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Nonchromosomal Antibiotic Resistance in Bacteria: Genetic Transformation of *Escherichia coli* by R-Factor DNA*

($CaCl_2$/extrachromosomal DNA/plasmid)

STANLEY N. COHEN, ANNIE C. Y. CHANG, AND LESLIE HSU

Division of Clinical Pharmacology, Department of Medicine, Stanford University School of Medicine, Stanford, California 94305

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ABSTRACT **Transformation of *E. coli* cells treated with $CaCl_2$ to multiple antibiotic resistance by purified R-factor DNA is reported. Drug resistance is expressed in a small fraction of the recipient bacterial population almost immediately after uptake of DNA, but full genetic expression of resistance requires subsequent incubation in drug-free medium before antibiotic challenge. Transformed bacteria acquire a closed circular, transferable DNA species having the resistance, fertility, and sedimentation characteristics of the parent R factor. Covalently-closed, catenated, and open (nicked) circular forms of R-factor DNA are all effective in transformation, but denaturation and sonication abolish the transforming ability of R-factor DNA in this system.**

Biochemical and genetic investigations in several different laboratories (1–9) have established that antibiotic resistance factors (R factors) of the *Enterobacteriacae* consist of autonomously replicating units of extrachromosomal DNA. Moreover, certain R factors are formed by reversible covalent linkage of separate plasmids that independently harbor either resistance or transfer functions (4–9). Recent electron microscope studies of heteroduplex formation between the DNA of various R factors, and between DNA of R factors and certain other bacterial plasmids, have indicated that these earlier conclusions about the molecular nature and structural composition of R factors are correct (ref. 10, and Sharp, Cohen & Davidson, manuscript in preparation). However, the functional interrelationships of genes located in separate regions of R-factor DNA molecules and the role of the various molecular forms of R-factor DNA in the replication, genetic expression, recombination, and transfer of these plasmids are still not well understood. Availability of a system for genetic transformation of host bacteria by purified molecular species of R-factor DNA would enable study of these and other important aspects of R-factor biology.

Although bacterial transformation has been widely investigated in *Pneumococcus*, *Haemophilus influenzae*, *Bacillus subtilis*, and certain other bacteria (11), attempts to transform *Escherichia coli* with bacterial DNA have been largely unsuccessful. In contrast, *transfection* of *E. coli* spheroplasts by ϕX174 (12), λ (13), and other bacteriophages has been accomplished with high efficiency, and transfection of intact *E. coli* cells by the DNA of lambdoid phages has been widely studied by the "helper phage" assay of Kaiser and Hogness (14). Survival of the transfected cells is not required in such experiments, since production of viable phage particles is assayed with an appropriate strain of indicator bacteria. Recently, Mandel and Higa (15) have reported that *E. coli* cells that have been treated with calcium chloride can take up phage λ DNA and can produce viable phage particles. We report here that treatment of *E. coli* cells with calcium chloride also renders them capable of taking up molecules of purified R-factor DNA. Moreover, we find that the introduced R-factor DNA can persist in such cells as an independently replicating plasmid, and can express both the fertility and antibiotic resistance functions of the parent R factor.

Abbreviation: R factor, antibiotic resistance factor.

* The previous paper in this series is ref. 18.

MATERIALS AND METHODS

Bacterial Strains and R Factors. The I-like R factor, R64-11 (16), which specifies resistance to tetracycline (Tc) and streptomycin (Sm), was obtained from R. Curtiss. R6 (17), an F-like R factor that carries resistance to kanamycin (Km), neomycin (Nm), chloramphenicol (Cm), sulphonamide (Su), streptomycin, and tetracycline, was obtained from T. Watanabe. R6-5, a spontaneous variant of R6 that lacks tetracycline resistance, was isolated in our laboratory (18). The bacterial strains used in these experiments have been described (7, 22).

