Article

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Reference

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Genetics

Transposition of R factor genes to bacteriophage λ

(R factor evolution/inverted repetition/kanamycin resistance/heteroduplex mapping/restriction endonuclease)

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ABSTRACT **Transpositions of segments of R factors (antibiotic resistance plasmids) to bacteriophage λ have been selected and characterized. Cells of *Escherichia coli* harboring R factors that determine kanamycin resistance were infected with phage λ, and λ*kan* transducing lines were obtained. Each of the three examined is unusual when compared to λ transducing phages containing *E. coli* chromosomal genes: the kan insertions (*a*) occur at several sites, each well removed from the integration region POP′, (*b*) are not associated with deletion of λ phage DNA, and (*c*) are separable from the λ genome during transduction or during lytic growth. Two insertions from the same R factor contain 1.5 kilobase sequences repeated in inverted order. The properties of the λ*kan* phages suggest that R factors contain systems capable of mediating genetic exchange in the absence of extensive DNA homology. It is suggested that such systems of exchange may have played important roles in R factor evolution.**

The recent widespread use of antibiotics in medicine and agriculture has exerted strong selective pressures on bacterial populations. In the preantibiotic era (more than 2 or 3 decades ago) antibiotic resistance was rare in clinical bacterial isolates; however, today many isolates are resistant to one or more clinically useful antibiotics. It is known that the resistance in the majority of such isolates is due to the presence of resistance plasmids (R factors) (1).

Just how R factors have evolved has been a subject of considerable curiosity and speculation. There is an enormous variety of R factors in terms of size ($\langle 10^7 - \rangle 10^8$ daltons), and combinations of traits (resistances to a variety of toxic agents, modifications of cell surfaces, capacity for conjugation, etc.). In general, a given R factor shows little or no homology with the chromosome of the bacterium in which it was first detected (2). However, most R factors can exist in a variety of different bacterial species, and it is believed that R factors may have "picked up" their genes from unknown ancestral host species. Since many of the antibiotic-producing species of actinomycetes synthesize antibiotic-modifying enzymes that appear similar to the enzymes responsible for plasmid-borne antibiotic resistance, it has been suggested that actinomycetes may have been one of the ancestral reservoirs from which R factor genes were drawn (3, 4).

As aids to the study of the evolution, and also the function and regulation of expression of the component genes of R factors, we have constructed λ phage derivatives containing small segments of R factor DNA. In starting this work we were encouraged by the knowledge that: (*a*) phage λ can insert into a large number of different DNA sequences, and after induction, give rise to transducing phage lines containing DNA segments that had been adjacent to the site of phage insertion (5); (*b*) phages (P1 and P22) containing R factor genes have already been obtained (6–8); (*c*) certain segments of R factors, for example, the ampicillin resistance, amp^R, segment of plasmid RP4, can be transposed to other replicons (9). We anticipated that the nature and function of R factor DNA segments cloned in λ phage genomes would be easily studied; many genetic and physical techniques are available for analyzing λ genomes, and the biology of the phage is well known (10).

The three λ.R hybrid phage lines we describe here carry genes encoding resistance to kanamycin as insertions at sites in the λ genome well removed from the integration region *POP′*. The behavior and structure of these phages suggest that genetic exchange events, many of which may be similar to the previously noted transposition of amp^R, may have played an important role in plasmid evolution by facilitating the "pick-up" of antibiotic resistance and other genes from ancestral hosts. λ.R phages prepared *in vivo* may provide valuable tools for understanding these exchange events.

MATERIALS AND METHODS

Phage and Bacterial Strains. Phage λ*bb* is λ*b*515 *b*519 *c*I857 Sam7 (from R. Weisberg), contains two small deletions to the left of *att*λ (Fig. 1), is lysis-defective in bacterial strains that are not *su*III$^+$, and is thermoinducible. λ*c*I857 Sam7 was used as the reference "wild-type" phage for physical measurements. λ*imm*21 phages carrying the amber mutations O8, P3, Q117, A121, G9, and J17 have been described previously (11).

Escherichia coli K12 strains were: DB729, *su*$^-$ *att*λBOB′Δ; DB594 *att*λBOB′$^+$, *su*$^-$; DB973 *su*III$^+$; CB0129, *thy*$^-$ *leu*$^-$ *su*II$^+$. R factor plasmids JR67 (I_w, Sm^R, Kan^R) and JR72 (F_{II}, Cb^R, Cm^R, Hg^R, Kan^R, $SmSp^R$, Tc^R) from the collection of J. Davies, were introduced into DB729 by conjugation.

