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Plasmid Co-Integrates of Prophage Lambda and R Factor R100

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Single and tandem insertions of prophage λ into R100 have been isolated. Insertions into the transfer genes, insertions into the transfer control gene *finO*, and insertions into regions that result in no detectable phenotypic change were found. From the last type, deletion mutants were isolated which established the sequence of antibiotic resistance genes as *tet-cml-fus-str-sul-mer* in R100. High-frequency transducing phage preparations λ*mer*, λ*sul str*, and λ*sul str cml* were also isolated from this type.

R factor R100 is a conjugative plasmid existing as a covalently closed circular deoxyribonucleic acid (DNA) molecule of 60×10^6 to 70×10^6 molecular weight in *Escherichia coli* K-12. The functions that this plasmid determines include: (i) the ability to replicate in a controlled fashion in the host cell, together with the incompatibility of this replication with that of related plasmids in the same cell; (ii) resistances to tetracycline, chloramphenicol, streptomycin (and spectinomycin, using the same adenylase [3, 9]), sulfonamides, mercuric ions, and fusidic acid (5); (iii) a transfer system that allows it to transfer its DNA by conjugation to a suitable recipient cell; and (iv) a two-stage system that controls expression of the transfer genes (7).

As a long-term goal, we are interested in elucidating the detailed genetic and physical structure of R100, including in particular the mechanisms whereby expression of the transfer genes is controlled. As a step towards that end, we first isolated a number of "co-integrates" of R100 and λ by using the techniques for λ insertion into "unusual" attachment sites pioneered by Shimada et al. (16). These λ insertions are frequently strongly polar and therefore help to identify operons. Second, from insertions of a λ*cI857* phage, deletion mutants can be selected as survivors to high temperatures. These can be used to locate the genes responsible for the various R factor functions, and they are also useful in the characterization of operons and the genes controlling them. Third, it is possible to isolate transducing phages from such insertions, which should allow gene amplification where it is required to purify a gene product, and serve as a source of DNA for measuring transcription of discrete regions of R100 using hybridization techniques.

This report presents the methods used to isolate R100 (λ) co-integrates and summarizes results showing the approximate locations of the prophage insertions. Deletion mutants from two integrates are described that have allowed a genetic map of the antibiotic resistance genes of R100 to be constructed.

(A preliminary report of this work was presented at the ICN Squaw Valley Conference on Plasmids, March 1975, and at the Annual Meeting of the American Society for Microbiology, New York, 27 April to 2 May 1975.)

MATERIALS AND METHODS

Bacterial strains. The host strain for R100 or R100-1 in which the λ insertions were generated was ED2149. This is a derivative of ED395 that is $T6^R$ and carries a deletion covering *nia, gal,* att_λ, and *bio* (ED395 is a $Lac_{\Delta U124}^-$ derivative of W3110). The att_λ deletion was transduced with P1 grown on CT10 (obtained from C. Town) into a (λ*cI857 susJ6 xis-1*)$^+$ lysogen of the parental att_λ^+ strain, since all spontaneous *gal* att_λ *bio* deletions also removed *uvrB*. The recipient strains used in matings, ED2144 and ED2145, were also originally derived from W3110. Both were His^- Trp^- $Lac_{\Delta X74}^-$ $T6^R\lambda^R$ $\phi80^R$ Str^R, and ED2145 was, in addition, lysogenic for wild-type λ. ED3814 is a related strain that is Su_{III}^+ $Lac_{\Delta X74}^-$ $T6^R$ Str^R. Strain DB10, in which fusidic acid resistance was determined (5), was obtained from J. Davies, as was CF1, a Fus^S $att\lambda^+$ strain.

Phage strains. The λ strain used for insertion into R100 was EDλ4, λ*cI857 susS7 b515 b519*. These two *b* deletions do not affect the phage integration functions (13) but should allow more plasmid DNA to be included in transducing phages. EDλ4 was constructed by crossing λ*cI857 susS7* (obtained from N. Murray) and λi^{434} *cIts susS7 b515 b519* (obtained from D. Berg). Pyrophosphate-resistant i^λ recombinants were selected. One such phage was purified and shown to have a density in CsCl gradients corre-

sponding to the expected 9.7% deletion. This phage, EDλ4, is referred to from here on as "λ."

Lysogens were selected by challenging with a mixture of λ*b2c*⁻ and λ*h*⁸⁰ *c*⁻∇*9*, both obtained from R. A. Weisberg via J. Gross (16).

Media. Most of the media were described previously (22), except that Oxoid nutrient agar was used. BBL bottom agar contained 5 g of NaCl, 10 g of BBL Trypticase, and 10 g of Difco agar per liter of water. BBL top agar was the same as the bottom agar, except that 6.5 g of Difco agar per liter was added. Phage buffer contained 3 g of KH_2PO_4, 7 g of Na_2HPO_4, 5 g of NaCl, 0.25 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $CaCl_2$, and 0.01 g of gelatin per liter of water.

Biotin and niacin were added to minimal medium at a final concentration of 1 μg/ml each. Antibiotics were used at the following concentrations: tetracycline hydrochloride, 20 μg/ml in nutrient medium and 10 μg/ml in minimal medium; chloramphenicol succinate, 50 μg/ml; sulfadimidine, 100 μg/ml; spectinomycin sulfate, 100 μg/ml; streptomycin, 200 μg/ml; and fusidic acid, 20 μg/ml. Mercuric chloride was added to a final concentration of 7.5×10^{-5} M.

The Spc^R phenotype was always used to test for the presence of the R100 *str* gene, whereas Str^R to a high level (200 μg/ml) of streptomycin was used to select recipient strains carrying a *strA* chromosomal mutation.

Lambda methodology. Phage λ stocks were titered by using ED3814 (Su_{III}^+) as the indicator strain and BBL medium.