DNA Preparations. In certain instances, covalently-closed R-factor DNA was isolated and purified from *E. coli* as described (6, 7). Alternatively, a Brij-lysis procedure (19) was used for initial R-factor DNA isolation, and preparations obtained by this method were subsequently purified by centrifugation in cesium chloride–ethidium bromide gradients. The catenated, closed circular, and noncircular forms of R-factor DNA used in experiments comparing the relative transforming ability of the various R-factor DNA species were isolated from *E. coli* minicells. R-factor DNA was denatured by heating it at 98° for 5 min in 15 mM NaCl–1.5 mM Na citrate followed by rapid cooling at 0°. Sonication of R-factor DNA to about 9S fragments was done for 15 sec at 0° by a Branson model W185 D sonicator, and the size of the R-factor DNA fragments was confirmed by sucrose gradient centrifugation (7).

Transformation Reaction Mixture. Transformation was done by a variation of the procedure of Mandel and Higa (15), as modified by Lobban, Masuda, and Kaiser (personal communication). *E. coli* strain C600 was grown at 37° in H1 medium (20) to an optical density of 0.85 at 590 nm. At this point, the cells

were chilled quickly, sedimented, and washed once in 0.5 volume 10 mM NaCl. After centrifugation, bacteria were resuspended in half the original volume of chilled 0.03 M $CaCl_2$, kept at 0° for 20 min, sedimented, and then resuspended in 0.1 the original volume of 0.03 M calcium chloride solution. Chilled DNA samples in TEN buffer [0.02 M Tris (pH 8.0)– 1 mM EDTA (pH 8.0)–0.02 M NaCl] were supplemented with 0.1 M calcium chloride to a final concentration of 0.03 M.

0.2 ml of competent cells treated with $CaCl_2$ was added to 0.1 ml of DNA solution with chilled pipettes, and an additional incubation was done for 60 min at 0°. This second incubation of bacteria at 0° resulted in a 4-fold increase in transformation frequency, whether or not R-factor DNA was present. Bacteria were then subjected to a heat pulse at 42° for 2 min to enable uptake of R-factor DNA, chilled, and then either plated directly onto nutrient agar containing appropriate antibiotics or, where indicated, diluted 10 times into L broth (21) and incubated at 37° before plating. Cell survival was greater than 50% after calcium chloride treatment and heat pulse. Drug resistance was assayed on nutrient agar plates with the antibiotics indicated in specific experiments. Drug concentrations used were: neomycin (25 μg/ml), streptomycin (10 μg/ml), tetracycline (25 μg/ml), kanamycin (25 μg/ml), and chloramphenicol (25 μg/ml).