Media. Tryptone broth contains 10 g of tryptone and 8 g of sodium chloride per liter; L broth contains 10 g of tryptone, 5 g of yeast extract, 0.5 g of sodium chloride per liter, adjusted to pH 7 with NaOH. Solid media were prepared from the above by the addition of 1% agar. Where needed, glucose (0.2%), maltose (0.2%), and kanamycin (20 μg/ml) were added.

Isolation of λ.R Hybrid Phage. This procedure is a modification of the procedure of Shimada *et al.* (5). *E. coli* strains DB729/JR67 and DB729/JR72 were grown to stationary phase in tryptone broth, infected with λ*bb* at a multiplicity of 10 phage per cell at 30°, diluted 20-fold into 200 ml of tryptone broth, and grown with aeration at 30° to stationary phase. The phage lytic cycle was initiated as follows: a 10 ml

Abbreviations: POP′, the region in phage λ used for integration into a bacterial genome; *att*λBOB′, the high frequency site of λ integration into the *E. coli* chromosome (see ref. 23); Δ, deletion. Resistances to antibiotics are indicated: streptomycin, Sm^R; kanamycin, Kan^R; carbenicillin, Cb^R; mercury, Hg^R; tetracycline, Tc^R; ampicillin, Amp^R; chloramphenicol, Cm^R; and joint streptomycin-spectinomycin resistance $SmSp^R$; genes determining these are given in corresponding italics. kb, kilobases.

aliquot of the stationary phase culture was diluted 10-fold in L broth, incubated for 1 hr at 30°, followed by 15 min at 42°, and 90 min at 37°. The cells were centrifuged, resuspended in 0.01 M $MgSO_4$ (5 ml), lysed with chloroform, and the cellular debris was removed by centrifugation. This phage stock was used to infect stationary phase tryptone broth cultures of *E. coli* DB973 at a multiplicity of five phage per cell at 30°. An aliquot of infected cells was diluted 10-fold into 50 ml of tryptone broth, grown to stationary phase at 30°, plated on L agar containing kanamycin, and incubated at 30°. Kanamycin-resistant (Kan^R) colonies (frequency = approximately 10^{-9}) were purified on the same agar, thermally induced for phage production, and the phage were plated on strain DB973 at 30°. Single isolated turbid plaques were picked and transferred to L agar kanamycin plates, incubated at 30°, and then the Kan^R lysogens were induced to make stocks for lysogenization of *E. coli* CB0129. The CB0129 lysogens were used to make high titer stocks for physical analyses.

Electron Microscope Heteroduplex Mapping. Heteroduplexes were prepared by denaturation and renaturation of purified phage DNA essentially as described by Davis *et al.* (12) using a DNA solution containing 40 mM Tris, 4 mM EDTA (pH 8.5), 40 μg/ml of cytochrome *c* in 50% formamide spread on a hypophase containing 1 mM Tris, 0.1 mM EDTA (pH 8.5) in 20% formamide. DNA molecules were examined in a Philips EM300 microscope, and the negatives were traced on a Nikon profile projector and lengths were measured with a Numonics electronic graphic calculator. Five pSC101 DNA molecules in the vicinity of the heteroduplex were measured and the average was used as an internal length standard. The length ratio between pSC101 and λ^+ DNA was found to be 0.190. A λ^+ DNA molecule has 46.5 kb (kilobases) and pSC101 DNA contains 8.84 kb (13, 14).

Restriction Endonuclease Digestion. Specific endonucleases were prepared and used as described previously using 1% agarose gel electrophoresis for analysis (15). The buffer used for electrophoresis was 20 mM Na acetate, 40 mM Tris acetate, pH 8.

RESULTS

Phages capable of transducing kanamycin resistance were obtained from lysates of R-factor-carrying strains infected with λ*bb* as described in *Materials and Methods*. These phages will be referred to as λ*bb kan* phages. λ*bb kan-1* and λ*bb kan-2* were obtained from *E. coli* DB729/JR67 and λ*bb kan-3* was obtained from *E. coli* DB729/JR72. These transducing phages have been studied and characterized in several ways.