Stocks of EDλ4, λ*b2c*⁻, and *h*⁸⁰*c*⁻∇*9* were made by a confluent plate lysis technique, using ED3814 as the host and Oxoid nutrient agar medium. The plates were overlaid with about 6 ml of phage buffer and stored at 5 C for 5 to 7 h; the buffer was then removed without disturbing the agar and filter sterilized. Titers ranged from 2×10^{10} to 10×10^{10}/ml.

Burst sizes and spontaneous curing frequencies of lysogens were measured as described by Shimada et al. (16), except that the lysogens were not made λ*vir* resistant but were used directly.

Isolation of λ lysogens. A method adapted from that of Shimada et al. (16) was used for λ lysogen isolation. Single colonies of plasmid-carrying derivatives of ED2149 were grown overnight at 37 C in L broth containing 0.2% maltose, without agitation. On the next day the cells were spun down, washed with 1/4 volume of M9 buffer, suspended in 1/5 volume of 0.01 M $MgSO_4$, and shaken for 1 h at 37 C. A 0.2-ml volume of the starved cells was mixed with 2×10^9 λ (multiplicity of infection [MOI], 1) and incubated for 30 min at 33 C. The mixture was then diluted 10- and 100-fold, and 0.1-ml portions spread together with 10^9 λ*b2c*⁻ and 10^9 λ*h*⁸⁰ *c*⁻∇*9* on minimal agar plates containing tetracycline.

After 2 days of incubation, 400 putative lysogens from each original starting clone were patched onto the same minimal medium and grown overnight. These plates were used to inoculate Oxoid nutrient plates by a replica plate technique and, after 8 to 10 h of incubation at 33 C, these nutrient plates were replica plate mated with ED2144 and ED2145, selecting Tet^R [Str^R] progeny. As is described below, this allowed clones carrying R100 (λ) co-integrates to be identified.

Mating conditions. Quantitative donor abilities were measured at 33 C by mixing 0.2 ml of an exponential culture of the donor strain at 2×10^8 cells/ml with 1.8 ml of a similar culture of the recipient strain. After incubation for 90 min, dilutions were plated on selective medium. A 90-min period of mating at 33 C gave approximately 100% efficiencies of transfer of R100-1 or F*lac* in control experiments.

Spontaneous deletion mutations from lysogens of R100 and R100-1. A single colony of a freshly reisolated strain carrying an R(λ) co-integrate was used to inoculate an overnight culture of L broth at 33 C. This was subcultured in the morning and grown with shaking to exponential phase, whereupon the culture was diluted to give 2×10^3 to 4×10^3 cells/ml. Portions of 0.1 ml of the diluted cultures were used to inoculate L broth overnight cultures incubated at 33 C. Other 0.1-ml portions were spread on two Oxoid nutrient agar plates. One plate was incubated at 33 C to obtain the viable count of the inoculum; the other was incubated at 42 C to detect the presence of any 42 C-resistant cells in the inoculum. On the next day, cultures found to have been made with inocula containing 42 C-resistant cells were discarded; the other cultures were diluted, plated onto Oxoid nutrient agar plates with or without an antibiotic, and incubated overnight at 42 C. Survivors were initially screened for their antibiotic resistance phenotypes by the replica plate method and for their transfer properties by replica plate mating. Final phenotyping was made with liquid cultures after the clones had been purified. Except for sulfadimidine resistance, antibiotic resistance was determined as the ability to grow as well-separated, single colonies on antibiotic-containing Oxoid nutrient agar. The sulfa resistance was determined with sulfadimidine in minimal agar plates. Fusidic acid resistance was determined after transfer to the sensitive host strain DB10 (5). Strains showing less than 1% of the transfer ability of R100 were designated transfer deficient.

Sucrose density gradients. In general, the methodology of Willetts and Bastarrachea (21) was used for sucrose density gradients. Cells were grown at 33 C in EM9, lysed with Sarkosyl by the method of Bazaral and Helinski (2), and "cleared" by centrifugation at 10,000 rpm for 15 min in an SS34 head of a Sorvall RC2B centrifuge. A 1:1 dilution (50 to 250 μl) of the cleared lysate(s) in TES buffer (22) was layered on top of a 5-ml 5 to 20% sucrose gradient in a polyallomer tube. ^{35}S-labeled R17 phage (kindly provided by W. Paranchych) was added where required as a reference marker. The tubes were centrifuged for 45 min at 3 C and 49,000 rpm in an SW50.1 head of a Beckman L2-65B ultracentrifuge. The tubes were then punctured, and 10-drop fractions were collected on 2.4-cm Whatman 3MM disks. After trichloroacetic acid precipitation, washing, and drying, the disks were immersed in a toluene butyl-PBD scintillant (CIBA; 0.5%, wt/vol) and counted in a Packard Tri-Carb scintillation counter.

Transduction with λ*mer* and λ*sul*. Low-frequency-transducing (LFT) lysates were prepared as follows. Overnight broth cultures of strains carrying EDR29 and EDR30 were subcultured into 20 ml of LB (22) at 33 C to a final density of 10^8 cells/ml.

After 60 min of vigorous aeration, the culture was shifted to 45 C for 15 to 20 min and then to 37 C for 1 to 2 h. Cells were collected, suspended in 4 ml of phage buffer, and mixed with 0.2 ml of chloroform to release the phage particles. After 15 to 20 min at room temperature, the mixture was agitated with a Vortex mixer and then centrifuged at 20,000 × *g* for 15 min. The supernatant solutions were filter sterilized and titered.

