JOURNAL OF BACTERIOLOGY, July 1975, p. 56-68
Vol. 123, No. 1


# Transition of the R Factor *NR1* in *Proteus mirabilis*: Level of Drug Resistance of Nontransitioned and Transitioned Cells

HAJIME HASHIMOTO[1] AND ROBERT H. ROWND*

*Laboratory of Molecular Biology and Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706*

Received for publication 29 January 1975

When *Proteus mirabilis* harboring the R factor *NR1* is cultured in Penassay broth containing 100 μg of chloramphenicol (CM) per ml, there is an amplification in the number of copies of the r-determinants per cell. Under these conditions, R factors harboring multiple tandem sequences of r-determinants are formed. Autonomous poly-r-determinants consisting of multiple copies of r-determinants are also formed. This phenomenon has been referred to as the "transition." Transitioned cells have considerably higher levels of resistance to CM and streptomycin (SM), but not to tetracycline (TC), than do nontransitioned cells and grow more rapidly in medium containing either CM or SM. There is essentially no difference in growth rates between transitioned and nontransitioned cells in drug-free medium. The higher level of resistance of transitioned cells to SM has made it possible to investigate the mechanism of the transition. Using replica plating, it has been possible to isolate spontaneously occurring transitioned cells from a nontransitioned population which appear to outgrow the nontransitioned cells during growth in medium containing 100 μg of CM per ml. If transitioned cells are subsequently cultured in drug-free medium, the cells return gradually to the nontransitioned state, which has been referred to as the "back-transition." The rate of appearance and disappearance of transitioned cells during the transition and back-transition was monitored by examining the level of resistance of the cells to SM. In both situations the cell populations were found to be heterogeneous, consisting of a mixture of nontransitioned and transitioned cells. Under the conditions of our experiments, the transition appeared to be due to the more rapid growth of a minor fraction of spontaneously occurring transitioned cells which outgrew the remainder of cells in the population. To obtain the transition, the drug resistance gene must reside on the r-determinants component of the R factor. The transition did not take place when the cells were cultured in medium containing high concentrations of TC. This indicates that the TC resistance genes reside on the resistance transfer factor component of the R factor, which is in agreement with physical studies on R factor deoxyribonucleic acid.

Genetic (3, 21, 22) and physical (4, 5, 14, 15, 17, 18) studies have shown that R factors are composed of two distinguishable units: a resistance transfer factor (RTF) and a unit that carries drug resistance genes (r-determinants). In *Escherichia coli* and *Serratia marcescens*, RTF and r-determinants appear to be stably associated to form a composite structure (an R factor) (4, 9, 14, 17). In *Proteus mirabilis*, there is now evidence that several different R factors can dissociate to varying degrees into RTF and r-determinants (4, 5, 14, 17, 18). In the case of the R factor *NR1*, we have presented evidence that the number of copies of r-determinants per cell can be amplified during growth in medium containing drugs to which r-determinants confer resistance (14, 15, 17, 18; D. Perlman and R. Rownd, submitted for publication). Under these conditions, R factors harboring multiple tandem sequences of r-determinants (poly-r-determinant R factors) are formed. Autonomous poly-r-determinants consisting of multiple copies of r-determinants may also be formed (Perlman and Rownd, submitted for publication). This mechanism of gene amplification, which has been referred to as the "transition,"

[1] Present address: Department of Microbiology, Gunma University School of Medicine, Maebashi, Japan.

can be monitored in a CsCl density gradient. The addition of multiple copies of r-determinants (1.718 g/ml) to an RTF (1.710 g/ml) results in an increase in the density and the relative amount of the R factor deoxyribonucleic acid (DNA). This process can be reversed if transitioned cells are subsequently cultured in drug-free medium; r-determinants are diluted from the cell population, presumably due to a decrease in their rate of replication when in the autonomous state (referred to as the "back-transition").

In this communication we describe a number of interrelated experiments on the level of drug resistance and the growth rates of nontransitioned and transitioned *P. mirabilis* cells and on the mechanism of the transition and the back-transition. A preliminary account of this work has been presented elsewhere (17).

## MATERIALS AND METHODS

**Bacterial strains.** φS-3 is a tetracycline (TC)-sensitive mutant of the *P. mirabilis* strain used in this laboratory whose origin has been described previously (11). φS-31 is a *gal*⁻ mutant of φS-3 and was isolated after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine by the method of Adelberg et al. (2). φS-38 in a thymine-requiring mutant of φS-31 which was isolated by D. Taylor in this laboratory. In a few experiments strain Pm17 (8) was used. *E. coli* K-12 W677 has been described previously (11, 16).

**R factors.** The R factor *NR1* confers resistance to chloramphenicol (CM), streptomycin/spectinomycin, sulfonamide, and TC and has been described previously (11, 16). R100-99 is a derivative of *NR1* (7) and has point mutations in the CM and TC resistance genes.

**Media.** Bacterial cells were cultured in Penassay broth (Difco) supplemented with drugs as described in the text. Penassay agar was used for the assay of the level of drug resistance on plates. To prevent swarming of *P. mirabilis*, 0.15% bile salts (Difco) was added to the plates. MacConkey agar base (Difco) containing 1% galactose was used in indicator plates to distinguish *gal*⁺ and *gal*⁻ strains. Appropriately supplemented M9 (1) agar plates were used to select for R⁺ recipient strains in mating experiments between *E. coli* and *P. mirabilis*.

**Drugs.** CM and streptomycin sulfate (SM) were purchased from Parke, Davis & Co., Detroit, Mich. and Merck & Co., Inc., Rahway, N. J., respectively. Tetracycline hydrochloride, sulfadiazine, and sulfathiazole were generously provided by American Cyanamide Co., Pearl River, N. Y.

**R factor transfer.** *E. coli* K-12 W677/*NR1* was used as the donor of the R factor *NR1* to the *P. mirabilis* strains. Overnight cultures of the donor and recipient strains were diluted 10-fold into fresh Penassay broth and incubated at 37 C with gentle shaking for 3 h. Equal volumes (1 ml) of the donor and recipient strains were then mixed, and the mixture was incubated for an additional 2 h with gentle shaking. Samples of the mating mixture were spread on selective plates consisting of M9 agar supplemented with 25 μg of TC per ml, 20 μg of nicotinic acid per ml, and 0.2% glucose.

**Isolation of DNA and density gradient centrifugation.** DNA was prepared from 3 ml of stationary-phase cultures and was examined in a CsCl density gradient as described previously (8, 13). The molecular weight of bacterial DNA prepared by this procedure is in the range $100 \times 10^6$ to $150 \times 10^6$ (10; Perlman and Rownd, submitted for publication).