They are not defective; single phages form plaques which at 30° contain lysogenic kan^R bacteria in the plaque centers. The lysogens are resistant to neomycin and kanamycin, and cell-free extracts contain the expected neomycin-kanamycin phosphotransferase activities (D. I. Smith and J. Davies, unpublished).

In preparing stocks of λ*bb kan-3* from single plaques we noticed that a high proportion of the progeny phage were no longer capable of transducing Kan^R. These derivatives of λ*bb kan-3* have lost DNA, probably the *kan* genes, since they, unlike the parental λ*bb kan-3*, are resistant to treatment with 0.01 M EDTA (pH 8.5) at 37° for 10 min. (Ref. 16 shows how resistance to chelating agents is a measure of the size of λ phage genomes.) In contrast, λ*bb kan-1* and λ*bb kan-2* are quite stable during lytic growth (Table 1A).

The Kan^R transductants resulting from λ*bb kan-3* infections were all lysogenic (Table 1B).

Table 1. Stability of *kan*–λ association

A. Lytic growth

Phage	Preparation	Proportion Kan^R transducing phage	No. phage tested
λ*bb kan-1*	induced lysogen	>0.99	150
λ*bb kan-2*	induced lysogen	>0.99	150
λ*bb kan-3*	induced lysogen	0.91	91
λ*bb kan-1*	plate stock	>0.98	70
λ*bb kan-2*	plate stock	0.98	73
λ*bb kan-3*	plate stock	0.37	94

B. Transduction to Kan^R

Phage	Bacteria	Proportion Kan^R transducing phage	No. transductants tested
λ*bb kan-1*	594 *att*BOB′$^+$	>0.99	125
λ*bb kan-2*	594 *att*BOB′$^+$	0.99	99
λ*bb kan-3*	594 *att*BOB′$^+$	>0.97	35
λ*bb kan-1*	729 *att*BOB′Δ	0.32	37
λ*bb kan-2*	729 *att*BOB′Δ	0.41	58
λ*bb kan-3*	729 *att*BOB′Δ	>0.99	119

(A) Lysogens of strain DB 973 were induced as described in *Materials and Methods*. Plate stocks were prepared at 37° using DB973 as the bacterial host. The phage from young (8 hr) plaques were adsorbed to DB973 and incubated in soft agar until lysis was complete, and then harvested. Progeny phage were plated with DB973 at 30°. Single well-separated turbid plaques were picked and inoculated sequentially on lawns of DB973 indicator bacteria, and to sterile L kanamycin agar plates and incubated at 30°. The phages which in this first test did not give rise to Kan^R transductants were retested using the areas of turbid phage growth on the "master" lawn.

(B) The strains to be transduced were grown in tryptone maltose broth to stationary phase, centrifuged, and resuspended in 0.01 M $MgSO_4$ at 10^9 cells per ml and aerated at 37°, 30 min. The cells were infected at a multiplicity of 10 phage per cell (15 min adsorption at 30°) and then diluted 10-fold into tryptone broth and aerated at 30° for 2 hr. They were then plated on LB–kanamycin maltose agar at 30°. Lysogeny was tested by cell killing at 42° and by immunity to λc.

Kan^R transductants of DB729 resulting from λ*bb kan-1* and λ*bb kan-2* infection (10 from each) which appeared nonlysogenic were tested for the presence of phage genes O, P, Q, A, G, and J by marker rescue using amber derivatives of phage λ *imm*21. None of the 20 isolates tested contained any of these phage genes.

In the cases of λ*bb kan-1* and λ*bb kan-2*, Kan^R transductants were λ lysogens provided that strain 594 (*att*λBOB′$^+$) was used as the recipient. By contrast, however, most Kan^R transductants of strain DB729 (*att*λBOB′Δ) were not λ lysogens, by the criteria of lack of immunity or of any of seven λ phage genes (Table 1B). Approximately 1% of the transductants exhibited new growth requirements. Thus, *kan-1* and *kan-2* are transposable from λ to other replicons such as the *E. coli* chromosome. Apparently similar phenomena involving transposition of other R factor genes have been studied independently by others (9, 17–19).