For transduction, an overnight standing culture (30 ml) of ED395 in L broth containing 0.2% maltose at 33 C was centrifuged, suspended in 15 ml of 0.01 M $MgSO_4$, and shaken for 1 h at 33 C. Portions of this culture were mixed with the lysates to give a ratio of approximately 1 λ plaque-forming unit per cell. After 15 min at 33 C, the mixtures were diluted 1:1 with double-strength LB and incubated another 30 min. The mixtures were then plated on both nutrient and glucose minimal agar plates containing mercuric chloride and on sulfadimidine agar plates. In a duplicate set of experiments, helper EDλ4 at an MOI of 3 to 5 was also present. It had no helping effect on the number of Mer^R clones isolated, but it did on the number of Sul^R clones. Accordingly, the clones purified for Mer^R were chosen from those prepared without added helper phage, and those for Sul^R were chosen from among clones prepared with added helper phage. Several clones arising after incubation at 33 C were purified by two consecutive single-colony isolations on the same media, and lysates from each were tested for their abilities to transduce strain ED3814 to Mer^R or Sul^R at high frequency, both in the presence and absence of helper EDλ4 at an MOI of 3 to 5. The procedures used were those described above for transduction of strain ED395. Strains of ED3814 that appeared to have been transduced to Mer^R or Sul^R at high frequency by this test were then used to prepare putative λ*mer* and λ*sul* stocks. Addition of helper phage in these transductions again did not increase the frequency of transduction of Mer^R but did increase the frequency of Sul^R transductants.

Transducing phage was prepared from purified stocks of these resistant ED3814 strains by growing the strains at 33 C and shifting them to 45 C for 20 min and then to 37 C for 2 h with vigorous shaking. The cultures were centrifuged, and the supernatant solutions were sterilized by filtration and titered. Most showed titers of 10^{10}/ml. To perform the transductions discussed in Results, these phage stocks were diluted with LB to 10^6 plaque-forming units/ml and then mixed with starved ED395 cells prepared as above to give an MOI of 0.01, with and without EDλ4 at an MOI of 10 to ~20. After 45 min at 33 C, the mixtures were diluted, spread on glucose M9 containing the different selective agents, and incubated at 33 C.

RESULTS

Detection of R100 (λ) co-integrates. The abnormal λ lysogens of ED2149 $(R100)^+$ selected by challenge with the λ*b2c*$^-$ and λh^{80} *c*$^-$∇9 phages were presumed to carry λ inserted either into the bacterial chromosome at one of the sites described by Shimada et al. (16) or into the R factor. Replica-plate mating tests were used to identify lysogens of the latter sort: chromosomal lysogens were expected to show no change in R factor transfer.

First, some clones transferred tetracycline resistance to the λ-lysogenic strain ED2145 at frequencies similar to that of R100 itself but not at all to the nonlysogenic strain ED2144. Such clones were presumed to carry λ inserted into R100 such that the transfer process was not affected; however, transfer of the R100 (λ) element to the nonlysogenic recipient would kill the cell by zygotic induction, so that no tetracycline-resistant progeny would be found. Eight mutants of this type were found among 11,000 clones tested.

Second, some clones transferred tetracycline resistance to ED2145 at a much higher frequency than R100 itself and again not to ED2144. Such strains were presumed to have λ inserted into one of the genes controlling the level of expression of the transfer genes, namely *finO* or *finP*. About 1% of the survivors were of this type: this high frequency suggests that the *finO* and/or *finP* genes contain a DNA sequence quite similar to the att_λ sequence. This high frequency made it unnecessary to impose any selective pressure for high-level donors among the lysogens. However, when it was found (see below) that all of the insertions affected only *finO* and not *finP*, attempts were made to select highly transfer-proficient R100 (λ) co-integrates from cells also carrying the $finO^+$ FI group plasmid R386. Since two copies of $finO^+$ were present in these experiments, this should have prevented R100 $finO^-$::λ insertion plasmids from transferring at high frequency, allowing selection of $finP^-$::λ insertions. However, 580 progeny from crosses between lysogens of this strain and a λ-immune Str^R recipient strain all proved to be $finP^+$.

Third, some clones failed to transfer tetracycline resistance either to ED2145 or to ED2144, and presumably in these the λ prophage was inserted into one of the *tra* genes, thereby inactivating it. However, because of the low frequency of transfer of R100 itself, such clones were difficult to detect and, instead, tra^-::λ insertions were sought starting with R100-1. R100-1 carries a $finO^-$ mutation and thus transfers very efficiently and is also fully sensitive to F-specific phages. Among 4,800 lysogens of ED2149 $(R100\text{-}1)^+$ tested, 21 transfer-deficient strains were found. This frequency was high enough to make selection of pililess R100-1 tra^-::λ mutants with F-specific phages unnecessary.

Fourth, some clones were $tet^s(\lambda)^+$ lysogens.

We tested 20 independent isolates of this kind for spontaneous tet^R revertants and found none. We concluded that none had a $tet^- ::\lambda$ genotype.

After the above presumptive identification of R100 (λ) lysogens, the strains were purified by two consecutive single-colony isolations, and their quantitative donor abilities were measured to confirm the original classifications. The results for some representative strains are shown in Table 1. Transfer at a much higher frequency to ED2145 than to the nonlysogenic ED2144 was taken as reasonable evidence that the strain carried an R100 (λ) co-integrate that killed ED2144 by zygotic induction. The relatively efficient transfer to ED2144 shown in the table was probably a reflection of the high spontaneous curing frequency for λ, since all the tetracycline-resistant ED2144 progeny tested were 42 C resistant.

For some strains, particularly those with λ insertions apparently in the transfer genes, a different test was used to confirm that λ prophage was indeed integrated into the R factor. Either R1-19 or ColB4, two plasmids incompatible with R100, were introduced into these strains, and the kanamycin-resistant or colicin-producing progeny were examined for their sensitivity to a temperature of 42 C and to tetracycline. Simultaneous loss of the 42 C-sensitive λ prophage and of the R100 tetracycline resistance was taken to indicate their covalent linkage in an R100 (λ) co-integrate.

