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Non-Chromosomal Antibiotic Resistance in Bacteria, III:* Isolation of the Discrete Transfer Unit of the R-Factor R1

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Abstract. A covalently-closed circular DNA species, banding at a buoyant density of $\rho = 1.709$ g/cm^3 in CsCl, has been identified in *antibiotic-sensitive* colonies of *E. coli* strain AB2463 (rec A^-) after mating with a *Proteus mirabilis* strain that carries the R-factor, R1. This plasmid, which represents a stable segregant of R1 that has lost all of the drug resistance determinants present on the parent R-factor but which has retained its ability to be transferred by conjugation, fulfills the functional definition of the R-factor transfer unit (RTF).

We have reported previously[1,2] that the R-factor R1, which carries resistance to chloramphenicol, streptomycin, ampicillin, kanamycin, and sulfonamide,[3] includes three independently-replicating species of covalently-closed circular DNA in *Proteus mirabilis;* in contrast, a single molecular species includes more than 98% of the closed circular DNA isolated from *Escherichia coli* containing the same R-factor. The predominant molecular class of DNA circles found in *E. coli* that carries R1 appears to express both resistance and transfer functions in that host, and is indistinguishable in buoyant density and molecular weight from the largest of the three R-factor species identified in *Proteus.* Moreover, the molecular weights, buoyant densities, and contour lengths of closed circular DNA molecules of R-factor isolated from *Proteus* suggested that the largest of the three species might represent a composite of the two smaller units.

Our earlier findings are consistent with the view that at least certain R-factors are formed by reversible covalent linkage of plasmids that separately harbor either resistance or transfer functions. As Campbell[4] has suggested, the apparently conflicting genetic experiments of Watanabe[5] on one hand and of Anderson[6] and Mitsuhashi[7] on the other can be reconciled by such a model, which is formally similar to the one proposed for integrative recombination of the temperate bacteriophage λ into the *E. coli* chromosome.[8] According to this scheme, the resistance transfer factor (RTF) unit is separately transferable as an independently-replicating plasmid, as well as in combination with another plasmid carrying resistance determinants. The resistance determinant (R-determinant) plasmid cannot ordinarily be transferred as a separate entity, although it functions as an independent replicon.

This model for the molecular nature of R-factors predicts that if an R^+ bacterial strain containing separable R-factor components is mated with an R^-

strain, and antibiotic resistant bacteria are not given a selective advantage, certain antibiotic sensitive cells might be isolated which have received only the RTF plasmid. If the complete R-factor, which specifies both resistance and transfer functions, and the RTF unit are transferred at approximately equal frequencies, half of all bacteria *carrying plasmids* would be expected to contain the RTF. Since the frequency of transfer is low,[9] most bacterial cells of the recipient strain would have received neither of these plasmids. However, repeated serial passage of such a culture might increase the frequency of both R^+ and RTF^+ bacteria in the recipient population,[6,10] and result in a consequent reduction in the percentage of cells which carry no plasmid. The R^+ and R^- cells could be easily differentiated by the presence or absence of antibiotic resistance, and DNA isolated from antibiotic sensitive cells could be examined by ultracentrifugation as described previously[1,2] for the presence of circular plasmid DNA representing the RTF unit.

Experiments of this design were used to obtain bacterial isolates carrying the separated RTF unit of the R-factor R1. The present report describes the results of these studies and demonstrates the presence of transferable, covalently-closed circular DNA in *antibiotic sensitive E. coli* recipient cells following mating with antibiotic-resistant *P. mirabilis* carrying R1. Such DNA, which has lost all drug resistance markers originally present on R1, fulfills the functional definition of the RTF units.

Materials and Methods. Strains and growth conditions: *P. mirabilis* strain PMZ, carrying the wild type R-factor R1,[3] was obtained from Dr. S. Falkow of Georgetown University. *E. coli* strain AB2463, rec A^-, str^-, arg^-, his^-, pro^-, thr^-, leu^- was obtained from Dr. J. Clark of the University of California. A mutant of AB2463 that was chromosomally resistant to 50 μg/ml of nalidixic acid (Sterling Laboratories) was selected by plating 10^{10} untreated cells on standard nutrient agar plates in the presence of this drug. The media and antibiotic concentrations used have been described.[1,2]

