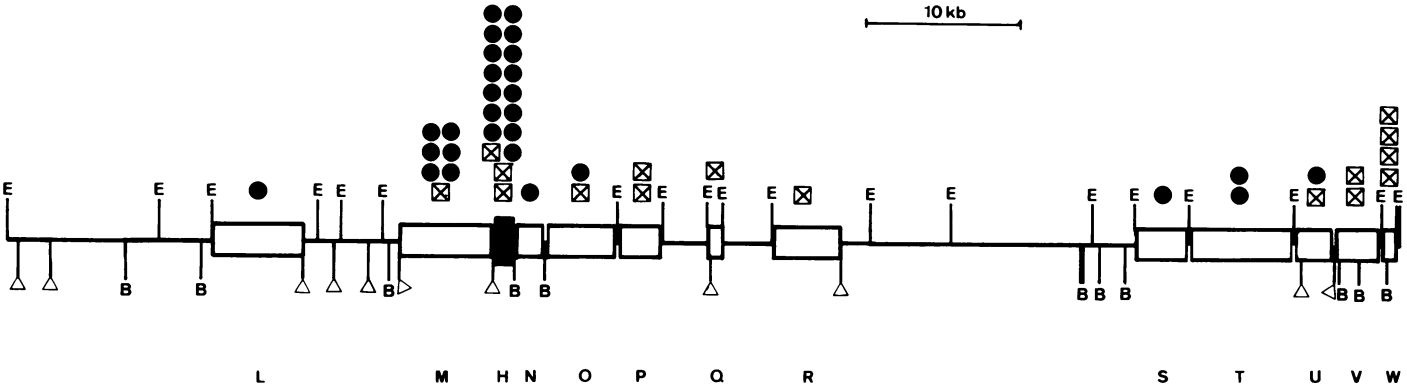

Fig. 2. Insertions in P1 and P1-15 DNA leading to defects in vegetative phage reproduction. The standard restriction map of the circular P1 genome is arbitrarily drawn linear. Restriction sites are: E: *Eco*RI, B: *Bam*HI, △: *Bgl*II. Not all restriction sites are shown. DNA of phage P1-15 is largely homologous to that of phage P1, except for short segments in the region labeled L and in the region just left of L. The symbol ● identifies IS2, and ⊠ other IS elements, shown to locate in the boxed regions (identified by letters) of the genomes of 44 independently isolated phage mutants. The hot region H of insertion carrying 18 insertions is shown in black.