All of the strains isolated were temperature sensitive as expected, due to the λ*cI857* mutation. The burst sizes and spontaneous curing frequencies of some representative strains are shown in Table 1. These are similar to those found for abnormal insertions of λ into the bacterial chromosome (16). Two main classes can be distinguished: those with burst sizes near to or less than unity, and those with burst sizes above 100. Based upon the work of Shimada et al. (16), these are presumed to be single and double (tandem) lysogens, respectively.

Physical confirmation of the insertion of one or two copies of λ prophage into R100 was obtained by comparing the rates of sedimentation of radioactively labeled plasmid DNA molecules in sucrose gradients. Two strains giving small burst sizes (carrying plasmids EDR20 and EDR29) and one giving a large burst size (carrying EDR35) were chosen.

First, ^{3}H-labeled plasmid DNA was co-centrifuged with ^{35}S-labeled R17 phage (Fig. 1); from these curves the sedimentation coefficients and hence the molecular weights of the plasmid molecules were calculated (Table 2). The molecular weights of EDR20 and EDR29 were approximately 28×10^6 greater than the molecular weight of R100 itself, and that of EDR35 was 57×10^6 greater. Since EDλ4 DNA has a molecular weight of 28×10^6, these are the expected values for single and double lysogens, respectively.

Second, mixed-label co-centrifugation experiments confirmed that EDR20, EDR29, and EDR35 plasmid DNA molecules all sedimented faster than R100 and that EDR35 sedimented faster than EDR29 (Fig. 2). We conclude that, in our strains, burst sizes of near to, or less than, unity indicate single insertions of λ prophage into R100, and burst sizes of 100 to 400 indicate tandem insertions of two λ prophages.

Location of the λ insertion points. For those insertions leading to a change in R100 or R100-1 phenotype, the point of insertion could be located by determining precisely which gene had been affected. Thus, the R100 $fin^- ::\lambda$ mutants

TABLE 1. *Properties of some R100 (λ) strains*[a]

Plasmid no.	Donor abilities to:		Burst size	Spontaneous curing frequencies
	ED2144	ED2145		
R100	0.15	0.14		
R100-1	166	159		
EDR20	14.3	500	3.2	3.8×10^{-4}
EDR21	19.5	175	3.6	2.1×10^{-4}
EDR22	9	226	2.0	8×10^{-4}
EDR23	11	146	162	3.5×10^{-4}
EDR24	13.4	172	0.02	7.5×10^{-4}
EDR25	4.1	84	4.5	2.1×10^{-4}
EDR29	7×10^{-4}	5×10^{-2}	0.2	7.6×10^{-4}
EDR30	7×10^{-3}	1.6×10^{-1}	1.0	2.9×10^{-4}
EDR31	6×10^{-3}	2.7×10^{-1}	2.4	3.9×10^{-4}
EDR35	2×10^{-5}	3×10^{-5}	103	8×10^{-5}
EDR40	$<1 \times 10^{-5}$	$<1 \times 10^{-5}$	0.01	5×10^{-4}

[a] EDR35 and EDR40 are R100-1::λ co-integrates. All of the other EDR plasmids in this table are R100::λ co-integrates.