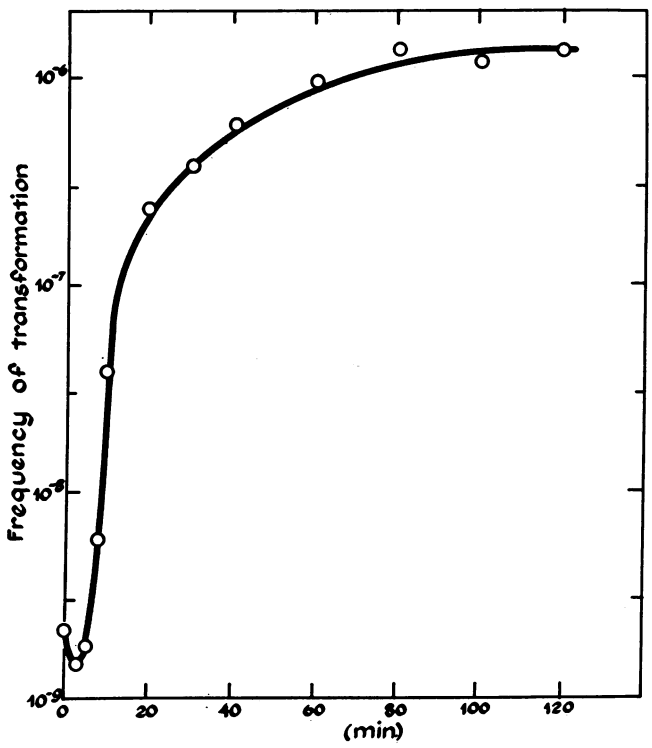

FIG. 1. Kinetics of expression of kanamycin resistance in transformed *E. coli*. After incubation of $CaCl_2$-treated cells with R6 DNA (0.6 μg/ml), bacteria were diluted 10-fold into antibiotic-free L broth. At the times shown, 0.1-ml samples of the bacterial culture were spread onto nutrient agar plates containing kanamycin and incubated overnight at 37° for determination of number of transformants. An identical sample was diluted appropriately, and plated on antibiotic-free nutrient agar to determine the total number of viable cells. Transformation frequency is expressed in terms of the number of kanamycin-resistant bacteria relative to the total number of viable cells.

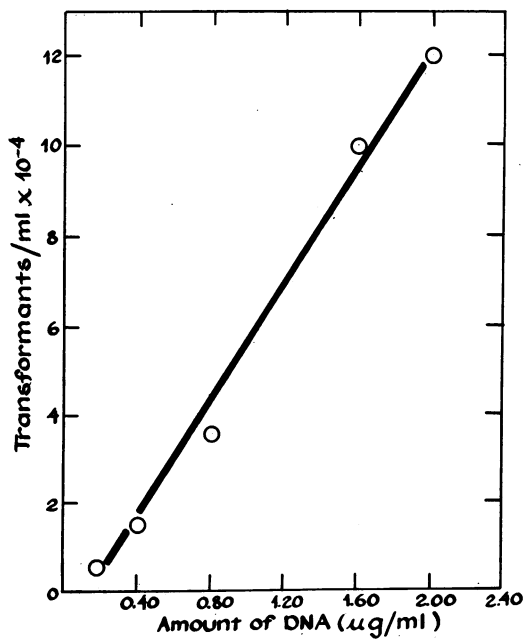

FIG. 2. Effect of concentration of R-factor DNA on transformation frequency. Various concentrations of covalently-closed R6 DNA were assayed for their ability to transform $CaCl_2$-treated *E. coli* to kanamycin resistance (see *Methods* and Fig. 1). Transformation frequency was determined after a 120-min incubation in antibiotic-free medium to allow complete expression of kanamycin resistance.

RESULTS

Expression of Antibiotic Resistance by Competent Cells. The kinetics of transformation of drug-sensitive *E. coli* to kanamycin resistance by covalently-closed R6 DNA is shown in Fig. 1. Resistance to kanamycin is expressed almost immediately by a small fraction of cells treated with $CaCl_2$ after uptake of R-factor DNA. The number of recipient bacteria exhibiting antibiotic resistance increases about 1000-fold during subsequent incubation in antibiotic-free medium and reaches a maximum in 1 hr.

Effect of DNA Concentration. The transforming ability of several different concentrations of closed circular R6 DNA is shown in Fig. 2. A linear relationship of transformation frequency to DNA concentration was observed throughout the range examined, and about 10^5 transformed bacteria were obtained per μg of R-factor DNA. Although the competence of cells treated with $CaCl_2$ to take up R-factor DNA varied in different experiments, the overall efficiency of transformation was of the same order of magnitude as the transfection efficiency reported for bacteriophage λ DNA in similarly treated cells (19) (about 2×10^5 transfectants/μg of λ DNA). In these experiments, bacteria were incubated in drug-free medium for 120 min after DNA uptake to allow complete expression of antibiotic resistance to occur.

Requirements for Transformation by R-Factor DNA. Since *E. coli* DNA does not appear to transform cells treated with $CaCl_2$ (15), we initially anticipated that covalent circularity of R-factor DNA might be required for its biological activity in the transformation system. However, the results of experiments presented in Table 1 indicate that closed circular, catenated, and nicked (open) circular forms of R6-5 DNA all possess transforming ability. Sonication of the R-factor DNA to 9S

fragments or denaturation of this DNA destroyed its ability to transform. Addition of DNase to reaction mixtures before the heat pulse (42°) also prevented transformation but had no effect on expression of antibiotic resistance when added after the 2-min incubation of competent bacteria with purified R-factor DNA. Moreover, phenol extraction of R-factor DNA preparations or treatment of them with either RNase or Pronase did not influence their transforming ability. The DNA of the I-like R factor, R64-11, was somewhat lower than that of the F-like R6-5 in transforming efficiency.