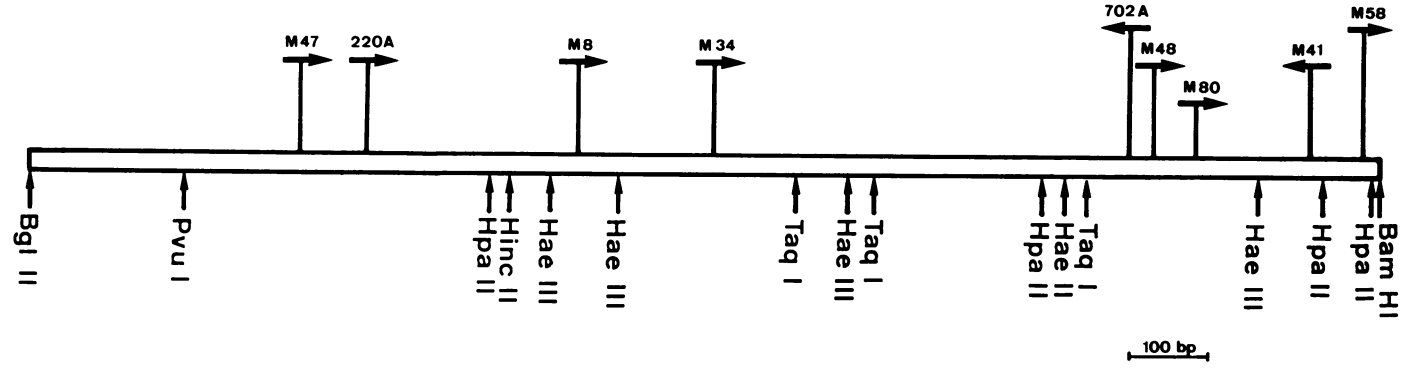

Fig. 3. Fine restriction map of the hot spot fragment and location of nine IS2 insertions within this fragment. Restriction sites are indicated below the bar that represents the hot spot fragment H of P1 DNA. The locations of nine studied IS2 insertions are given above the bar. The orientation of IS2 elements is represented by arrows that show in the direction from nucleotide 1 – 1327 of IS2.

1982). The remaining seven IS2 elements are scattered over six locations indicating that some genome regions serve only occasionally as targets for IS2 transposition.

Terminal sequences of three IS2 elements carried in the hot spot fragment

The terminal IS2 sequences of the three mutants M8, M34 and M47 were obtained following the sequencing strategy schematized in Figure 4. Comparing these sequences with those of the 1327 bp long IS2 in *galOP*-308 (Ghosal *et al.*, 1979), some differences were observed: M8, M34 and M47 show a one nucleotide insertion (C) between positions 1219 and 1222. Furthermore M8 has a T inserted between positions eight and nine, i.e. within the 41 bp long terminal inverted repeat at the left end; it has another T inserted between positions 1290 and 1292, i.e., in the inverted repeat at the right end of the element (Figure 5). The numbering of nucleotides was adjusted to the system of Ghosal *et al.*, but we are aware that the lengths of the variants may differ. None of these alterations affect any of the two large reading frames of IS2 (Ghosal *et al.*, 1979). The two sequenced variants of IS2 belong to mutants that were picked up in the same set of experiments. This may reflect the presence of different variants in the *E. coli* WA921 chromosome and indicate that more than one of these may participate in transposition to P1-15. Another variant of IS2 is carried in λr32 (Ghosal *et al.*, 1979).

As expected from previous studies on IS2 insertion sites (Ghosal *et al.*, 1979), 5 bp of target material has been duplicated upon the integration of each of the three IS2 into the phage DNA to produce the mutants M8, M34 and M47.

Discussion

The only criterion that had served for the isolation of the P1 and P1-15 phage mutations under investigation was a defect in vegetative phage reproduction. In other terms, the mutant prophages studied represent a population of lethal mutations of an entire viral genome rather than a set of mutations affecting a particular gene or operon. These mutations were isolated under what one can consider as normal growth conditions for the P1-lysogenic bacteria: growth in tryptone broth at 30°C, with periods of exponential growth interrupted by periods in the stationary phase. It was shown previously that a majority of prophage mutants thus isolated were IS mediated (Arber *et al.*, 1978, 1980). We now show that more than half of the IS elements that were independently trapped on the phage genome are IS2. This allows the conclusion that IS2 transposition from the host chromosome to the P1 and P1-15 prophage represents an efficient, natural mutagenic power.

For the growth conditions decribed above, IS2-mediated mutagenization of the P1 genome occurs with a frequency of 3×10^{-5} per cell per generation. We are aware that this frequency is probably an overestimate of IS2 transposition in exponentially growing cultures, and the influence of the intermittent cycles of exponential growth and stationary phase requires a more profound analysis.

We do not know if extrapolation of our results to the spontaneous mutagenesis of the host bacteria, *E. coli* K12, is justified. However, we think that the findings with P1 are suggestive of an important role of the resident IS2 elements in the natural mutation of K12 strains.