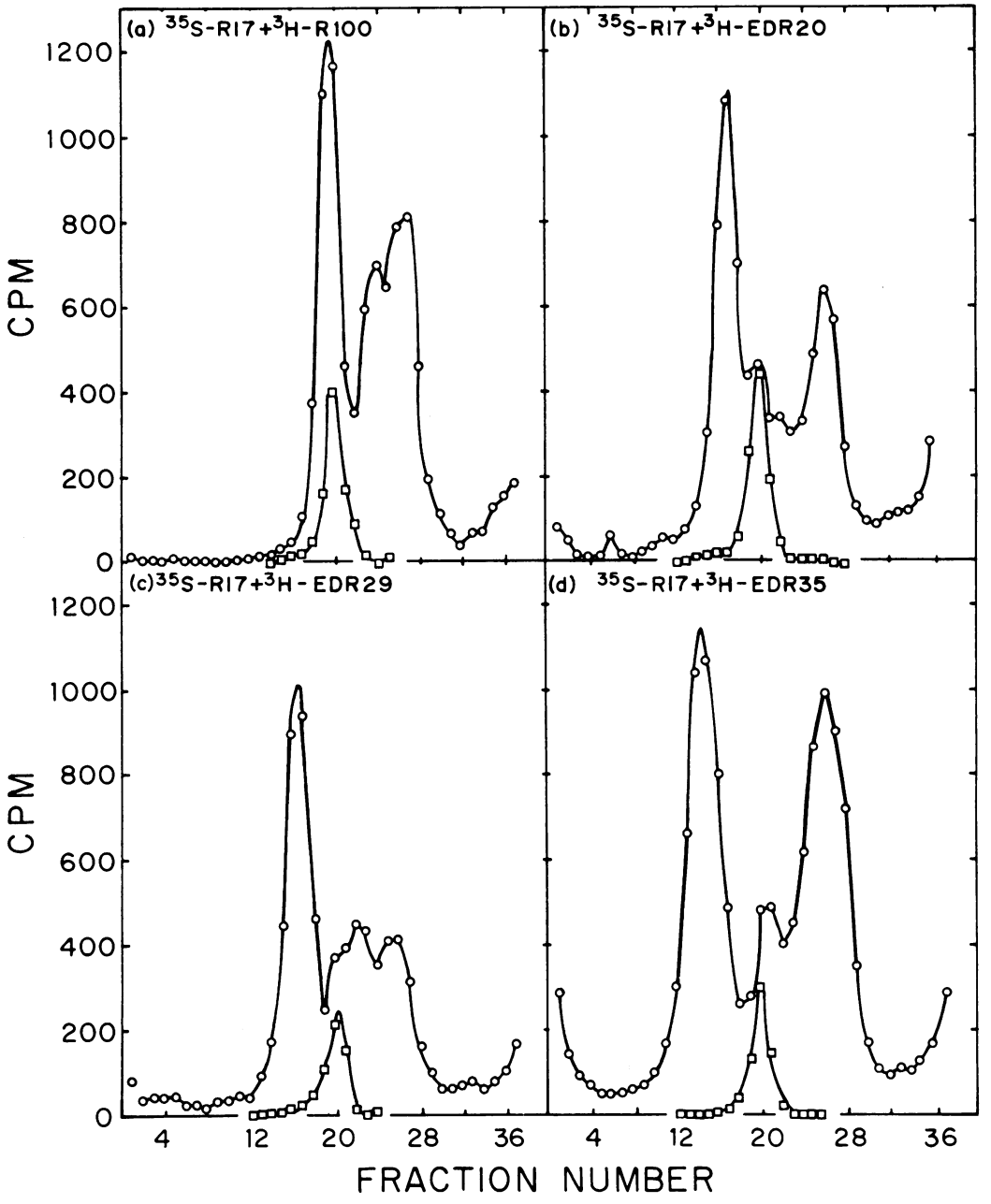

FIG. 1. *Neutral sucrose density gradient elution profiles of different R100 (λ) co-integrates. Symbols: ○, ^{3}H-labeled R100 derivatives; □, ^{35}S-labeled R17 phage. The sedimentation values calculated from these data were for covalently closed circular DNA form of the plasmid represented by the peaks at the bottom of the tube. Direction of the sedimentation was from right to left. The large peak of material of lower sedimentation value in each case may represent linear chromosomal DNA contaminants.*

were all shown to have the FinO^{-} phenotype since they did not inhibit F*lac* transfer, whereas their own transfer was inhibited by the *finO*$^{+}$ FI group plasmid R386, which cannot supply a *finP* product able to replace that of R100 (6). The R100-1 *tra*$^{-}$::λ mutants were analyzed by complementation with a series of F*lac* *tra*$^{-}$ mutants (1), and insertions into *traA* and *traD* have been identified so far. Analysis of the *fin* and *tra* insertions is continuing and will be reported in later publications.

The λ insertion points can also be located by

an analysis of a series of deletion mutants generated from each R100 (λ) co-integrate; in fact, this is the only genetic method available for insertions leading to no apparent change in R100 phenotype.

Analysis of R100 deletion mutants. From each insertion mutant it was possible to isolate deletion mutants (together with spontaneous λ⁻ revertants) as survivors at 42 C. (The techniques used are described in Materials and Methods.) It should be noted that so far only mutants retaining at least one resistance marker have been tested, for convenience in handling.

Most of the information concerning deletions derived from R100-1 *tra*⁻::λ and R100 *fin*⁻::λ co-integrates and from EDR31 will be described in later publications. However, a preliminary map of the R100 genes, as constructed from the data obtained, is presented in the Discussion (see Fig. 4). This also summarizes the locations of the various λ integration points.

Here we wish to present data concerning deletions derived from two insertions into R100 that caused no apparent change in phenotype, EDR30 and EDR29. Deletions from these two strains had several different phenotypes which allowed not only the λ insertion points to be mapped, but also all the antibiotic resistance markers carried by R100. The phenotypes of the deletion mutants are described in Table 3 and interpreted in Fig. 3. The isolation of single *mer*⁻ and *sul*⁻ deletions from EDR29 indicated that the λ insertion point was between *mer* and *sul*. Insertion in EDR30 could have been on either side of *mer*. The deletions established the order of the antibiotic resistance markers as *-mer-sul-str-fus-cml-tet-*.

Not unexpectedly, certain types of deletion predominated in these studies and the frequencies with which the different types were observed are also given in Table 3. Both of the plasmids yielded many derivatives, such as EDR52 and EDR55, that had lost *mer sul str fus cml* in a single step. The segregation of these genes from the "RTF" in *Proteus* is well-documented (11) and probably occurs by reciprocal recombination between the two Isl sequences located at the two junctions of the RTF and the "r determinant" that carries the antibiotic resistance markers (10, 15). This can presumably also happen, at a low frequency, in *E. coli*. However, none of our conclusions would be altered if in some deletions from EDR30 all of these antibiotic resistance genes were lost spontaneously, and other genes were lost together with the inserted λ as a second event.

TABLE 2. *Molecular weights of R100 (λ) co-integrates*

Plasmid	S[a]	Mol wt (× 10⁻⁶)[b]
R100	80	70
EDR20	92	97
EDR29	93	100
EDR35	101	127

[a] These were estimated for the covalently closed circular form of the plasmid DNA from the curves shown in Fig. 1, taking the sedimentation coefficient of R17 phage to be 78S (12).

[b] The sedimentation coefficient of the linear plasmid molecule was first calculated from that of the superhelical form by using the relationship derived by Fukatsu and Kurata (8). The molecular weight was then calculated by using the Studier formula (17).

All of the deletions were wild-type with respect to the *fin* and *tra* genes. In particular, *cml*⁺ *tra*⁻ deletions were not obtained among about 1,000 temperature-resistant survivors from each of EDR29 and EDR30. This could indicate the presence of a "forbidden region" for deletion, containing essential replication genes, between *tra* and *mer*. That a replication gene(s), required for integrative suppression, lies in this approximate position was suggested previously by Yoshikawa (23). Accordingly, we tentatively located a replication gene, *rep*A, between *tra* and *mer*. Another replication gene(s) may be located near *tet* since plasmids carrying deletions removing *tet*, such as EDR59, are unstable. A similar phenomenon was observed by Yoshikawa (23), and we followed his designation of the gene responsible for stability of inheritance as *repB*.

Finally, attempts were made to determine the orientation of the λ prophage insertion by testing the deletion mutants to determine whether any retained the λ *J* gene. This entailed marker rescue experiments using a λ*susJ6* phage. Most of the deletions from EDR29 had lost *J*, but EDR58, a Mer^R Sul^S Str^S Cml^S Tet^R deletion plasmid, retained it. The orientation of λ in EDR29 must therefore be *mer (J . . . cI . . . int) sul*. All the deletions from EDR30 had lost *J*, and the orientation of this prophage remains to be determined.