Expression of Different Drug Resistance Markers in Transformed Bacteria. Transformation of *E. coli* for various antibiotic resistance markers carried by R6 is shown in Table 2.

TABLE 1. *Requirements for transformation by R-factor DNA*

DNA species	Transformants/μgDNA
R6-5 (F-like)	
Closed circular	9.2×10^4
+DNase (before)	<0.3
+DNase (after)	7.7×10^4
+RNase (before)	9.6×10^4
+Pronase (before)	8.1×10^4
Phenol extraction	9.9×10^4
Isolated from transformed bacteria	7.0×10^4
Catenated	3.2×10^4
Open-circular	5.6×10^4
Denatured	<0.3
Sonicated	<0.3
No DNA	*
No bacteria	<0.3
R64-11	
Closed circular	9.4×10^3
No DNA	*
No bacteria	<0.3

Transforming ability of different molecular forms of R-factor DNA was assayed (see *Methods* and Fig. 1). All R6-5 DNA species used in this experiment were isolated from *E. coli* minicells, and DNA concentration ranged from 0.6–5 μg/ml. The sample labeled *open-circular DNA* was obtained from peak 3 of a cesium chloride–ethidium bromide gradient containing R-factor DNA isolated from minicells and was free from significant contamination by (noncircular) chromosomal DNA (22). This fraction was composed of about 85–90% nicked (open) circular R-factor DNA, and 10–15% noncircular R-factor DNA molecules (23). Transformation efficiency was determined after a 120-min incubation in antibiotic-free medium. Where indicated, R-factor DNA preparations were incubated with pancreatic DNase (10 μg/ml), pancreatic RNase (20 μg/ml, Worthington), or Pronase (100 μg/ml, Calbiochem) at 37° for 5 min before use in the transformation assay. DNase treatment was done in the presence of 10 mM $MgCl_2$; the RNase preparations were previously heated to destroy DNase activity (6). Pronase was self-digested for 37° for 1 hr and at 80° for 2 min, and then chilled rapidly before use. Phenol extraction of DNA was done as described (7). The terms *before* and *after* refer to the period of incubation of R-factor DNA with $CaCl_2$-treated cells at 42°. Since the competence of bacteria varied somewhat in different experiments, the effects of R-factor DNA structure on transformation were determined with a single batch of $CaCl_2$-treated cells.

* No colonies were observed when 10^9 bacteria were assayed in the absence of DNA.

As seen in this table, R^+ transformants could be selected with Km, Nm, Cm, or Tc, but no transformants were obtained when initial selection was carried out with Sm. Our inability to select R6 transformants with streptomycin was surprising since persistance of the Sm marker on the R factor was demonstrated by subsequent expression of streptomycin resistance in nearly all of the clones that had been initially selected with the other antibiotics (Table 2). However, the observation that streptomycin resistance is the last drug marker to be phenotypically expressed by bacteria that have received an R factor by conjugation (23, 24) suggests that expression of the Sm marker in transformed bacteria may require more extensive incubation in antibiotic-free medium than was used in these experiments.

R-Factor DNA Species Isolated from Transformed Bacteria. DNA isolated from a transformed *E. coli* clone carrying all of the antibiotic resistance determinants originally present on R6 was subjected to cesium chloride–ethidium bromide gradient centrifugation. As seen in Fig. 3 (*top*), a closed circular DNA peak (25) (peak *A*) was identified in this preparation, in addition to a peak (peak *B*) characteristic of DNA preparations isolated from the R^- *E. coli* recipient strain (7). R-factor DNA collected from peak A was treated with isopropanol to remove ethidium bromide (26), dialyzed against 0.02 M Tris (pH 8)–1 mM EDTA (pH 8)–0.02 M NaCl, and a sample of this DNA was then centrifuged in neutral sucrose in the presence of 34S λ DNA marker (7). As seen in Fig. 3 (*bottom*), this R-factor peak was composed of DNA having the same S value as the closed circular DNA of the parent R factor (75S) (7); a small amount of the 52S open circular species of R6 DNA was also observed in this preparation.