Isolation of RTF unit: Mating was performed as described by Watanabe and Fukasawa.[9] Donor and recipient cells were grown separately in Penassay broth to a cell density of 5×10^8/ml, diluted together into nonselective medium, and incubated in stationary flasks for 18 hr at 37°C. Mating mixtures were diluted 10^4 times into Penassay broth which contained nalidixic acid (50 μg/ml), but which included none of the antibiotics for which the R-factor specified resistance. Cultures were grown to stationary phase at 37°C with very gentle shaking, and then diluted 100 times into identical medium; ten such serial transfers were carried out. Antibiotic resistant and antibiotic sensitive cells present in cultures during these serial transfers were assayed by plating appropriate dilutions on nutrient agar plates and then replica-plating colonies onto identical plates containing nalidixic acid (50 μg/ml) and either chloramphenicol (25 mg/ml) or streptomycin (10 μg/ml). All colonies scored tentatively as R^- by this method were tested additionally with Difco or Bioquest discs containing the antibiotic potencies: ampicillin, 10 μg/ml; chloramphenicol, 30 μg/ml; kanamycin, 30 μg/ml; and streptomycin, 10 μg/ml before being examined for the presence of closed circular DNA.

Isolation of RTF and R-factor DNA: For analytical centrifugation on CsCl gradients, and zone centrifugation in neutral sucrose, DNA was isolated from *E. coli* carrying the complete R1 R-factor or the RTF unit after lysis of spheroplasts[1,2] with Brij-58[11] (Van Waters and Rogers). Extraction of DNA for alkaline sucrose gradient centrifugation and analysis of samples was performed essentially as reported by Freifelder.[13] DNA was obtained for direct use in preparative CsCl gradients by sarcosyl (Geigy) lysis of spheroplasts.[1,2,12]

Denaturation of DNA, to be used for CsCl gradient centrifugation or for subsequent purification by bulk nitrocellulose adsorption, was at pH 12.3 as previously described.[1]

Centrifugation of DNA: Analytical ultracentrifugation of DNA was performed in 12-mm 4°C sector cells using a Beckman model E ultracentrifuge equipped with an ultraviolet light source and monochrometer. dAT (kindly donated by Mr. Paul Modrich) was used as a density marker (ρ = 1.679 g/cm^3)[14] in CsCl gradients adjusted to a ρ = 1.700 g/cm^3 using a Zeiss refractometer. Centrifugation was for 20–24 hr at 44,000 rpm at 25°C.

Covalently-closed circular DNA was separated from nicked or linear DNA[15] by centrifugation in CsCl gradients (ρ = 1.555 g/cm^3) containing EDTA (pH 8.0, final concentration = 0.01 M) and ethidium bromide (2,7-diamine-10-ethyl-9-phenylphenanthridium bromide, Calbiochem, final concentration 800 μg/ml). Ethidium bromide was removed from DNA fractions by equilibration with isopropyl alcohol.[16] Alternatively, denatured noncircular DNA was separated from nondenaturable DNA by adsorption to bulk nitrocellulose.[1]

RTF DNA, isolated by CsCl gradient centrifugation or nitrocellulose adsorption of denatured noncircular DNA, was centrifuged in 5–20% linear neutral sucrose gradients[1,2] or in identical gradients containing 0.3 N NaOH. Fractions (0.15 ml) were collected through a hole pierced in the bottom of the tube, precipitated with 2 ml of cold 5% trichloroacetic acid, washed with 10 ml of 2% trichloroacetic acid, collected on Whatman GF/C glass fiber filters, and counted in a standard Toluene-PPO-POPOP phosphor.[17]

TABLE 1. *Fraction of antibiotic resistant-bacteria after repeated serial transfer of recipient strain.*

Transfer number	% Antibiotic resistant
1	0·93
2	1·7
3	1·3
4	2·4
5	1·9
6	4·0
7	5·5
8	11·3
9	12·0
10	11·6

The conditions employed for bacterial mating, subsequent serial transfer of the recipient strain, and identification of antibiotic resistant bacteria are described in the text. The numbers shown represent an average obtained from 3 or 4 separate determinations at appropriate dilutions after each bacterial transfer.

Results. Isolation of RTF1: After mating of *Proteus* strain PM1-R1 with *E. coli* strain AB2463 and selection for the *E. coli* strain, ten serial transfers were performed, and the percent of antibiotic resistant bacteria was determined after each transfer. In the experiment shown in Table 1, the fraction of resistant cells reached a maximum of 12% of the total after 8–10 transfers; in other experiments, from 6 to 25% of the total recipient strain population were drug resistant after the same number of passages in antibiotic-free medium.