The study of IS2-mediated mutations reveals a surprising target specificity for IS2 insertion. Of the 28 mapped, independent IS2 insertions, 22 are located in a segment comprising ~10% of the P1 genome only, and a 1.7-kb subfragment that is <2% of the genome, contains 15 of these mutations. Nine of them were mapped precisely, and each is located at a separate site. We do not yet understand the reasons for the quasi-randomness of these nine IS2 insertions in the hot spot fragment given the obvious non-random use of the entire P1 genome as target for IS2 insertion. Nucleotide sequence studies in progress hopefully will answer this question. For the time being, the following arguments justify the statement that the P1 genome is not used randomly as a target for IS2 transposition. First, Figure 1 shows that segments of the P1 genome not having served for IS2 insertion have been used as targets for the insertion of other IS elements which caused the loss of essential phage functions. Secondly, a comparison of Figure 1 with the relatively densely populated genetic map of phage P1 (Walker and Walker, 1976; Yarmolinsky, 1982) reveals that additional regions carry essential P1 genes, which have not so far been found to serve as targets for IS transposition.

The sequence data show small differences in the IS2 elements participating in the mutagenization of the P1 genome. One variant, M8, has a one nucleotide insertion in both the left and in the right 41 bp long terminal inverted repeats, and these insertions are carried at different locations with respect

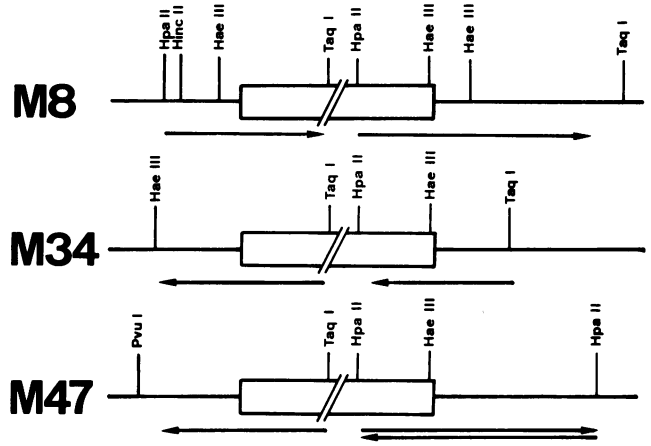

Fig. 4. Sequencing strategy for the variant IS2 ends. Horizontal lines correspond to phage DNA, the ends of the IS2 elements are indicated by boxes. The orientation of the elements is the same as in Figure 3. Sequencing was performed as indicated by the arrows.

	1	5	10	1220	1225	1290	1295
gal OP-308	T G G A T T T G C C . . .			G C C T A A G . . .		A A G T G A T A . . .	
M8	T G G A T T T G$_T$C C C . . .			G C$_C$C T A A G . . .		A A G T$_T$G A T A . . .	
M34	T G G A T T T G C C C . . .			G C$_C$C T A A G . . .		A A G T G A T A . . .	
M47	T G G A T T T G C C C . . .			G C$_C$C T A A G . . .		A A G T G A T A . . .	

Fig. 5. Terminal sequences of IS2 variants. Only the parts with identified differences to the published 1327 bp long IS2 sequence in *galOP*-308 (Ghosal *et al.*, 1979) are listed. The length of the available sequence is 109 bp at the right end of each variant and 78, 114, and 105 bp at the left end of M8, M34 and M47, respectively.

to the sequence homology in the repeat. Upon transposition, this variant duplicates 5 bp of target material at the site of insertion, as does the prototype IS2. The detected variations therefore seem not to influence either transposition *per se*, because they presumably occurred before the element transposed to P1, or the mode of interaction with the target. In contrast, an IS1 variant with a sequence alteration in the terminal inverted repeat had been described to cause an 8-bp rather than the usual 9-bp duplication of target sequence (Iida *et al.*, 1981).

The available material is expected eventually to reveal still hidden rules for target selection in IS2 transposition. Comparing the P1 DNA sequences around the sites of insertions of several IS2 elements in the hot spot region, some curious regularities have begun to emerge. More sequencing data is however necessary before we will be able to decide whether or not IS2 recognizes specific sequences in the target DNA.

Materials and methods

Media

Tryptone: 10 g Bacto trypton Difco, 5 g NaCl, 1 l H_2O, pH 7.2. LB: 10 g Bacto trypton Difco, 5 g yeast extract Difco, 10 g NaCl, 1 l H_2O, pH 7.2 completed after sterilization with 0.1% glucose. LBMg contains, in addition, 10 mM $MgSO_4$. LA plates contain, in addition to LB medium, 2.5 mM $CaCl_2$ and 15 g Bacto agar Difco per liter.