## RESULTS

**Comparison of the level of drug resistance of nontransitioned and transitioned P. mirabilis harboring NR1.** The R factor *NR1* was transferred by bacterial mating to *P. mirabilis* strains φS-3 (*gal*⁺) and φS-31 (*gal*⁻), using TC as the drug in the selective plates. When these R⁺ strains were cultured in drug-free Penassay broth, *NR1* DNA appeared as a satellite band of density 1.712 g/ml to the *P. mirabilis* chromosomal DNA (1.700 g/ml) (Fig. 1A). The percent-

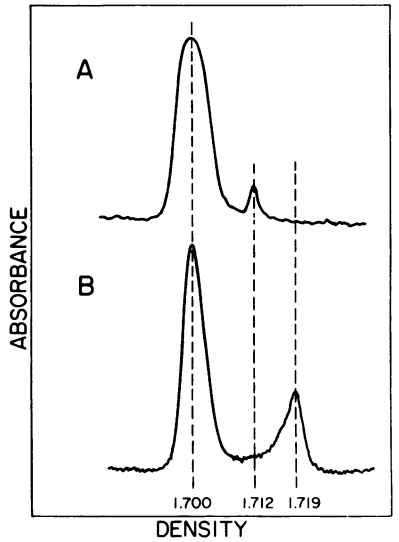


FIG. 1. *Density profiles of R factor NR1 DNA in nontransitioned and transitioned P. mirabilis. (A) Density profile of DNA isolated from nontransitioned φS-3/NR1 cells that had been cultured in drug-free Penassay broth. (B) Density profile of DNA isolated from transitioned φS-3/NR1 cells that had been cultured in Penassay broth containing 50 μg of SM per ml. The 1.719-g/ml NR1 DNA represents 37% of the chromosomal DNA (1.700 g/ml). A similar NR1 DNA density profile was observed when the cells were cultured in Penassay broth containing 100 μg of CM per ml; in this case the 1.719-g/ml NR1 DNA represented 21% of the chromosomal DNA (1.700 g/ml).*

age of the *NR1* DNA relative to the host chromosomal DNA in stationary-phase cultures was about 7% for φS-3/*NR1* and about 5% for φS-31/*NR1*. φS-3/*NR1* was cultured in Penassay broth containing either 50 μg of SM or 100 μg of CM per ml. The *NR1* DNA from these cultures had a density of 1.718 to 1.719 g/ml (Fig. 1B); the 1.712-g/ml *NR1* DNA band was no longer observed in the R factor DNA density profile. The amount of the *NR1* DNA also increased to 37% (SM culture) and 21% (CM culture) of the host DNA (1.700 g/ml), respectively. In similar experiments using Penassay broth containing various concentrations of TC, the density of *NR1* DNA in *P. mirabilis* did not increase even after prolonged growth in medium containing up to 200 μg of TC per ml.

As described previously, the increase in the density of *NR1* DNA as a result of growth in medium containing appropriate concentrations of CM and SM is due to the incorporation of multiple copies of r-determinants (1.718 g/ml) into individual R factors to form R factors with repeated sequences of r-determinants (poly-r-determinant R factors) (14, 15, 17, 18; Perlman and Rownd, submitted for publication). Autonomous poly-r-determinants consisting of multiple copies of r-determinants themselves may also be formed. This results in a large amplification in the number of copies of the drug resistance genes that reside on r-determinants in the cells.

To compare the level of drug resistance of nontransitioned and transitioned $R^+$ *P. mirabilis*, a nontransitioned culture and cultures of φS-3/*NR1* that had been transitioned by using either CM or SM were each diluted $10^2$-, $10^4$-, and $10^6$-fold. A 0.02-ml amount of each of the original cultures and their respective dilutions were spotted on plates containing either no drugs or various concentrations of CM, SM, or TC. After 20 h of incubation, the growth of each of the dilutions on the different types of plates was recorded. Transitioned cells had a much higher level of resistance to CM and SM than did nontransitioned cells (Table 1). This difference was apparent even for dilutions producing confluent growth on drug-free plates because of the large number of cells inoculated. However, the difference was most evident when higher dilutions of the cells were inoculated so that individual clones could be counted. The numbers recorded in Table 1 refer to cases in which the number of clones appearing on the plates could be counted. Individual nontransitioned cells can only form colonies on plates containing a maximum of 100 μg of CM or 12.5 μg of SM per ml. However, individual transitioned cells can form colonies on plates containing up to 400 μg of CM or up to 200 μg of SM per ml. The increased level of resistance to both CM and SM was observed for transitioned cells produced by growth in either of the drugs. Thus plates containing 200 to 400 μg of CM or 25 to 50 μg of SM per ml can be used to distinguish between nontransitioned and transitioned cells when only about 100 cells are inoculated onto plates. There was no reproducible difference observed in the growth of nontransitioned and transitioned cells on plates containing various concentrations of TC.

The minimal inhibitory concentrations of CM, SM, and TC for the formation of single colonies by cells of $R^-$ φS-3 are less than 5, 5, and 1 μg/ml, respectively. Since the same number of colonies of nontransitioned cells are formed on plates containing 100 μg of CM or 12.5 μg of SM per ml as on drug-free plates, all of the cells in the nontransitioned cultures must be $R^+$. *NR1* is very stable in the strain of *P. mirabilis* used in our experiments, and $R^-$ segregants of nontransitioned cells are rarely observed (segregation frequency of less than $10^{-4}$).

**Relative growth rates of nontransitioned and transitioned cells.** The previous results suggested that transitioned cells would grow more rapidly than nontransitioned cells in medium containing either CM or SM due to their higher level of drug resistance. To examine this point further, transitioned cells of φS-3/*NR1* (*gal*$^+$) and φS-31/*NR1* (*gal*$^-$) were prepared by growth in Penassay broth containing 100 μg of CM per ml. Transitioned stationary-phase φS-3/*NR1* (*gal*$^+$) cells were mixed with nontransitioned stationary-phase φS-31/*NR1* (*gal*$^-$) cells, and the cell mixture was diluted $10^2$-, $10^4$-, $10^6$-, and $10^8$-fold into either drug-free Penassay broth or Penassay broth containing 100 μg of CM or 50 μg of SM per ml. After growth of these cultures into stationary phase, appropriate dilutions of the cells were plated on galactose indicator plates. The transitioned cells rapidly outgrew the nontransitioned cells in medium containing CM or SM (Table 2). In Table 3 is shown the effect of CM or SM concentration in the growth medium on the proportion of transitioned cells in a mixing experiment similar to the one just described. (The proportion of transitioned to nontransitioned cells differed in the original mixture of cells in these two experiments.) To exclude the possibility that the ability of the cells to ferment galactose affected the growth rates of the