After the tenth serial passage, ten antibiotic-sensitive colonies were identified; each was inoculated into 50 ml of Penassay broth and grown to a cell concentration of 8 × 10^8/ml. The DNA was isolated, brought to pH 12.3 for 10 min at 2°C, neutralized, and centrifuged analytically in CsCl to detect the presence of nondenaturable (circular) DNA. In the experiment reported in Table 1, five of ten antibiotic sensitive colonies selected for further examination contained nondenaturable DNA. The resistance to alkaline denaturation of a fraction of the DNA isolated from one of these colonies is demonstrated in Fig. 1*B;* the bulk of the DNA showed an increase in buoyant density of 0.015 g/cm^3 as a consequence[18] of alkali treatment, while a fraction (which has been designated RTF1), representing less than 5% of the total DNA, remained at a buoyant

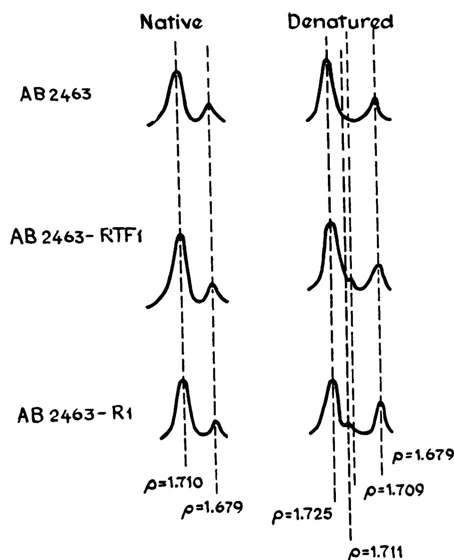

FIG. 1. Microdensitometer tracings of photographs taken during analytical centrifugation of purified DNA samples in neutral CsCl. Growth of bacteria, isolation of DNA by detergent lysis of spheroplasts and phenol extraction, and alkaline denaturation were performed as described in *Materials and Methods*. Samples were centrifuged in solutions of optical grade CsCl (ρ = 1.700) for 21 hr at 44,000 rpm at 4°C in a Spinco model E analytical ultracentrifuge in the presence of dAT polynucleotide as a density marker (ρ = 1.679).

(*Top*) DNA isolated from strain AB2463 before mating. DNA from 5 of 10 antibiotic sensitive colonies selected following mating showed a centrifugation pattern identical to the parental strain.

(*Middle*) DNA obtained from an antibiotic-sensitive colony of *E. coli* strain AB2463 after mating with *P. mirabilis* strain PM1 carrying the R-factor R1, and subsequent passage as described in text.

(*Bottom*) DNA isolated from antibiotic resistant *E. coli* AB2463 carrying the parent R-factor R1.

density of ρ = 1.709 g/cm^3. In contrast, only a single class of (denaturable) DNA, that was identical in buoyant density to that isolated from the *E. coli* strain before mating (Fig. 1*A*), was obtained from the remaining five of the ten selected colonies. Furthermore, no nondenaturable DNA was detected in *E. coli* that had been selected and serially passed after "mating" with *Proteus* strain PM1 that lacked the R-factor.

DNA preparations isolated from several antibiotic-resistant colonies of AB-2463-R1 were also treated with alkali and were then centrifuged analytically in CsCl. As illustrated in Fig. 1*C*, such preparations contained a small peak of native DNA banding at a buoyant density of ρ = 1.711 g/cm,^{3} in addition to a major band of denatured DNA at ρ = 1.725 g/cm^3. The nondenaturable DNA species having a buoyant density of ρ = 1.711 g/cm^3 appears to carry both the resistance and transfer functions of R1 in *E. coli;* presumably this species represents the "complete" R-factor. This interpretation is consistent with our earlier conclusion that the DNA species banding at this buoyant density represents the "complete" R1 isolated from *P. mirabilis*.[2]

In order to more rigorously establish the presence of covalently-closed circular DNA in antibiotic sensitive colonies of *E. coli* after mating with *Proteus* that carries R1, ^{3}H-labeled DNA, obtained from the isolate of Fig. 1*B*, was centrifuged in CsCl in the presence of ethidium bromide. As demonstrated by Vinograd and his collaborators,[15] the interaction of closed-circular DNA with ethidium bromide enables it to be separated from linear DNA or from circular DNA having one or more single-strand scissions. As illustrated in Fig. 2*A*, the antibiotic sensitive isolate contained a ^{3}H-labeled DNA peak banding at a position that is characteristic of covalently-closed circular DNA centrifuged under these conditions.[15] CsCl-ethidium bromide centrifugation of ^{3}H-labeled