R-factor DNA isolated from transformants could transform other bacteria at an efficiency comparable to that shown by the parent R factor (Table 1). In addition, *E. coli* cells that were transformed to antibiotic resistance by R6 DNA transferred this resistance at the same frequency (about 10^{-4}) as bacteria that had received the R factor by conjugation.

DISCUSSION AND SUMMARY

The experiments we have reported here indicate that purified R-factor DNA can transform *E. coli* treated with $CaCl_2$ to

TABLE 2. *Expression of different drug resistance markers in transformed bacteria*

Selected by	No. of colonies	Number resistant to				
		Km	Nm	Cm	Tc	Sm
Km	30	30	30	30	30	27
Nm	52	52	52	52	52	48
Cm	35	35	35	35	35	33
Tc	36	36	36	36	36	36
Sm	0					

After transformation of $CaCl_2$-treated *E. coli* by R6, as indicated in Table 1, 0.1-ml samples of bacterial cultures were spread onto plates containing the antibiotic indicated, and numbers of antibiotic bacteria were determined. Separate bacterial clones isolated from each antibiotic plate were examined for the presence of other antibiotic resistance determinants by stabbing colony samples onto appropriate drug-containing plates.

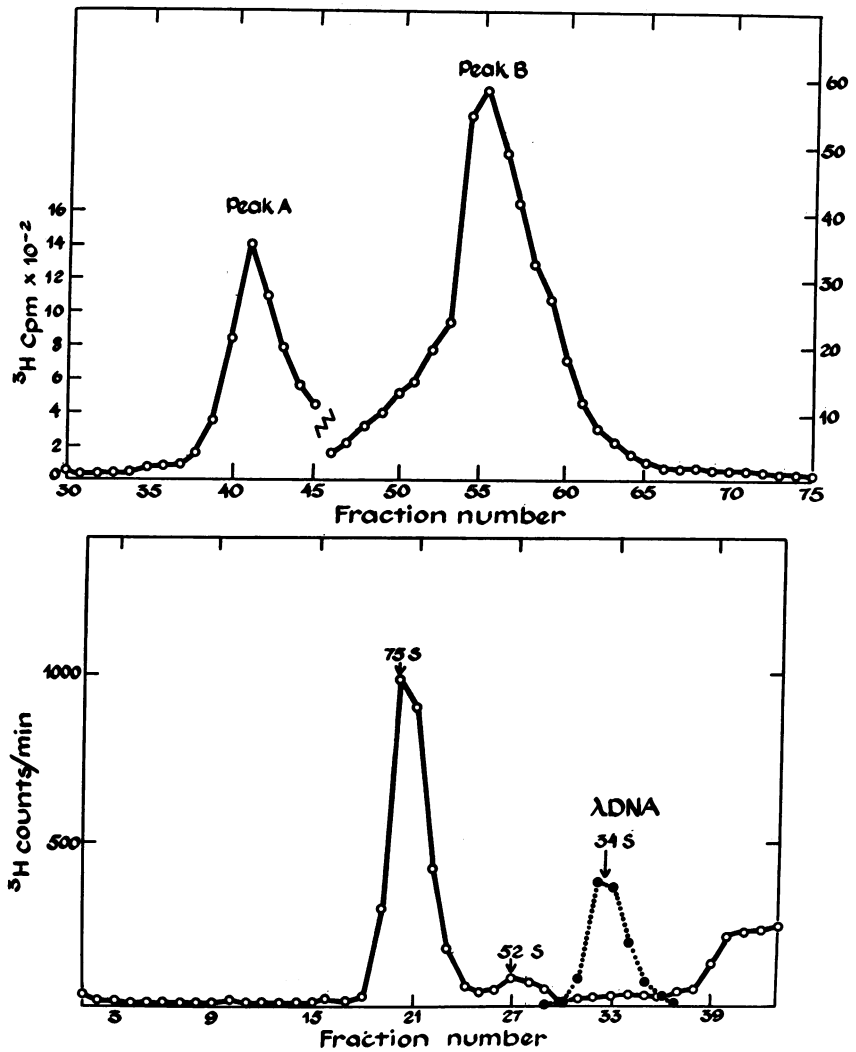

FIG. 3. Centrifugation analysis of R-factor DNA present in transformed cells. (*Top*) Tritium-labeled R-factor DNA was prepared by growth of bacteria in modified M9 medium (2) containing [^{3}H]thymidine (5 μCi/ml) and deoxyadenosine (250 μg/ml) as described (23). Bacterial cultures were harvested and lysed, and centrifuged for 42 hr at 39.5 rpm at 20° in cesium chloride (ρ = 1.5570) containing ethidium bromide (1 mg/ml) in a Spinco 50 Ti rotor. Collection of fractions and assay of a 0.01-ml sample of each fraction for radioactivity was done as described (7). Note the change in units for *peak B*, which is drawn according to the scale shown at the right of this figure. (*Bottom*) After removal of ethidium bromide from fractions 40–43 of *peak A* in Fig. 2*A* by equilibration with isopropyl alcohol (26), DNA was dialyzed against 0.02 M Tris (pH 8.0)–1 mM EDTA–0.02 M NaCl and a 0.1-ml aliquot was layered onto a 5–20% linear sucrose gradient containing 1mM EDTA, (pH 8.0)–1 M NaCl–0.02 M Tris(pH 7.4). The gradient was centrifuged for 50 min at 39.5 rpm in a Spinco SW50.1 rotor at 20° in the presence of ^{14}C-labeled λ DNA marker (34S) that had previously been heated to 75° and rapidly cooled to insure its linearity. Fractions (0.12 ml) were collected, precipitated with cold 5% trichloroacetic acid, and counted (7).