The λ*bb kan* DNAs were examined by electron microscopy. Single DNA strands, and also heteroduplexes between λ*bb kan-1* and λ*bb kan-2* showed prominent "foldback" (double-stranded stem plus single-stranded terminal loop) structures at positions 70.7 and 76.1, respectively, on the λ physical map (Fig. 1a and b; Table 2). Heteroduplexes of

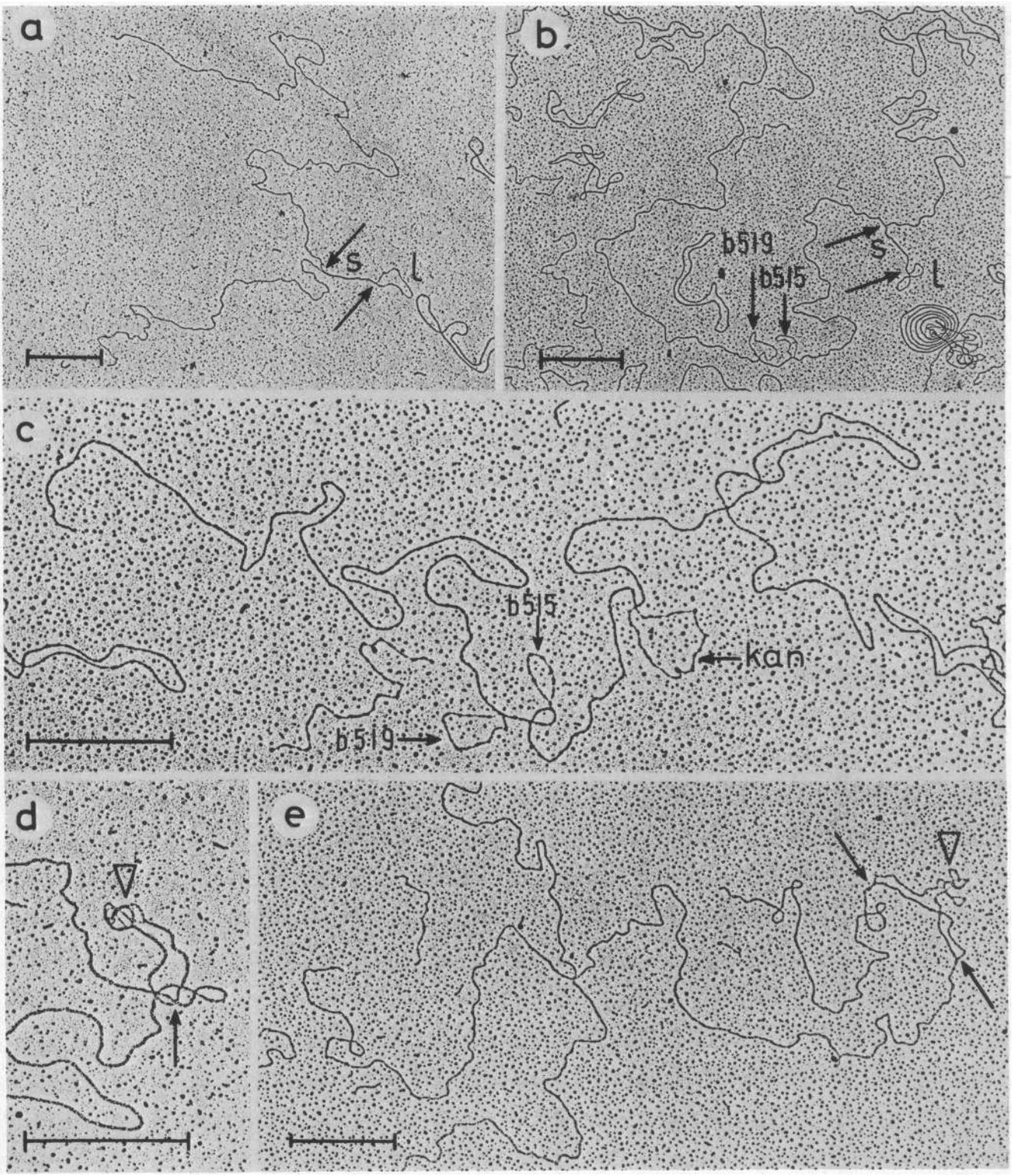

FIG. 1. (a) Single stranded λ*bb kan-1* DNA molecule displaying snapback region "S" (between the two arrows) and the single-stranded loop "l". (b) Heteroduplex of λ*bb kan-2*/λ : "S" and "l" denote the same as in (a). The small circles are pSC101 molecules. The single-stranded loops corresponding to the *b*515 and *b*519 deletions are indicated. (c) Heteroduplex of λ*bb kan-3*/λ. The single-stranded loop corresponding to the *kan-3* segment is indicated. (d) Homoduplex of λ*bb kan-1*/λ*bb kan-1*. Arrow shows insertion point of the two stem regions into λ; ▽ indicates the partially annealed loops. (e) Heteroduplex of λ*bb kan-1*/λ*bb kan-2*. Arrows show the two insertion points of the *kan-1* and *kan-2* fragments; ▽ indicates the partially annealed loops.

λ*bb kan-3*/λ show an insertion at 65.0 and no foldback structures were seen in single DNA strands or in heteroduplexes (Fig. 1c).

The heteroduplexes were examined for the presence of single-stranded DNA loops opposite the *kan-1*, *kan-2*, and *kan-3* insertions; none was found. The absence of such loops

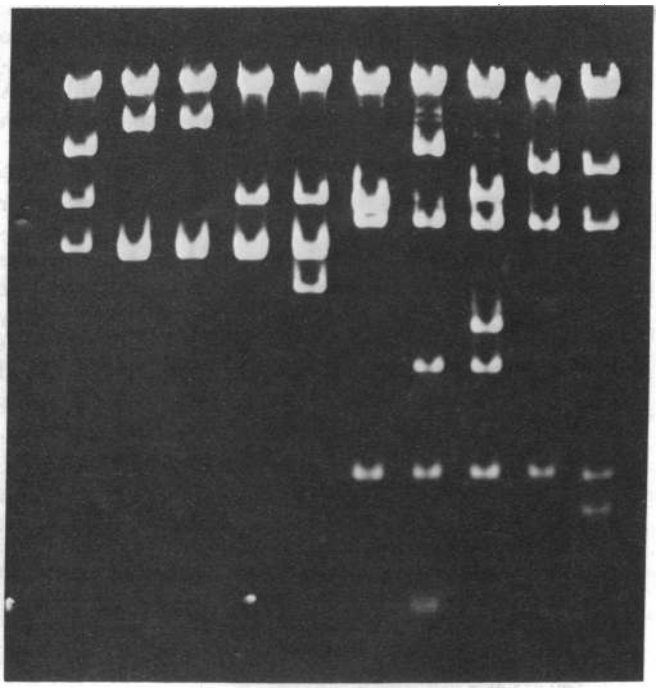