TABLE 1. *Level of drug resistance of nontransitioned and transitioned P. mirabilis φS-3/NR1*[a]

| State of transition | Decimal dilution of culture inoculated | Growth of bacteria on Penassay agar plates containing: | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | No drug | CM (μg/ml) | | | | SM (μg/ml) | | | | | | TC (μg/ml) | | | |
| | | | 50 | 100 | 200 | 400 | 12.5 | 25 | 50 | 100 | 200 | 400 | 12.5 | 25 | 50 | 100 |
| Nontransitioned | 0 | +++[b] | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | ++ | 102 | +++ | +++ | 52 | 18 |
| | −2 | +++ | +++ | +++ | +++ | — | +++ | +++ | ++ | 34 | 2 | — | +++ | +++ | 44 | — |
| | −4 | ++ | ++ | ++ | 4 | — | ++ | ++ | — | — | — | — | ++ | ++ | — | — |
| | −6 | 77 | 81 | 79 | — | — | 82 | — | — | — | — | — | 49 | 22 | — | — |
| Transitioned with CM | 0 | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | ++ | 48 |
| | −2 | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | 7 | +++ | +++ | + | — |
| | −4 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | 2 | 1 | ++ | ++ | — | — |
| | −6 | 144 | 137 | 133 | 127 | 97 | 138 | 140 | 97 | 32 | — | — | 125 | 121 | — | — |
| Transitioned with SM | 0 | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | 35 | 18 |
| | −2 | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | 35 | +++ | ++ | 1 | — |
| | −4 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | 1 | ++ | + | — | — |
| | −6 | 66 | 57 | 90 | 80 | 77 | 62 | 73 | 78 | 63 | 37 | — | 76 | 9 | — | — |
| R⁻ φS-3[c] | 0 | +++ | — | — | — | — | ++ | ++ | ++ | + | — | — | — | — | — | — |
| | −2 | +++ | — | — | — | — | — | — | 11 | 2 | — | — | — | — | — | — |

[a] Overnight nontransitioned and transitioned cultures were diluted 10-fold into Penassay broth and incubated for 3 h at 37 C. Hundred-fold serial dilutions were made from each culture, and 0.02 ml of the original cultures and their hundred-fold serial dilutions were spotted onto Penassay agar plates containing either no drugs or CM, SM, or TC at the concentrations given above. The plates were incubated at 37 C for 18 to 20 h and the number of colonies on the plates was scored. The level of resistance of an R⁻ φS-3 culture was also examined in similar experiments.

[b] +++, Heavy confluent growth; ++, light confluent growth; +, isolated colonies; —, no observable growth. Numbers refer to number of isolated colonies observed.

[c] Minimal inhibitory concentrations of CM, SM, and TC for the formation of colonies by single cells of R⁻ φS-3 are less than 5, 5, and 1 μg/ml, respectively.

TABLE 2. *Relative growth rates of nontransitioned and transitioned cells in medium containing CM or SM*[a]

| Increase in no. of cells | Percent transitioned cells after growth in medium containing: | |
|---|---|---|
| | CM (100 μg/ml) | SM (50 μg/ml) |
| $10^2$ | 73 | 94 |
| $10^4$ | 99 | 100 |
| $10^6$ | 100 | 100 |
| $10^8$ | 100 | 100 |

[a] φS-31/*NR1* (*gal*⁻) nontransitioned cells and φS-3/*NR1* (*gal*⁺) transitioned cells were mixed and the cultures were given hundred-fold serial dilutions into Penassay broth containing 100 μg of CM or 50 μg of SM per ml. After growth into stationary phase, the cultures were plated onto MacConkey indicator plates containing 1% galactose. After overnight incubation, the percentage of the cells that were *gal*⁺ (transitioned cells) was determined. More than 600 colonies were scored in the determination of the percentages listed. The initial mixture of cells contained approximately 10% transitioned cells.

cells in medium containing CM or SM, similar experiments were carried out in which *gal*⁻ transitioned cells were mixed with *gal*⁺ nontransitioned cells and 100-fold serial dilutions of the mixture of cells were cultured in either drug-free medium or medium containing either CM or SM. In agreement with the previous experiments, the transitioned cells grew more rapidly in medium containing either CM or SM (data not shown).

When mixtures of transitioned φS-3/*NR1* (*gal*⁺) and nontransitioned φS-31/*NR1* (*gal*⁻) cells were cultured in drug-free Penassay broth, there was only a slight increase in the percentage of *gal*⁺ cells even after a $10^8$-fold increase in the total number of cells (Table 4). A comparable increase in the percentage of *gal*⁺ cells was also observed in similar mixing experiments with R⁻ φS-3 (*gal*⁺) and R⁻ φS-31 (*gal*⁻) cells (Table 4). Thus, φS-3 (*gal*⁺) has a slightly faster growth rate than φS-31 (*gal*⁻), which was derived from it. These experiments suggest that there is essentially no difference in growth rate between nontransitioned and transitioned cells in drug-free medium.

These findings were confirmed in experiments in which the growth rates of transitioned and nontransitioned *P. mirabilis* cells were determined by turbidity measurements at 650 nm. Both transitioned and nontransitioned cells had a doubling time of 43 min in drug-free medium. In medium containing 100 μg of CM per ml the transitioned culture had a doubling time of 45 min, whereas the doubling time of the nontransitioned culture was 126 min in this medium. The growth rate of nontransitioned cells (and probably of transitioned cells) would, of course, be expected to depend on the drug concentration in the medium as well as the extent of inactivation of the drug by the cells.

TABLE 3. *Effect of concentration of CM or SM on growth of transitioned cells*[a]

| Concn of drug in growth medium (μg/ml) | Percent transitioned cells after the indicated increase in the number of cells | | | |
|---|---|---|---|---|
| | CM | | SM | |
| | $10^2$ | $10^4$ | $10^2$ | $10^4$ |
| 0 | 17.7 | 21.5 | 9.5 | 10.5 |
| 6.25 | | 17.0 | | 15.2 |
| 12.5 | | 31.4 | | 82.3 |
| 25 | | 50.5 | | 100 |
| 50 | | 86.3 | 61.2 | |
| 100 | 47.2 | 98.6 | 99.1 | |
| 200 | 80.6 | | 100 | |
| 600 | 98.0 | | 100 | |
| 800 | No growth | | 100 | |

[a] Mixtures of nontransitioned (*gal*⁻) and transitioned (*gal*⁺) cells were diluted either $10^2$ or $10^4$ into Penassay broth containing varying concentrations of either CM or SM. After growth into stationary phase, the percentage of transitioned cells was determined as described in Table 2. The initial mixture of cells contained a different ratio of nontransitioned and transitioned cells in the two experiments.

TABLE 4. *Relative growth rates of nontransitioned and transitioned cells in drug-free medium*

| Increase in no. of cells | Percent transitioned φS-3/*NR1* (*gal*⁺) cells[a] | | | Percent R⁻ φS-3 (*gal*⁺) cells[b] | | |
|---|---|---|---|---|---|---|
| | 1[c] | 2 | 3 | 1 | 2 | 3 |
| $10^2$ | 18.2 | 13.9 | 7.1 | 12.1 | 13.2 | 15.5 |
| $10^4$ | 15.5 | 21.5 | 11.0 | 14.8 | 12.2 | 19.4 |
| $10^6$ | 11.8 | 12.8 | 9.6 | 13.2 | 16.5 | 14.2 |
| $10^8$ | 16.3 | 16.4 | 15.2 | 16.2 | 18.6 | 16.5 |

[a] φS-31/*NR1* (*gal*⁻) nontransitioned and φS-3/*NR1* (*gal*⁺) transitioned cells were mixed, diluted, cultured, and analyzed as described in Table 2. The initial mixture of cells contained approximately 10% transitioned cells in the three experiments.