Transducing phages from EDR29 and EDR30. As demonstrated above, the λ prophages in EDR29 and EDR30 are probably located on either side of *mer*. Attempts were made therefore to prepare LFT lysates from strains carrying these plasmids, which were able to transduce nearby antibiotic resistance markers to the plasmid-free recipient strain ED395. Both lysates gave transduction to Mer^R with a high frequency, to Sul^R with a moderate frequency, and to Spc^R (i.e., transduction of the *str* gene of the R factor) very rarely.

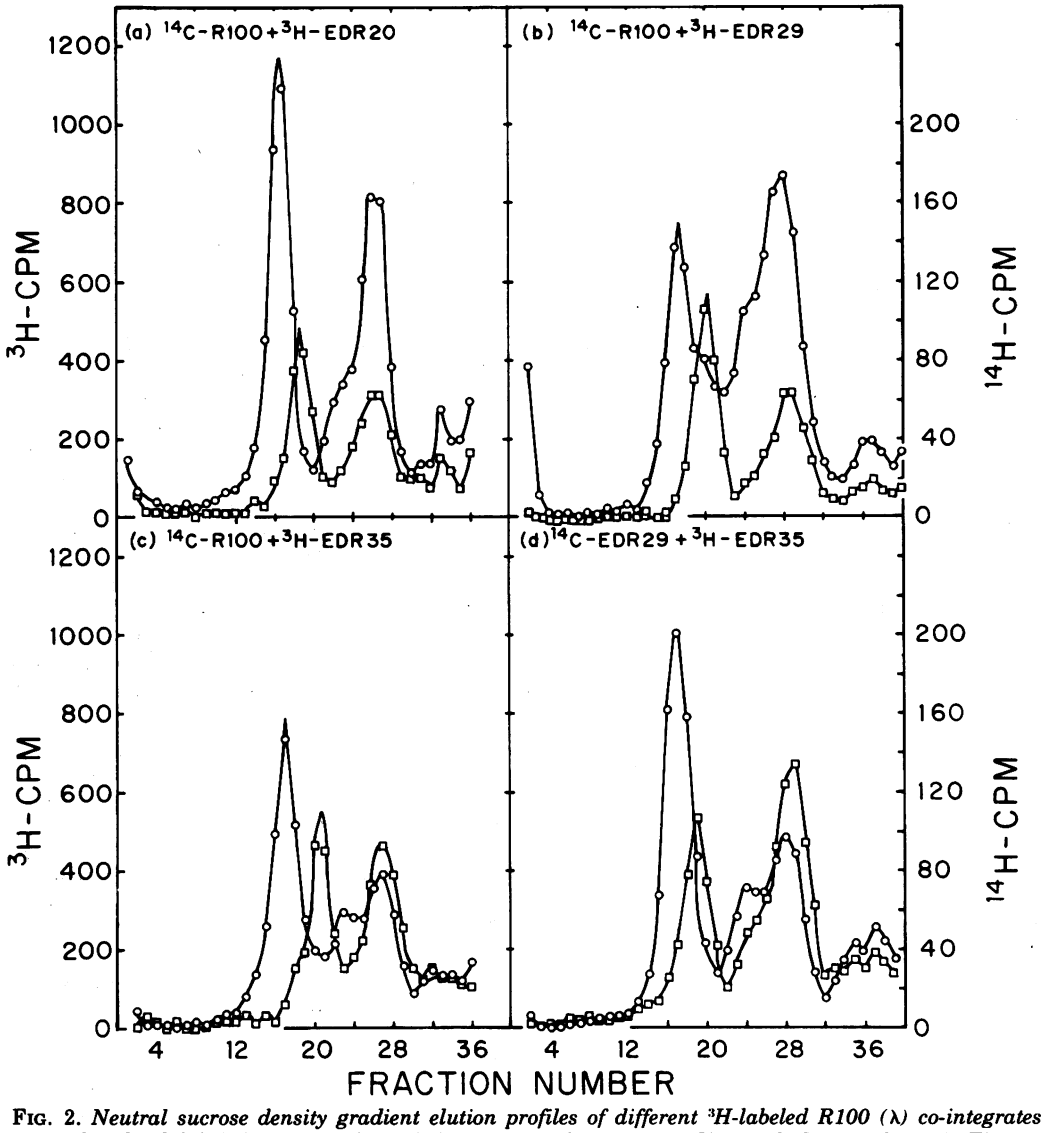

FIG. 2. *Neutral sucrose density gradient elution profiles of different ^{3}H-labeled R100 (λ) co-integrates compared with ^{14}C-labeled R100 or ^{14}C-labeled EDR29(d). Symbols: ○, ^{3}H; □, ^{14}C. Remainder as in Fig. 1.*

Six resistant clones for each type of antibiotic resistance were purified for each of the two LFT lysates and used to prepare further lysates. The antibiotic resistance-transducing frequencies per plaque-forming unit of these lysates were then measured, with ED3814 as the recipient. All.12 Mer^{R} derivatives of ED395 appeared to produce high-frequency-transducing (HFT) lysates for *mer*, whereas only 1 of the 12 Sul^{R} derivatives (from the EDR29 LFT) did so for *sul*. The 11 other Sul^{R} strains apparently gave LFT lysates that transduced all the antibiotic resistance markers of R100 at an equal but low frequency (10^{-7}): we have not tested these strains further. None of the 12 Spc^{R} derivatives, nor of 14 others then tested, gave an HFT for *str*.