FIG. 2. Endonuclease digests of DNA from phage λ, λ*bb*, λ*bb kan-1*, λ*bb kan-2*, and λ*bb kan-3*. Digestion, separation, and identification of the fragments on agarose gels was carried out as described previously. Reading from the right, samples 1–5 were digested with endonuclease *Hind*III, and samples 6–10 were digested with endonuclease *Eco*RI. The samples are: (1) λ, (2) λ*bb*, (3) λ*bb kan-1*, (4) λ*bb kan-2*, (5) λ*bb kan-3*, (6) λ, (7) λ*bb*, (8) λ*bb kan-1*, (9) λ*bb-kan-2*, and (10) λ*bb kan-3*. In the *Hind*III digest of λ*bb* (sample 2) (*1*) the slowest moving fragment includes genes for the late region of the phage, (*2*) the next fragment (lower molecular weight) extends from a site in the b2 region to a site in the immunity region, (*3*) the third fragment contains genes to the right of the immunity region, (*4*) the fourth (faint) fragment contains the right end of linear λ DNA and (*5*) the fifth (fastest moving) fragment contains part of the b2 region, between fragments (1) and (2). In the *Eco*RI digest of λ*bb* (sample 7), (*1*) the slowest moving band contains the DNA of the late genes, (*2*) the faster moving band contains the immunity region, (*3*) the next band includes the *nin* region, (*4*) the fastest moving band, forming a doublet with band (*3*) contains genes to the left of the immunity fragment. A band of molecular weight 2.3×10^6 is not seen in any of the *Eco*RI digests of λ DNA because it is associated with fragment 1 through "sticky end" homology.

indicates that insertion of the kan DNA segments into the λ genome was not accompanied by significant deletion of any portion (i.e., more than 100 base pairs) of the λ genome.