In selection for transformants, ampicillin was added to a concentration of 200 μg/ml.

Bacterial and bacteriophage strains

The *E. coli* K12 strains used are WA921 (Wood, 1966), C600 (Appleyard, 1954), AB1157 (Howard-Flanders and Theriot, 1966), W3350 (Arber, 1960) and DW103 (Walker and Walker, 1975). Bacteriophage P1 (Lennox, 1955) is from our collection, and its restriction cleavage map is published (Bächi and Arber, 1977; Iida and Arber, 1979). The phage P1-15 is hybrid 2 between P1 and plasmid p15B, and its restriction cleavage map was recently established (Arber and Wauters-Willems, 1970; Arber *et al.*, 1980; Meyer *et al.*, 1983; Iida *et al.*, 1983). All P1 and P1-15 phages carried the *c1*ts225 allele (P1*c1*ts225 was originally obtained from J. Scott). The phage mutants studied in this work are listed in Table I.

Isolation of P1 plasmids

The procedure described by Humphreys *et al.* (1975) was modified as follows for the preparation of cleared lysates: overnight 1 liter cultures in LB were harvested by centrifugation and the bacteria resuspended in 25% sucrose, 10 mM Tris pH 8, 1 mM EDTA. The cells were treated with lysozyme and then lysed with 2% SDS in the presence of 50 mM EDTA.

Isolation of DNA from phage particles

Some of the investigated mutants still produce phage particles upon thermoinduction, although these particles do not form plaques. They can serve for isolation of phage DNA. Lysogenic bacteria were grown at 30°C in 200 ml LBMg to ~2 x 10^8 cells/ml. The culture was then incubated for 30 min at 42°C and for a further 90 min at 37°C. After adding $CHCl_3$, the debris was removed by centrifugation. Phage was concentrated with 10% PEG and 0.75 M NaCl (Iida and Arber, 1977) and purified in a CsCl step gradient. Phage particles were dialyzed and the DNA purified by several phenol extractions, followed by extensive dialysis against 10 mM Tris-HCl, pH 8, 1 mM EDTA.

Mapping restriction cleavage sites

This was carried out using the method of Smith and Birnstiel (1976). The plasmid pAW30 containing the 1.7-kb hot spot fragment was linearized by *Bam*HI digestion, both ends were radioactively labeled. Then the fragment was asymmetrically cleaved by *Sal*I, the digestion products were separated on an agarose gel and a 2-kb fragment containing the hot spot, now labeled at one end, was isolated from the gel. This fragment was partially digested with one of the restriction enzymes, the partial digestion products were separated on a 1.5% agarose gel and the gel was autoradiographed.

Hybridizations to IS2 probes

Appropriate digests (*Bgl*I, *Bgl*II, *Bam*HI) of mutant phage DNA were electrophoresed on 0.6% or 1.2% agarose gels in TBE buffer (90 mM Tris pH 8.3, 90 mM H_3BO_3, 2.5 mM EDTA), the DNA was transferred to nitrocellulose by the method of Smith and Summers (1980). The nitrocellulose sheets were hybridized at 65°C overnight with an alkali-denatured, nick-translated (Maniatis *et al.*, 1975) probe in 1% SDS, 0.72 M NaCl, 90 mM Na-citrate, 78 mM KH_2PO_4, 6 mM EDTA, pH 7. Hybridization probes were either phage λr32 DNA (Brachet *et al.*, 1970) or the plasmid pAW14. pAW14 was constructed by cloning the *Bam*HI/*Eco*RI fragment of λr32, which contains the IS2 element, into pBR322 (Bolivar *et al.*, 1977).

End-labeling and sequencing of DNA fragments

Purified DNA fragments extracted from low melting agarose or polyacrylamide gels were labeled at the 3′ end by Klenow polymerase and sequenced as described by Maxam and Gilbert (1977).

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