None of the 12 HFT lysates for *mer* transduced any other antibiotic resistance marker, and none of the Mer^{R} transductants carried any other resistance. One representative of each, derived from the LFT lysates from EDR29 and EDR30, was chosen therefore for further testing. The HFT lysate for *sul* derived from EDR29, however, also transduced *str* but not *cml*, and Sul^{R} clones were Spc^{R} and Cml^{S}. *fus* could not be tested since DB10 was λ^{R}.

Lysates from these three ED3814 derivatives

were prepared, and the frequencies of transduction of the appropriate antibiotic resistance markers to ED395 were measured with and without helper phage (Table 4). Both λ*mer* lysates gave a very high frequency of transduction, and helper phage was not required, showing that these phages were not defective in any integration function. The λ*sul str* lysate gave high frequencies of transduction of *sul* or *str* only with helper, and this phage may lack *int* and/or *att*. Further details of the λ*mer*, λ*sul str*, and other antibiotic resistance-transducing phages will be the subject of a later publication.

The method of Schrenk and Weisberg (14) was then used to screen 1,000 additional Str^R transductants for efficient transducing phage production. Spectinomycin-resistant clones for this procedure were obtained by infecting starved cells of strain ED395 at an MOI of 1 with LFT lysates prepared from strains carrying EDR29 and EDR30 and with helper EDλ4 at an MOI of 10. Procedures of plating and incubating were as above. By this method we identified 25 strains capable of producing transduc-

TABLE 3. *Antibiotic resistance properties of deletions from EDR29 and EDR30*

Plasmid no.	Resistance property						No. with this phenotype[a]
	Mer	Sul	Spc	Fus	Cml	Tet	
Deletions from EDR30							
None	R	R	R	R	R	R	10
EDR50	S	R	R	R	R	R	3
EDR105	S	S	R	R	R	R	1
EDR51	S	S	S	R	R	R	1
EDR104	S	S	S	S	R	R	1
EDR52	S	S	S	S	S	R	10
Deletions from EDR29							
None	R	R	R	R	R	R	30
EDR53	S	R	R	R	R	R	2
EDR54	S	S	R	R	R	R	1
EDR106	S	S	S	R	R	R	1
EDR55	S	S	S	S	S	R	7
EDR56	R	S	R	R	R	R	1
EDR57	R	S	S	R	R	R	2
EDR58	R	S	S	S	S	R	5
EDR59	R	S	S	S	S	S	1

[a] The number of times that this phenotype was independently isolated.

TABLE 4. *Transduction of antibiotic and mercury resistance in strain ED395*

Transducing phage	Parental plasmid	Burst size[a]	Marker transduced	Transductants per 100 PFU[b]	
				No helper	EDλ4 helper
EDλ25	EDR29	0.6[a]	*sul*	0.03	9
			str	0.03	17
EDλ28[c]	EDR29	110[d]	*sul*	0.13	8.2
			str	0.02	6.8
			cml	0.07	7.5
EDλ29	EDR30	56[d]	*sul*	0.18	14
			str	0.03	13
EDλ26	EDR29	80[a]	*mer*	100	58
EDλ27	EDR30	90[a]	*mer*	32	12

[a] Measured in the transducing phage lysogen of ED3814.
[b] Measured as described in Materials and Methods, with ED395 as the recipient. PFU, Plaque-forming unit.
[c] This phage also transduced *fus* when tested in CF1.
[d] Measured in the transducing phage lysogen of ED395.

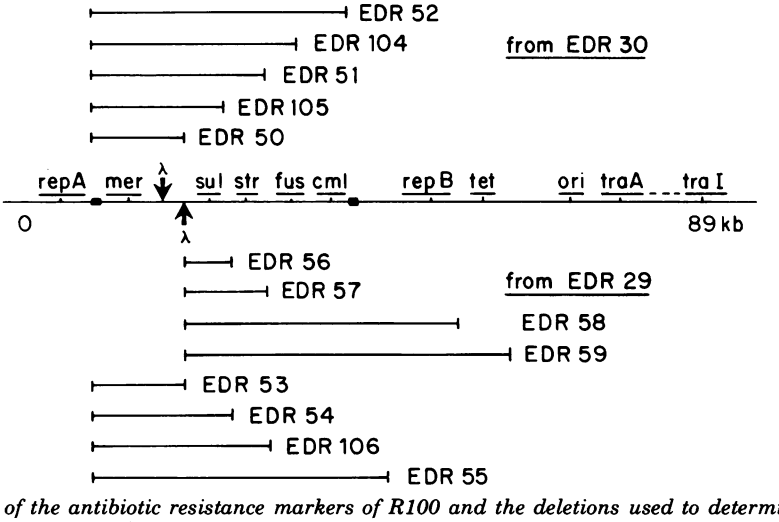

FIG. 3. *Map of the antibiotic resistance markers of R100 and the deletions used to determine the order shown. The lines identified with EDR numbers represent the deletions associated with the respective plasmids. No attempt is made in this map to represent map distances precisely. The linear sequence of the genes is correct, however, as far as present data allow.*

ing phages. The properties of phages from two of these, EDλ28 and EDλ29, resemble those of the λ*sul str* phage EDλ25 (Table 4).

DISCUSSION

The most immediate result of the work presented here is that the gene order for the antibiotic resistance genes of R100 is: *mer-sul-str-fus-cml-tet-tra*. Earlier work by Watanabe and Fukasawa (20), based upon P22 transduction of *Salmonella* strains bearing R222 (R222≡R100) had suggested the sequence: *sul-str-cml-(tra, tet)* for this R factor. In a later article (19) Watanabe pointed out that his gene sequence was based upon untested assumptions about the integration of drug resistance genes in the recipients and was therefore to some degree uncertain. Nevertheless, the gene sequence *sul-str-cml* he proposed (20) has been proved to be correct by our deletion mapping of these genes. In addition, our data extend the map to include the gene for mercury resistance *(mer)* and fusidic acid resistance *(fus)*.