The *kan-1* and *kan-2* insertions are probably the same DNA segment of the ancestral plasmid JR67, although inserted into different regions of the λ genome. Their lengths are the same (stem = 1.46 kb; loop = 2.32 kb) (Table 2), and a similar structure with the same dimensions in stem and loop regions is found in single DNA strands of the ancestral plasmid JR67 (unpublished). The terminal loops in homoduplexes, and in λ*bb kan-1*/*kan-2* heteroduplexes are partially annealed with each other (11 of 12, and 17 of 24 molecules scored, respectively) (Fig. 1d and e). Such annealing is not seen with any other single-stranded DNA segments. The double strandedness of the stem region in λ*bb kan-1* and λ*bb kan-2* indicates that the R factor DNA sequences at one junction with λ DNA are repeated (in inverted order) at the other junction. We interpret the partially annealed terminal loops as being completely homologous; partial annealing

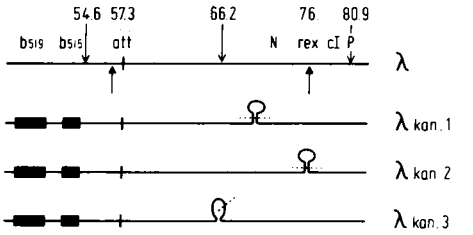

FIG. 3. The top figure indicates the central portion of phage λ DNA. *b*515, *b*519 indicate the position of the two deletions marked in black on the lower figures. The numbers indicate map position in percent and several λ genes and the attachment site *att* are shown. Open arrows (↓) show points of cleavage by endonuclease *Eco*RI and closed arrows (↑) are sites of cleavage by endonuclease *Hind*III. The broken lines (- - -) on the lower three figures indicate probable cleavage sites by *Hind*III.

would occur when the intramolecular association of complementary sequences in the stem region precedes the intermolecular reaction between the loops.

Digestion of the transducing phage DNAs with specific endonucleases confirmed and extended the conclusions obtained from electron microscopy. As shown in Fig. 2, the gel electrophoresis patterns of *Eco*RI fragments of λ*bb*, λ*bb kan-1* and λ*bb kan-2* were indistinguishable, with the exception that fragment 2 was replaced by a slower moving (higher molecular weight) fragment. This clearly indicated that λ*bb kan-1* and λ*bb kan-2* contain extra DNA added between the map positions 66.2 and 80.9 (Fig. 3), and that this extra DNA is not cleaved by *Eco*RI. The size of the fragments inserted into the two phages is identical, which is in agreement with the data obtained by electron microscopy.

From the analysis of *Eco*RI fragments of λ*bb kan-3* we deduced that this phage contained an insertion in *Eco*RI fragment 4, between map positions 54.6 and 66.2, and that this insertion, also, is not cleaved by the *Eco*RI enzyme.

Digestion of the three transducing phages with endonuclease *Hind*III fails to generate a fragment (designated 2 in Fig. 2) that extends between map positions 56.8 and 77.5. In the case of λ*bb kan-1* and λ*bb kan-2* three new fragments are produced (compared to λ*bb*); one of these fragments is common to both phage DNAs. This indicates that the DNA

Table 2. Electron microscope determination of position and size of *kan* DNA segments

Fragment	Position ± SD on λ map	Size in kb ± SD, estimate 1	Size in kb ± SD, estimate 2
kan-1	70.7 ± 1.0 (7)	5.3 ± 0.3	5.9 ± 0.5
kan-2	76.1 ± 0.3 (9)	5.3 ± 0.3	6.0 ± 0.9
kan-3	65.0 ± 0.3 (16)	4.1 ± 0.3 (17)	–

The numbers in parentheses refer to the number of molecules measured. Estimate 1 is derived by measurements of heteroduplexes (2 × size of stem region S + 1 × size of single-stranded loop l, cf. Fig. 1a and b). The stem and loop regions were 1.45 ± 0.08 kb (16) and 2.33 ± 0.15 kb (8) for λ*bb kan-1*, and 1.46 ± 0.08 kb (27) and 2.32 ± 0.14 kb (8) for λ*bb kan-2*, respectively. Estimate 2 is derived by subtraction of the size of *Eco*RI DNA fragment (2) of λ$^+$ (= 7.0 ± 0.1 kb) from the size of *Eco*RI DNA fragment (2) of λ*bb kan-1* [= 12.9 ± 0.4 kb (7)] and of λ*bb kan-2* [= 13.0 ± 0.9 kb (33)]. Only molecules whose size was within two standard deviations from the mean of the size distribution were considered. DNA molecules were spread and examined as described in *Materials and Methods*. The single-stranded loops in the heteroduplexes corresponding to the deletions *b*515 and *b*519 were used as internal length standards for single-stranded DNA.