[b] R⁻ φS-31 (*gal*⁻) and R⁻ φS-3 (*gal*⁺) cells were mixed, diluted, cultured, and analyzed as described in Table 2. The initial mixture of R⁻ cells contained 9.6, 12.0, and 14.6% φS-3 (*gal*⁺) cells, respectively, in the three experiments.

[c] Experiment number.

We have not examined these points in any detail by using turbidity measurements.

**Selective outgrowth of transitioned cells.** Since nontransitioned cells are unable to form colonies on plates containing 25 or 50 μg of SM per ml, it should be possible to monitor the appearance of "transitioned" cells in a nontransitioned population by plating the cells on plates containing either 25 or 50 μg of SM per ml. In the experiment shown in Fig. 2, nontransitioned cells of φS-3/*NR1* were diluted $10^6$-fold into Penassay broth containing 100 μg of CM per ml. After incubation at 37 C for various periods, the cells were plated on either drug-free plates or plates containing 25 or 50 μg of SM per ml. There was no increase in the number of viable cells for the first 4 h when monitored on drug-free plates; the cells then began to divide with a doubling time of approximately 55 min (Fig. 2). After 4 to 6 h, a small fraction of the cells could be detected that could form colonies on plates containing 25 μg of SM per ml; a still smaller fraction could form colonies on plates containing 50 μg of SM per ml. The doubling times of both of these more resistant cell subpopulations was about 36 min. The proportion of cells resistant to either 25 or 50 μg of SM per ml increased with time, presumably as a result of their faster growth rate in Penassay broth containing 100 μg of CM per ml. When initially detected 4 to 6 h after the dilution, these cells represented approximately 1 and 0.1% of the cell population, respectively. After growth of the culture into stationary phase, their proportion had increased to 68 and 10%, respectively.

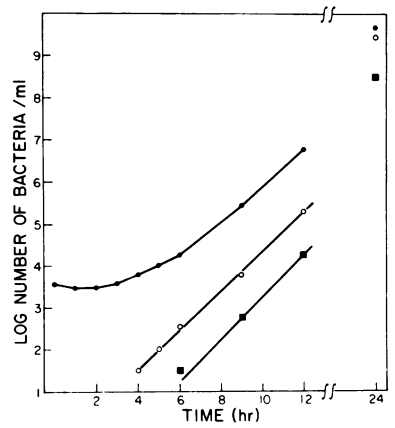


FIG. 2. *Growth rates of nontransitioned and transitioned cells in Penassay broth containing 100 μg of CM per ml. An overnight culture of nontransitioned φS-3/NR1 cells was diluted $10^6$-fold into 10 ml of Penassay broth containing 100 μg of CM per ml. The culture was incubated at 37 C, and the number of viable cells was monitored by plating on Penassay agar plates containing either no drugs* (●), *25 μg of SM per ml* (○), *or 50 μg of SM per ml* (■).

**Comparison of the density profiles of NR1 DNA and the fraction of more resistant cells.** In these experiments a stationary-phase culture of nontransitioned cells was given various dilutions into Penassay broth containing 100 μg of CM per ml. Each dilution was grown into stationary phase and DNA was prepared from samples of each culture. The percentage of the cells that could form colonies on plates containing 25 μg of SM per ml (SM25-resistant cells) was also determined in each culture. In the nontransitioned culture used to begin this experiment, *NR1* DNA appeared as a small satellite band of density 1.712 g/ml. After a $10^4$-fold dilution and subsequent growth into stationary phase, there was no apparent change in the *NR1* density profile (Fig. 3A) although the percentage of SM25-resistant cells had increased to 2%. After growth into stationary phase after a $10^6$ dilution, a small band of density 1.718 g/ml was observed in the *NR1* density profile (Fig. 3B) and the amount of SM25-resistant cells had increased to 13%. The culture that had been diluted $10^6$-fold and grown into stationary phase was diluted $10^4$-fold and grown into stationary phase. When the DNA prepared from this culture was examined, a broad and diffuse band of density intermediate between 1.712 and 1.718 g/ml was now present in addition to the 1.718-g/ml band. The 1.718-g/ml band had increased in proportion so that it now represented about 15% of the chromosomal DNA (Fig. 3C). A distinct 1.712-g/ml band was no longer observed in the density profile of the *NR1* DNA. The percentage of SM25-resistant cells had increased to 57%. After continued dilution and growth into stationary phase, the percentage of the intermediate density *NR1* DNA decreased and eventually only appeared as a skewing of the 1.718-g/ml band toward the less dense side (Fig. 3D, E). By this time, 100% of the cells could form colonies on plates containing 25 μg of SM per ml.

**Decrease in the proportion of transitioned cells during growth in drug-free medium.** If cells harboring *NR1* DNA in the 1.718-g/ml form are cultured in drug-free medium, there is a decrease in the percentage and the density of the *NR1* DNA (14, 17, 18). After several hundred generations of growth, only the 1.712-g/ml *NR1* DNA band appears in the *NR1* density profile. This phenomenon has been referred to as the "back-transition." To examine the mechanism of the back-transition in further detail,

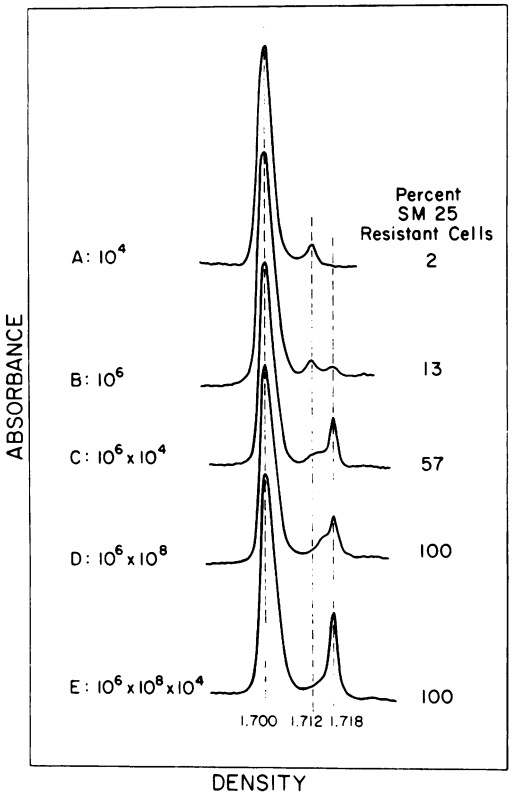


FIG. 3. *Comparison of the density profile of R factor NR1 DNA and the fraction of more highly resistant cells at various stages of the transition. Nontransitioned cells of φS-38/NR1 were given various dilutions into Penassay broth containing 100 μg of CM per ml. DNA was prepared from samples of the cells after growth into stationary phase. The numbers following the letters which identify the various density profiles refer to the total increase in the number of cells after the dilutions. In some cases, cells which had been diluted and grown into stationary phase were given further dilutions and regrown into stationary phase. For example, in C, the cells were diluted $10^6$-fold, grown into stationary phase, and then diluted $10^4$-fold and grown into stationary phase. The fraction of cells in the population that were resistant to 25 μg of SM per ml was determined as described in the legend to Fig. 2.*

we have analyzed the density profile of *NR1* DNA and the percentage of cells resistant to 25 μg of SM per ml as a function of the number of generations of growth of a previously transitioned culture (Fig. 4A) in drug-free medium. Initially 100% of the cells were resistant to 25 μg of SM per ml. After a $10^8$ dilution and subsequent growth into stationary phase, the percentage of the total *NR1* DNA decreased to about one-half of its initial value, and about 50% of the *NR1* DNA now had a density intermediate between 1.712 and 1.718 g/ml. Seventy-one percent of the cells were resistant to 25 μg of SM per ml (Fig. 4B). After a second $10^8$ dilution, there was a further decrease in the density of the *NR1* DNA and only 24% of the cells were resistant to 25 μg of SM per ml (Fig. 4C). After a third $10^8$ dilution, most of the *NR1* DNA was present in the 1.712-g/ml form; only 17% of the cells were resistant to 25 μg of SM per ml (Fig. 4D).