The gene order *cml (tra, tet)*, uncertain from earlier work (4, 20), appears to be resolved to *cml-tet-tra* by our finding of deletion mutants terminating among these genes. A single R100 deletion mutant, EDR59, establishes the order as *cml-tet-tra* because it is Tet^S but still able to transfer *mer* (transfer data not shown). Some small uncertainty does in fact remain in the sequence *cml-tet-tra* from our work alone because we have only a single mutant of this key phenotype, and we have not rigorously excluded the possibility that it is Tet^S by virtue of a genetic event independent of the initial act of deletion.

The gene order *cml-tet-tra* has also been clearly suggested by the heteroduplex mapping of R100-1 performed by Sharp et al. (15) and by Hu et al. (10). These studies (10, 15) likewise confirmed the earlier observation (20) that *tet* resided apart from the other resistance genes.

The map of Yoshikawa (23) includes, in addition, two replication genes, *repA* (needed for integrative suppression) and *repB*, and agrees with our mapping of the *tra, rep,* and antibiotic resistance genes. Recently, an EcoR1 fragment from R6-5 carrying replication genes has been located between coordinates 88 and 1 kb (18). This is homologous to the region between coordinates 84 and 8 kb on R100, and therefore

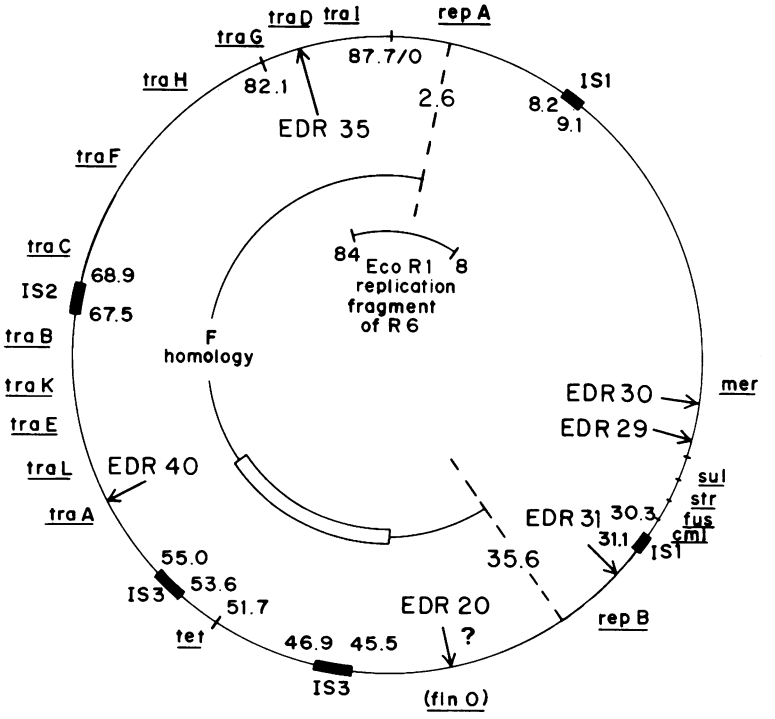

FIG. 4. *Genetic map of R100. The kilobase designations and approximate positions indicated by tic marks are derived in part from our interpretation of data of Sharp et al. (15) and in part by redrawing the R100-1 map of Hu et al. (10). The relative positions of the other markers derive from data presented herein and, earlier data from this lab. The marks next to the EDR numbers refer to the most likely insertion points of EDλ4 in the plasmids with those numbers. These insertion points and the relative positions of the mer and fus genes are the only new contributions to the R100 map.*

agrees with our location of the gene(s) essential for R100 replication (and including *repA*) here.

Some other genetic markers of R100, as well as structural features such as insertion sequences and regions of homology with related plasmids, have been located recently during an electron microscope heteroduplex analysis of F-like R factors (10, 15). In particular, it was shown that R100-1 is 87.7-kb long, that *tet* and *traG* are located at coordinates 51.7 and 82.1 kb, respectively, and that the "r determinant" carrying all of the other antibiotic resistance markers lies between two IS1 sequences at coordinates of approximately 9 and 30 kb. Furthermore, the region of R100-1 that is homologous with F (35.6 to 45.5 kb and 55.0 to 2.6 kb) carries the *tra* genes of both plasmids.

In Fig. 4 a synthesis is presented of the results of the heteroduplex mapping and of our results, including those given above and those to be described in future publications. On this map of R100, the locations of all the λ insertion points are also indicated. The position of the insertion in EDR30 is slightly uncertain. We mark it proximal to *sul* because we have isolated λ*sul str* transducing phages from EDR30. However, we have not found any deletions of antibiotic resistance genes in EDR30 that did not also delete *mer*, suggesting a λ prophage location distal to *sul*. With regard to other genes, it should be noted that the precise coordinates of most of the R100 genes are still unknown; however, it should be possible to determine at least some of these by heteroduplex mapping of λ insertions into various *tra* genes and of various R100 deletion plasmids.

Some of the other uses of plasmid-lambda co-integrates have been mentioned in the text. To summarize, we expect that such co-integrates and the deletion mutants and transducing phages derived from them will be useful not only for the genetic and physical mapping of the R100, but also for studying the operon structures of the *tra* and other genes and the control of their expression. Furthermore, the transducing phages, and possibly fusions to the highly efficient λ promoter p_L, will facilitate purification of gene products of particular interest. They will also allow construction of stable diploids for the various R100 genes, as well as serving as sources of discrete segments of R100 DNA for the analysis by hybridization of R100 transcription.

These methods are applicable to all the plasmids (i.e., the vast majority) that can be transmitted to *E. coli* K-12, and plasmid-lambda co-integrates should therefore prove a valuable tool in their genetic analysis. Co-integrates may prove more versatile for the dissection of bacterial plasmids than techniques involving restriction enzymes and in vitro recombination since they are not subject to such stringent site specificity limitations.

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