fragment inserted into λ*bb kan-1* and λ*bb kan-2* is cleaved twice by *Hind*III. The size distribution of the new *Hind*III fragments produced from λ*bb kan-1* and λ*bb kan-2* is compatible with the conclusion that the same piece of DNA (from JR67) was inserted into both transducing phages, at different sites. *Hind*III digestion of λ*bb kan-3* produced only two new fragments that form a doublet; this indicates that the DNA inserted into λ*bb kan-3* (from JR72) is cleaved once and at a site equidistant from the *Hind*III sites at map positions 56.3 and 76.2.

Similar analyses with other restriction endonucleases, including *Hind*, *Hpa*II, and *Hae*III have allowed us to locate the various sites of insertion with more precision. The fragments coming from within the *kan-1* and *kan-2* insertions were identical for each enzyme.

DISCUSSION

The λ*bb kan* phages are unusual among λ transducing phage lines by three criteria: (*1*) The foreign (R factor) DNA they carry is inserted into regions of the λ genome distant from POP′. (*2*) The *kan* segments are *not* substitutions which replace λ DNA. (*3*) The *kan*–λ association is unstable during lytic growth (*kan-3*) or during insertion into chromosomes which lack *att*λBOB′ (*kan-1* and *2*). These characteristics make it seem unlikely that the λ*bb kan* phages arose by the usual mode of λ transducing phage formation; namely the insertion of λ into another DNA molecule by cleavage of the λ genome at *att*λ, followed by aberrant excision of λ from the recipient DNA molecule. The *kan* DNA segments may have been transposed to phage λ by the insertion of the R factors into λ, followed by the excision of all R sequences except those of the *kan* segments. Alternatively, the *kan* segments may have been transposed directly to λ without any R factor–λ fusion intermediate.

Transposability may be a general characteristic of genes found on R factors. The apparent identity of *kan-1* and *kan-2*, based on criteria of behavior in transduction, the inverted repeat stem and homologous loop structures, the distribution of restriction enzyme cleavage sites, their similarity to a portion of the ancestral JR67 plasmid, and their sites of insertion distant from *att*λ, suggests that *kan-1*(2) may comprise an intact unit of transposition.

Recent work has uncovered other R-factor-derived antibiotic resistance genes which behave either like *kan-1*(2) or *kan-3*. Tc^R and Amp^R can be transposed to any of a large number of sites on other replicons (9, 17–19). λCm, like λ*kan-3*, is unstable during lytic growth (L. Rosner, personal communication).

What processes might be involved in transposition? Models may be drawn from our understanding of the insertion–excision systems used by temperate phages such as mu-1, P2, and λ. Phage DNA is inserted into, and excised from, bacterial DNA by cleavages at special sites on the phage genome. The specificity of this cleavage is governed by the products of genes found very close to the special sites on the bacteriophage chromosome (20). Recently, small DNA elements resembling phage integration gene complexes have been identified in bacterial plasmids and chromosomes. These elements [designated IS (insertion sequence)] insert into genes, and thus cause mutation; several distinct species are known to exist. IS elements are present in several copies in the bacterial chromosome, in R factors, and in related plasmids. They appear to be important in integration of plasmids into the chromosome, pick-up of chromosomal genes by plasmids, and recombination within and among plasmid DNA molecules. IS2 and IS3 are similar in size to the 1.5 kb stem region of *kan-1*(2) (21, 22).

Regardless of the mechanisms used, it seems that plasmid-encoded systems which facilitate transposition and other types of genetic exchange in the absence of extensive homology must play important roles in plasmid evolution; one role would be in the "pick-up" of genes from ancestral hosts. The low frequency of transposition that we have observed may indicate that such exchange events are rare in nature. This might be expected, since the stability of an R factor, and therefore the activity of its exchange systems is subject to natural selection.

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