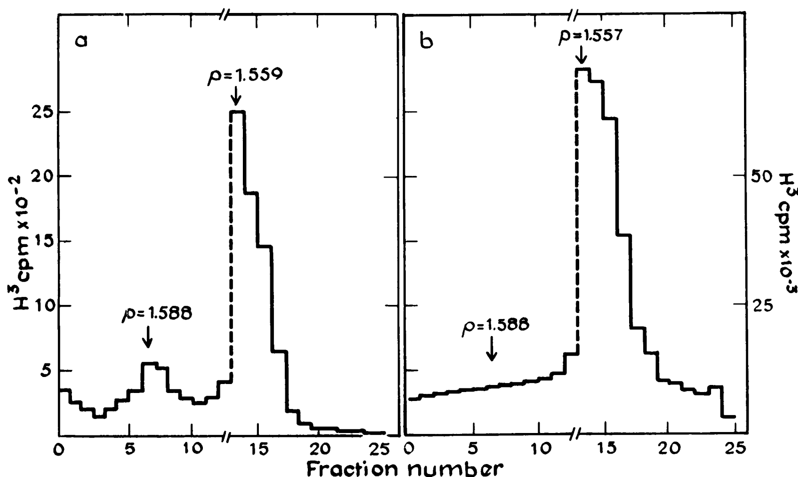

FIG. 2. Preparative ultracentrifugation of AB2463-RTF1 DNA labeled with ^3H and isolated by sarcosyl lysis as described in *Materials and Methods*. Centrifugation of DNA obtained from 20 ml of culture, at a cell density of 8×10^8/ml, was performed in 10 ml CsCl (ρ = 1.565) at 25°C. Samples were centrifuged for 55 hr at 40,000 rpm in the Spinco SW50.1 rotor in a model L2-65B centrifuge with 800 μg/ml ethidium bromide. Fractions (0.2 ml) were collected through a hole pierced in the bottom of the tube, and a sample (0.02 ml) of each fraction was precipitated with 5% trichloroacetic acid. The precipitates were collected on glass filters (GF/C), dried, and counted as described in *Materials and Methods*. (*A*) DNA sample from AB2463-RTF1. (*B*) DNA sample isolated from parental strain.

DNA isolated from the parental strain before mating, or from colonies that showed no nondenaturable DNA after mating, revealed only a single (non-circular) band at an appropriate buoyant density (Fig. 2*B*).

Sedimentation of the same ^3H-labeled DNA, in 5-20% linear sucrose gradients containing 0.3 N NaOH, confirmed the existence of circular DNA in antibiotic sensitive *E. coli* (Fig. 3*A*). A rapidly sedimenting peak characteristic of closed-circular DNA centrifuged in alkaline sucrose gradients[18] was observed; the parental strain contained no rapidly sedimenting DNA species (Fig. 3*B*).

RTF1 was transferred from the *E. coli* host to *P. mirabilis* strain PM1 by conjugation, and *Proteus* cells were selected in the absence of antibiotics.[2] Eight serial passages of *Proteus* cultures were performed as described above for *E. coli*, and DNA isolated from *Proteus* was centrifuged analytically in CsCl. As seen in Fig. 4*A*, only a single satellite peak of DNA banding at a buoyant density of ρ = 1.709 g/cm^3 is associated with the presence of RTF1 in *Proteus*, whereas in this host an additional discrete DNA species banding at ρ = 1.718 g/cm^3 (Fig. 4B) is associated with the presence of the "complete" R-factor (R1), as previously reported.[2] No satellite peak was observed in DNA preparations isolated from the parent *Proteus* strain lacking the R-factor (Fig. 4*C*), and no closed-circular DNA species could be isolated from such a culture.