These experiments have shown that there is a decrease in the percentage of cells resistant to 25 μg of SM per ml which accompanies the decrease in the density of *NR1* DNA to the nontransitioned value of 1.712 g/ml. Since *NR1* density profiles consisting of 1.718- and 1.712-g/ml DNA species (and also intermediate density *NR1* DNA) were observed, it was of interest to examine whether such profiles repre-

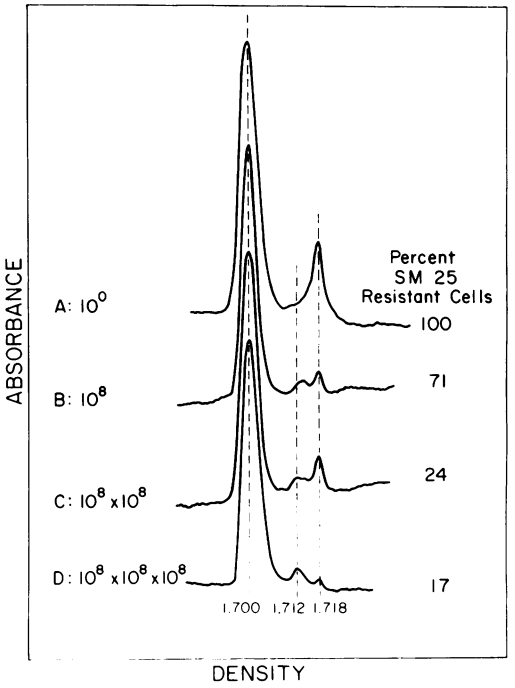


FIG. 4. *Comparison of the density profile of R factor NR1 DNA and the fraction of more highly resistant cells during the back-transition. A transitioned culture of φS-38/NR1 (A) was given successive $10^8$-fold dilutions into drug-free Penassay broth and grown into stationary phase. At various stages of the experiment DNA was prepared from samples of the cells, and the fraction of the cells that were resistant to 25 μg of SM per ml was determined as described in the legend to Fig. 2. The numbers following the letters which identify the various density profiles refer to the total increase in the number of cells after the dilutions.*

sented individual cells harboring both DNA species or a heterogeneous mixture of cells harboring primarily one type of DNA species or the other. To distinguish between these possibilities, some of the single clones that grew on drug-free plates inoculated from cultures in various stages of the back-transition experiment shown in Fig. 4 were examined for their level of resistance to SM and their DNA was examined in a CsCl gradient. Three different types of clones could be distinguished (Table 5). Type A clones grew well on plates containing 50 μg of SM per ml. Analysis of the DNA prepared from type A clones showed that most of the *NR1* DNA had a density corresponding to the transitioned state. Type B clones only formed a few colonies on plates containing 25 μg of SM per ml although they were resistant to 12.5 μg of SM per ml. The density of *NR1* DNA in these clones was 1.712 to 1.713 g/ml, corresponding to the nontransitioned state. Type C clones, which represented only a very small fraction of the total, formed only a few colonies on plates containing 12.5 μg of SM per ml. This indicated that the majority of cells in type C clones were $R^-$. Consistent with this view, type C clones did not show a detectable satellite band when their DNA was examined in a CsCl gradient. However, when all of the cells of type C clones were streaked onto plates containing 12.5 μg of SM per ml, some of the cells were found to be drug resistant. This indicates that type C clones originated from $R^+$ cells that gave rise to $R^-$ progeny during the early stages of colony formation.

To examine the rate of accumulation of these three types of clones in the back-transition experiment shown in Fig. 4, 10 of the clones from samples of the cells that had been plated on drug-free plates at various stages of the experiment were examined for their level of resistance to SM. It appeared that some cells returned almost completely to the nontransitioned state (type B) during growth in drug-free medium, whereas others remained in the transitioned state (type A) (Table 6). Thus, *NR1* density profiles such as those shown in Fig. 4B and 4C would appear to reflect a heterogeneous mixture of cells, some of which harbor 1.712-g/ml *NR1* DNA whereas the others harbor 1.718-g/ml *NR1* DNA. After prolonged growth in drug-free medium, however, all of the *NR1* DNA had a density of 1.712 g/ml, which is characteristic of nontransitioned *NR1* DNA (14, 15).

**Isolation of a minor fraction of partially transitioned cells from a nontransitioned population.** The experiment shown in Fig. 2 is consistent with the view that a minor fraction of the cells in a nontransitioned population can outgrow other cells during growth in medium containing appropriate drugs. This fraction of the cells could be regarded as spontaneously transitioned cells in the nontransitioned population. Alternatively, it could be imagined that the presence of drug in the growth medium somehow induces the conversion of nontransitioned cells to transitioned cells, which therefore accumulate during growth in the presence of drug. If the former explanation is correct, it should be possible to isolate transitioned cells from a nontransitioned population by using replica plating. Nontransitioned cells were plated onto drug-free plates and about 5,000 clones were examined by replica plating onto plates containing 25 μg of SM per ml. Although the majority of the replicated clones showed only sparse growth on the SM25 plates, 32 clones showed extensive growth. A number of these clones were picked from the drug-free

TABLE 5. *Types of cells observed during the back-transition*[a]

| Type of clone | Pattern of cell growth on plates containing: | | | | Density pattern of *NR1* DNA |
|---|---|---|---|---|---|
| | No drugs | SM (12.5 μg/ml) | SM (25 μg/ml) | SM (50 μg/ml) | |
| A | +++[b] | +++ | ++ | ++ | Two bands of density 1.715 and 1.718 g/ml |
| B | +++ | ++ | ca. 20 | — | Single band of density 1.712 to 1.713 g/ml |
| C | +++ | ca. 10 | — | — | No observable *NR1* DNA bands |

[a] Single clones which resulted from the plating of the cells on drug-free plates during the back-transition experiment shown in Fig. 3 were transferred into 0.2 ml of sterile buffer with a sterile toothpick and vortexed. The suspension of cells was streaked onto Penassay agar plates containing either no drugs or various concentrations of SM. After the plates were incubated at 37 C for 20 h, the pattern of cell growth was monitored. DNA was prepared from the lawn of cells which grew on the drug-free plates after resuspending them in an appropriate volume of buffer.