multiple antibiotic resistance. R-factor DNA is rapidly taken up by competent cells, and drug resistance is expressed almost immediately in certain of the recipient bacteria. In the case of kanamycin resistance, the number of transformants continues to increase for about 1 hr after DNA uptake and subsequently forms a plateau. Since the kanamycin resistance of R6 is believed to be dependent on synthesis of the enzyme, kanamycin monophosphotransferase, in order to inactivate the drug (27), the observed rapid expression of antibiotic resistance in a fraction of the transformed cells is somewhat surprising. Presumably, uptake of growth inhibitory concentrations of kanamycin by such cells is delayed until sufficient quantities of the enzyme have been formed to allow bacterial multiplication to occur in the presence of the antibiotic.

Although prior treatment of R-factor DNA with pancreatic DNase prevents transformation, addition of DNase to transformation reaction mixtures after a 2-min incubation of competent bacteria with R-factor DNA at 42° has no effect on the frequency of transformation. Furthermore, transformation is sharply reduced by incubation of cells treated with $CaCl_2$ at 42° for 2 min before addition of the R-factor DNA. These latter results suggest that bacteria treated with $CaCl_2$ remain competent to take up R-factor DNA for only a very brief period at elevated temperature. This interpretation is consistent with the results of earlier experiments with λ DNA by Mandel and Higa (15) and by Lobban (personal communication).

Closed and open-circular forms of R-factor DNA are both

capable of transforming recipient cells. However, denaturation and sonication eliminate the transforming activity of R-factor DNA in this system. *E. coli* cells that have been transformed by R-factor DNA acquire an independently replicating closed circular DNA species indistinguishable from the parent R factor, and can transfer this DNA normally to other cells by conjugation. The general usefulness of this system for study of the biological properties of bacterial plasmids is suggested by our observation that DNA from both F-like and I-like R factors is capable of transforming *E. coli*. Moreover, the observation (van Embden and Cohen, in preparation) that DNA obtained from a *nontransmissible* plasmid coding for Tc resistance is able to transform *E. coli* indicates the additional use of this method for the investigation of nontransferable, independently replicating species of plasmid DNA.

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