Discussion. The existence of a transfer unit which is linearly and covalently linked to resistance determinants was originally postulated by Watanabe and Fukasawa on the basis of transduction experiments.[5] Later, genetic studies by

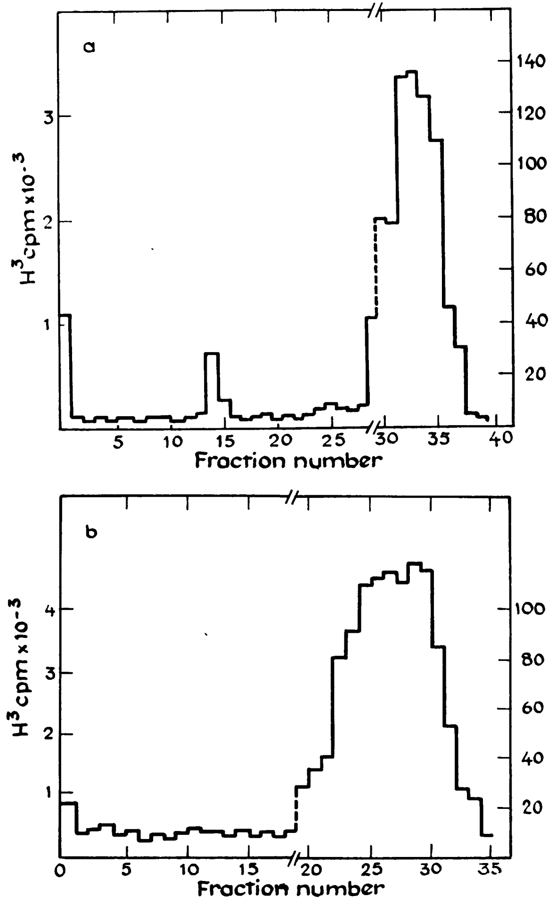

Fig. 3. Alkaline sucrose gradient sedimentation analysis of DNA isolated from AB2463-RTF1 and from parental strain. Conditions of DNA isolation were by a modification of the procedure described by Freifelder[13]. [^{3}H]DNA samples were layered onto a 5–20% linear sucrose gradient containing 0.3 N NaOH, 0.001 M EDTA, 0.7 M NaCl, and 1.0% sodium dodecyl sarcosinate and centrifuged in a SW41 rotor for 35 min at 20°C at 30,000 rpm. The rotor was allowed to stop without braking, and fractions (0.2 ml) were collected through a hole pierced in the bottom of the tube and a sample (0.02 ml) of each fraction was precipitated with 5% trichloroacetic acid. The precipitates were treated as indicated in Fig. 2.

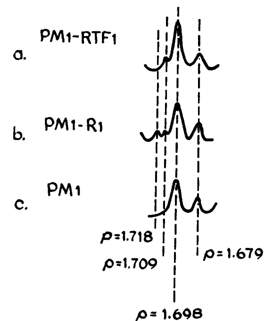

Fig. 4. Analytical ultracentrifugation of DNA isolated from *P. mirabilis* strain PM1. Conditions for mating, serial passage of bacteria, and isolation of DNA are described in the text; centrifugation was performed with dAT polynucleotide density marker (ρ = 1.679) as indicated in Fig. 1. All *Proteus* cultures were harvested in late logarithmic stage of growth.

(*a*) DNA isolated from PM1 that has been mated with *E. coli* AB2463-RTF1.

(*b*) DNA isolated from antibiotic resistant PM1 containing the "complete" R-factor, R1.

(*c*) DNA isolated from the parent *Proteus* strain, PM1, before introduction of R1 or RTF1.

Anderson and his collaborators in *Salmonella* species[6] and by Mitsuhashi[7] indicated that the transfer unit of at least certain classes of R-factors can be transmitted alone, as well as in combination with resistance determinants. These findings led to the proposal that units specifying transferability and resistance were independent plasmids which could reversibly associate with each other, and that transfer of the R-determinant unit was made possible as a result

of this association. Our earlier investigations[1,2] indicated that the transfer unit and resistance determinant unit of R-factors R1 and R6 can associate by covalent linkage, and that a larger plasmid comprising the complete R-factor is consequently formed. A similar conclusion has been reached independently for other R-factors by Nisioka *et al.*[19] and for R1 by Silver and Falkow.[20]

It has long been recognized on the basis of genetic experiments that RTF can exist as a functionally discrete unit. The present investigations provide evidence of a molecular nature that the transfer unit of the R-factor R1 can exist as a separate, stable species of covalently-closed circular DNA which has a buoyant density of $\rho = 1.709$ g/cm^3 in CsCl. The plasmids we have identified in antibiotic-sensitive *E. coli* are segregants of R1 which have retained their capacity for transfer into R^- bacteria, but which have lost all of the drug resistance markers present on the parent R-factor; they thus fulfill the functional definition of the transfer unit.

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Abbreviation: RTF, resistance transfer factor.

* For the preceding paper in this series see ref. no. 2.

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