[b] The notation for the pattern of cell growth is as described in Table 1.

TABLE 6. *Clonal analysis of change in level of resistance of cells at various stages of the back-transition*[a]

| Increase in no. of cells during growth in drug-free medium | Resistance levels (types) of clones examined | | |
|---|---|---|---|
| | A | B | C |
| — | 9 | 1 | 0 |
| $10^8$ | 7 | 3 | 0 |
| $10^8 \times 10^8$ | 6 | 3 | 1 |
| $10^8 \times 10^8 \times 10^8$ | 3 | 6 | 1 |

[a] Level of resistance of 10 clones which resulted from the plating of the cells on drug-free plates during various stages of the back-transition experiment shown in Fig. 3 was determined as described in Table 5.

master plates and found to be resistant to at least 25 μg of SM per ml. These clones were generally unstable. If they were streaked onto drug-free plates, both more highly SM-resistant progeny clones and less resistant (nontransitioned) progeny clones could be isolated. Occasional $R^-$ progeny clones were also observed.

The level of SM resistance of these clones was also reflected in the density profile of their DNA. Whereas *NR1* DNA in the original nontransitioned population of cells had a density of 1.712 g/ml (Fig. 5A), the more highly SM-resistant clones isolated by replica plating from the nontrasitioned population also contained *NR1* DNA of density 1.718 g/ml as well as intermediate density *NR1* DNA (Fig. 5B, C). The density of some of the DNA of these clones was thus characteristic of the transitioned state. In general, clones with higher levels of SM resistance contained a larger amount of *NR1* DNA of density greater than 1.712 g/ml. More highly SM-resistant progeny clones derived from the SM25-resistant clones formed on the original drug-free master plates also had a larger amount of more dense *NR1* DNA than the original clones from which they were derived. For example, the DNA density profile shown in Fig. 5C corresponds to a more highly resistant clone that was isolated from the SM25-resistant culture whose DNA density profile is shown in Fig. 5B. The occasional $R^-$ progeny clones that were observed did not have detectable satellite DNA bands. It should be emphasized that all of these experiments were carried out with drug-free medium so that the changes in the level of SM resistance and the *NR1* DNA density profile were not caused by the presence of antibiotics.

In the experiments shown in Fig. 5, the proportion of the *NR1* DNA species of density greater than 1.712 g/ml was considerably smaller than that observed after prolonged growth in medium containing appropriate drugs (as in Fig. 3E). Thus, it seems that these more highly resistant cells in a nontransitioned population are most properly designated as "partially transitioned." The frequency of occurrence of such partially transitioned cells is estimated to be about 1 in $10^3$ cells, although this figure must be regarded as only approximate. It is conceivable that less partially transitioned cells might occur in higher proportion in the cell population; if these cells had only slightly higher levels of resistance than nontransitioned cells, they may not have been detected by the replica plating procedure.

To show conclusively that these spontaneously occurring "partially transitioned" cells

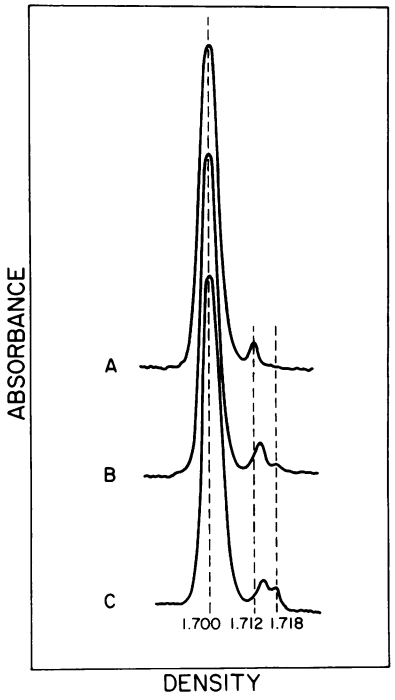


FIG. 5. *Density profiles of DNA prepared from spontaneous partially transitioned cells. Spontaneously occurring clones of φS-38/NR1 having an increased level of resistance to SM were isolated by replica plating as described in the text. The cells were cultured in drug-free Penassay broth. DNA was prepared from stationary-phase cells and examined in a CsCl density gradient. (A) Density profile of original nontransitioned cell population; (B) density profile of a culture prepared from a clone resistant to 25 μg of SM per ml that was isolated from the original nontransitioned population by replica plating; (C) density profile of a culture prepared from a more highly resistant progeny clone isolated from the culture whose DNA density profile is shown in B.*

can actually outgrow nontransitioned cells in a cell population, a reconstruction experiment was carried out. In several experiments, a minority of the φS-38 (*gal*⁻) spontaneously transitioned cells described above were mixed with φS-3 (*gal*⁺) nontransitioned cells and the mixture of cells was diluted to various extents into medium containing 100 μg of CM per ml. The spontaneously transitioned cells rapidly outgrew the nontransitioned cells (Table 7). These results clearly indicate that the minor fraction of preexisting partially transitioned cells in the cell population outgrow the nontransitioned cells to cause the change in the *NR1* DNA density profiles as well as the increase in the fraction of more highly resistant cells under the conditions of our experiments.

**Drug resistance genes must reside on the R factor in order to obtain the transition.** Since the transition of the R factor *NR1* appears to be due to the selective outgrowth of a minority of partially transitioned cells under the conditions of our experiments, it seemed likely that the drug resistance genes must reside on the R factor if the transition is to occur during growth in medium containing appropriate drugs. To examine this possibility, a mutant of φS-38 was isolated that was CM resistant due to a chromosomal mutation. Chromosomal CM resistance in *P. mirabilis* is due to the synthesis of a chloramphenicol acetyltransferase that appears to be quite closely related to the R factor chloramphenicol acetyltransferase (20). A CM-sensitive derivative of *NR1* (R100-99) was transferred to φS-38 CMʳ. φS-38 CMʳ/R100-99 was incubated for prolonged periods in Penassay broth containing various concentrations of CM (up to 800 μg/ml). Examination of the DNA prepared from these different cultures showed only a single R factor DNA band of density 1.712 g/ml. Thus the transition did not occur. φS-38 CMʳ/R100-99 was found to undergo the transition after a $10^6$ dilution and subsequent growth into stationary phase in medium containing 50 μg of SM per ml. In another experiment, a CM-resistant revertant of R100-99 was isolated in *E. coli* and transferred to φS-38 CMʳ. Analysis of the DNA prepared from this R⁺ *P. mirabilis* strain after incubation in medium containing appropriate concentrations of CM showed that the R factor DNA was in the transitioned state. These experiments clearly show that a drug resistance gene must reside on the R factor in order to obtain the transition during growth in medium containing the drug in question.

TABLE 7. *More rapid growth of spontaneously transitioned cells than nontransitioned cells in Penassay broth containing 100 μg of CM per ml*[a]

| Experiment no. | Increase in no. of cells | Percent spontaneously transitioned (*gal*⁻) cells | |
|---|---|---|---|
| | | Initial mixture of cells | After growth in Penassay broth containing CM (100 μg/ml) |
| 1 | $10^4$ | 0.33 | 86 |
| 2 | $10^6$ | 0.33 | 100 |

[a] A spontaneously transitioned clone of φS-38/*NR1* (*gal*⁻) which had a higher level of resistance to SM and which was isolated by replica plating as described in the text was mixed with a nontransitioned population of φS-3/*NR1* (*gal*⁺) and diluted into Penassay broth containing 100 μg of CM per ml. After growth into stationary phase, appropriate dilutions of the mixture of cells were plated onto MacConkey plates containing 1% galactose to determine the percentage of *gal*⁺ and *gal*⁻ cells in the population.

## DISCUSSION

Our characterization of R factor *NR1* DNA from nontransitioned and transitioned cultures of *P. mirabilis* by sedimentation in sucrose gradients and electron microscopy has verified a number of aspects of the transition model (17; Perlman and Rownd, submitted for publication). In the transitioned state the major fraction of *NR1* DNA is linear in structure, heterogeneous in size, and of molecular weight similar to *P. mirabilis* chromosomal DNA that has been isolated under the same conditions ($100 \times 10^6$ to $150 \times 10^6$). Since the linear chromosomal DNA most likely results from the fragmentation of the bacterial chromosome during DNA isolation, these findings suggest that the large, linear 1.718-g/ml *NR1* DNA molecules are probably breakage products of larger circular R factor molecules. The observed large size of 1.718-g/ml *NR1* DNA is not unexpected since the changes in the density of *NR1* DNA during the transition indicate that 10 to 20 copies of r-determinants may be incorporated into individual R factors. The resulting poly-r-determinant R factors would have molecular weights in the range 200 to 400 million and would be subject to shear breakage during isolation and handling, just as is the *P. mirabilis* chromosomal DNA. The intermediate-density *NR1* DNA from transitioned cultures should be of lower molecular weight since these molecules would contain fewer copies of r-determinants. Using electron microscopy, it has been possible to identify large circular *NR1* DNA molecules (which therefore cannot be breakage products) that fit

the size distribution expected for molecules consisting of an RTF-TC + $n$ r-determinants, where $n$ is an integer having values as high as six. Circular molecules consisting of an integral number of copies of r-determinants have also been observed in the 1.718-g/ml region of the *NR1* density profile. One basic modification of our original model is that *NR1* dissociation into the RTF-TC and r-determinants in *P-mirabilis* is very infrequent in the nontransitioned state. Almost 100% of nontransitioned *NR1* DNA exists as a composite structure of the RTF-TC and r-determinants, just as in *E. coli*.

The results of this study have also verified a number of predictions of the transition model. In agreement with our previous findings that transitioned cells have much higher specific activities of chloramphenicol acetyltransferase (14, 18), our present experiments have shown that transitioned cells have much higher levels of resistance to CM and SM than do nontransitioned cells. As a result, transitioned cells grow more rapidly in medium containing these drugs. Since growth in medium containing either CM or SM results in increased resistance to both drugs, it would appear that both the CM and SM drug resistance genes are simultaneously amplified. This suggests that both the CM and SM resistance genes reside on the same physical structure, that is, on the same r-determinants. In contrast, the level of resistance of transitioned cells to TC is not increased. Growth in medium containing TC also does not result in an increase in the density of *NR1* DNA. Both of these findings have led to the suggestion that the TC resistance genes reside on the RTF component of *NR1*. According to our model, none of the genes which reside on the RTF component are amplified in the transitioned state. Heteroduplex studies (19) and denaturation mapping studies (manuscript in preparation) on TC deletion mutants of the R factor *NR1* have unambiguously shown that the TC resistance genes reside on the RTF component of the R factor. Biochemical studies on the level of TC resistance of transitioned cells have also led to this conclusion (6).

Our observations that individual transitioned cells can form clones on plates containing 25 $\mu$g of SM per ml have made it possible to identify and to isolate a minor fraction of more highly resistant cells in a nontransitioned population. The *NR1* DNA of these cells is in a partially transitioned state even during growth in drug-free medium. These transitioned *NR1* DNA species were not observed in our physical characterization of nontransitioned *NR1* DNA, probably because these cells represent only a very small fraction of the nontransitioned cell population. As mentioned previously, essentially all of the *NR1* DNA from nontransitioned cells is in the form of the composite structure of the RTF-TC and r-determinants. A systematic analysis of the fraction of these more highly resistant cells and the density profile of *NR1* DNA suggests that these cells outgrow the nontransitioned cells in the population during growth in medium containing 100 $\mu$g of CM per ml. This results in the observed changes in the *NR1* DNA density profile. This interpretation is also clearly supported by the reconstruction experiment in which these partially transitioned cells were mixed with nontransitioned cells and the proportion of each cell type was monitored during growth in medium containing appropriate drugs. Thus, under the conditions of our experiments, it appears that *NR1* DNA density profiles consisting of both 1.712- and 1.718-g/ml DNA bands reflect a heterogeneous population of cells consisting of a mixture of highly transitioned (1.718 g/ml) and nontransitioned (1.712 g/ml) cells. After prolonged growth in medium containing appropriate drugs, the highly transitioned cells would have outgrown the nontransitioned cells and only an *NR1* DNA band of density 1.718 g/ml would be observed in the R factor DNA density profile.

Our analysis of the DNA prepared from several of the clones in a nontransitioned culture that are more highly resistant to SM has shown that most of the *NR1* DNA has a density intermediate between 1.712 and 1.718 g/ml and that the proportion of the *NR1* DNA is considerably less than the DNA from a highly transitioned culture. This suggests that the R factors in these cells harbor only a few copies of r-determinants. It would appear that further amplification in the number of copies of r-determinants must occur to account for the higher density and proportion of *NR1* DNA after longer periods of growth in medium containing appropriate drugs. Thus the minor fraction of more highly resistant cells in a nontransitioned population would seem to be in a partially transitioned state.

Since the partially transitioned cells in a nontransitioned population represent only a small fraction of the cells, it is important to exclude the possibility that this minor fraction of the cells resulted from previous exposure to drugs. It was for this reason that TC was used in the selective plates when the R factor *NR1* was transferred from *E. coli* to *P. mirabilis* by bacterial mating (see Materials and Methods).

As discussed previously, TC does not cause the transition in *P. mirabilis*. The instability of partially transitioned cells when subcultured on drug-free plates also shows that the number of copies of r-determinants in this minor fraction of the cells can be amplified without exposure to antibiotics. Both more highly resistant and less resistant progeny clones could be isolated from partially transitioned clones, and these changes in the level of drug resistance were also reflected in the *NR1* DNA density profiles of the cells that were cultured in drug-free medium (Fig. 5).

The decrease in the proportion of the 1.718-g/ml *NR1* DNA band and the appearance of *NR1* DNA of lower density that accompanies the growth of transitioned cells in drug-free medium can be explained by the dissociation of copies of r-determinants from poly-r-determinant R factors and the gradual dilution of these autonomous r-determinants from the cell population. This would also explain the appearance of cells having the lower levels of drug resistance characteristic of nontransitioned cells. It is unlikely that these changes arise from differences in growth rates among cells within the population. Both the nontransitioned and transitioned cells have essentially the same growth rate in drug-free medium (Table 4). Thus, the dilution of copies of r-determinants during growth in drug-free medium would seem to be due primarily to a lower frequency of replication in the autonomous state. In our original model (14, 15, 18) we postulated that autonomous r-determinants replicated under a stringent replication control that permitted only one round of replication during the bacterial division cycle. When attached to an RTF, however, r-determinants could replicate under the control of the RTF replication system which allowed multiple rounds of replication each generation. This would explain why there would be selection for R factors harboring multiple copies of r-determinants during growth in medium containing appropriate drugs rather than just having r-determinants themselves existing in an autonomous state. The replication of r-determinants is probably more complex than originally considered and much remains to be learned about their replication. In any event, to explain the dilution of r-determinants from the cell population during the back-transition it is sufficient to postulate that, during balanced growth in drug-free medium, r-determinants undergo fewer rounds of replication in the autonomous state than when attached to an RTF to form an R factor.

Although the transition results from the selective outgrowth of partially transitioned cells in the population under the conditions of the experiments described here, the transition appears to occur by a different mechanism at much higher concentrations of CM (17; Perlman and Rownd, manuscript in preparation). Under these conditions a much larger fraction of the cells undergoes the transition after a 20- to 30-h lag period.

It is curious that $R^-$ cells are observed during the back-transition at a frequency that is considerably higher than that observed in the case of nontransitioned *P. mirabilis* cells when cultured in drug-free Penassay broth. The partially transitioned cells isolated from a nontransitioned population are also unstable and give rise to $R^-$ progeny. The origin of the $R^-$ cells is presently not understood in either of these situations. It is interesting to note, however, that spontaneous $R^-$ cells are observed in a nontransitioned population at about the same frequency as the partially transitioned cells observed in our experiments. Perhaps the $R^-$ cells in nontransitioned *P. mirabilis* cultures arise from the small fraction of partially transitioned cells in the population.

## ACKNOWLEDGMENTS

We thank John Anderegg for use of the Joyce-Loebl microdensitometer.

This investigation was supported by Public Health Service research grant GM-14398 and Research Career Development Award GM-19206 (to R.R.) from the National Institute of General Medical Sciences.

## LITERATURE CITED

1. Adams, M. H. 1959. Bacteriophages. Interscience Publishers, Inc., New York.
2. Adelberg, E. A., M. Mandel, and G. C. C. Chen. 1965. Optimal conditions for mutagenesis by N-methyl-N'-nitro-N-nitrosoguanidine in *Escherichia coli* K-12. Biochem. Biophys. Res. Commun., **18**:788–795.
3. Anderson, E. S. 1968. The ecology of transferable drug resistance in the Enterobacteriaceae. Annu. Rev. Microbiol. **22**:131–180.
4. Cohen, S., and C. Miller. 1970. Non-chromosomal antibiotic resistance in bacteria. II. Molecular nature of R factors isolated from *Proteus mirabilis* and *Escherichia coli*. J. Mol. Biol. **50**:671–687.
5. Falkow, S., D. K. Haapala, and R. P. Silver. 1969. Relationships between extrachromosomal elements, p. 136–158. *In* G. E. Wolstenholme and M. O'Connor (ed.), Ciba Foundation symposium on bacterial episomes and plasmids. Little, Brown & Co., Boston.
6. Franklin, T. J., and R. Rownd. 1973. R-factor-mediated resistance to tetracycline in *Proteus mirabilis*. J. Bacteriol. **115**:235–242.
7. Hashimoto, H., and Y. Hirota. 1966. Gene recombination and segregation of resistance factor R in *Escherichia coli*. J. Bacteriol. **91**:51–62.
8. Kasamatsu, H., and R. Rownd. 1970. Replication of R factors in *Proteus mirabilis*: replication under relaxed control. J. Mol. Biol. **51**:473–489.
9. Kontomichalou, P., M. Mitani, and R. Clowes. 1970.

Circular R factor molecules controlling penicillinase synthesis, replicating in *Escherichia coli* under either relaxed or stringent control. J. Bacteriol. **104**:34-44.
10. Morris, C. F., and R. Rownd. 1974. Transition of the R factor R12 in *Proteus mirabilis*. J. Bacteriol. **118**:867-879.
11. Nakaya, R., and R. Rownd. 1971. Transduction of R factors by a *Proteus mirabilis* bacteriophage. J. Bacteriol. **106**:773-783.
12. Rownd, R. 1967. The molecular nature and the control of the replication of R factors, p. 17-33. *In* S. Falkow (ed.), Symposium on infectious multiple drug resistance. U.S. Government Printing Office, Washington, D.C.
13. Rownd, R. 1969. Replication of a bacterial episome under relaxed control. J. Mol. Biol. **44**:387-402.
14. Rownd, R., H. Kasamatsu, and S. Mickel. 1971. The molecular nature and replication of drug resistance factors of the Enterobacteriaceae. Ann. N. Y. Acad. Sci. **182**:188-206.
15. Rownd, R., and S. Mickel. 1971. Dissociation and reassociation of RTF and r-determinants of the R factor *NR1* in *Proteus mirabilis*. Nature (London) New Biol. **234**:40-43.
16. Rownd, R., R. Nakaya, and A. Nakamura. 1966. Molecular nature of the drug-resistance factors of the Enterobacteriaceae. J. Mol. Biol. **17**:376-393.
17. Rownd, R., D. Perlman, H. Hashimoto, S. Mickel, E. Applebaum, and D. Taylor. 1973. Dissociation and reassociation of the transfer factor and resistance determinants of R factors as a mechanism of gene amplification in bacteria, p. 115-128. *In* R. F. Beers, Jr., and R. C. Tilghman (ed.), Cellular modification and genetic transformation by exogenous nucleic acids. Proc. 6th Miles Int. Symp. Mol. Biol. Johns Hopkins Press, Baltimore.
18. Rownd, R., H. Watanabe, S. Mickel, R. Nakaya, and B. Gargan. 1970. The molecular nature and the control of the replication of R factors, p. 535-544. *In* H. Umezawa (ed.), Progress in antimicrobial and anticancer chemotherapy, vol. 2. University of Tokyo Press, Tokyo.
19. Sharp, P. N., S. N. Cohen, and N. Davidson. 1973. Electron microscope heteroduplex studies of sequence relations among plasmids of *Escherichia coli*. II. Structure of drug resistance (R) factors and F factors. J. Mol. Biol. **75**:235-255.
20. Shaw, W. V. 1971. Comparative enzymology of chloramphenicol resistance. Ann. N.Y. Acad. Sci. **182**:234-242.
21. Watanabe, T. 1963. Infective heredity of multiple drug resistance in bacteria. Bacteriol. Rev. **27**:87-115.
22. Watanabe, T. 1967. The concept of R factors, p. 5-16. *In* S. Falkow (ed.), Symposium on infectious multiple drug resistance. U.S. Government Printing Office, Washington, D.C.