THE EVOLUTION OF PLASMIDS CARRYING MULTIPLE RESISTANCE GENES: THE ROLE OF SEGREGATION, TRANSPOSITION, AND HOMOLOGOUS RECOMBINATION

RICHARD CONDIT* AND BRUCE R. LEVIN

Department of Zoology, University of Massachusetts, Amherst, Massachusetts 01003

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The evolution of resistance to antibiotics in bacteria is an intriguing (and frightening) illustration of the power of natural selection. As a consequence of the clinical, prophylactic, and agricultural uses of antibiotics, the frequency of antibiotic resistance in certain bacterial populations rose from undetectable levels to 50% or more in less than 20 years (Watanabe 1963; Anderson 1968; Falkow 1975). Even outside the arenas of intensive antibiotic use, resistant bacteria are prevalent. For example, the majority of fecal samples from healthy people in the Boston area contain substantial numbers of bacteria resistant to one or more antibiotics (Levy et al. 1985; Levy 1986). Not only has the frequency of resistant bacteria increased during the antibiotic era, but there has also been an increase in the proportion of bacteria resistant to several antibiotics (Kagiwada et al. 1960; Watanabe 1963; Anderson 1968; Falkow 1975; Levy 1986). In the study by Levy and his colleagues, about half of the fecal samples contained organisms resistant to two or more antibiotics.

Most clinically important resistance to antibiotics in the gram-negative enteric bacteria is coded for by genes on *plasmids*, which are small extrachromosomal genetic elements sometimes called *resistance factors*. Often, plasmid-borne resistance genes are associated with transposable elements, or *transposons* (Watanabe 1963; Anderson 1968; Falkow 1975; Broda 1979; Levy 1986), which are sections of DNA capable of changing location within the genome of a single cell. Indeed, the rise of antibiotic resistance can be viewed as plasmid and transposon evolution rather than bacterial evolution (Reanney 1976; Levin and Lenski 1983). The majority of well-characterized resistance factors isolated from gram-negative bacteria are self-transmissible (*conjugative*) and carry more than one resistance gene. Plasmids coding for resistance to five different antibiotics are not uncommon (see Bukhari et al. 1977, App. B).

Although plasmids and antibiotic-resistance genes existed before the human use of antimicrobial drugs (Davies and Smith 1978; Datta and Hughes 1983), most multiple-resistance plasmids probably evolved recently in response to the human

* Present address: Department of Biology, Princeton University, Princeton, New Jersey 08544.

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use of antibiotics (Falkow 1975). In this investigation, we examine some population processes and genetic mechanisms that might account for the evolution of multiple-resistance plasmids. We present and analyze the properties of a mathematical model of selection for multiple-resistance plasmids, and we compare theoretical predictions with the results of experimental studies with populations of *Escherichia coli* and conjugative plasmids.

We model the following scenario. Two plasmids occur together in a bacterial population, each carrying a different antibiotic-resistance gene, which the bacteria lack. The plasmids are *incompatible*: they do not stably coexist in one bacterial clone because they compete for replication and inheritance. The plasmids are conjugative, however, and cells losing one plasmid type can be reinfected with another copy of the same element. This maintains a population of cells carrying both plasmids. In one particular habitat, both antibiotics are present, and cells carrying both plasmids have a considerable selective advantage over cells with only one. Our conjecture is that selection favors a cell in which the two resistance genes occur on a single plasmid, because all of that cell's descendants would have both resistances and maintain maximum fitness, whereas many of the descendants of the unstable line would lose one plasmid and hence lose fitness. In the Discussion, we argue that this is a realistic scenario for the origin and evolution of plasmids carrying several antibiotic-resistance genes.

THEORETICAL CONSIDERATIONS

A Model for the Evolution of Multiple-Resistance Plasmids

The model used here is an extension of those employed in our earlier studies of the population dynamics of plasmids and transposons (Stewart and Levin 1977; Condit et al. 1988). We consider a single host strain and three different plasmids labeled 1, 2, and X; the X plasmid carries two resistance genes that are borne separately on the 1 and 2 plasmids. There are seven cell types in the model, distinguished by the plasmids they carry. Plasmid-free cells are designated N; those with single plasmids are N_1, N_2, and N_X; and those carrying two different plasmid types are N_{12}, N_{1X}, and N_{2X}. Cells with two different plasmids are referred to as *heteroplasmid*. For simplicity, we neglect the movement of resistance genes from plasmids to the chromosome and the carriage of more than two plasmids.

These populations grow at rates Ψ, which depend on the concentration of a limiting resource, r;

$$\Psi_i(r) = (1 - s_i)Vr/(K + r), \tag{1}$$

where s_i is the selection coefficient ($i = 0, 1, 2, X, 12, 1X, 2X$), V is the hypothetical maximum growth rate, and K is the concentration of the resource at which growth is half the maximum rate (Monod 1949). Resource is taken up at a rate equal to the product of total cell density, the growth function, and a yield parameter, ϵ. We assume a serial transfer mode of population maintenance, with resource at concentration R made available at the start of each transfer (Stewart and Levin 1973). The populations grow until the resource is depleted, at which time a fraction δ survive and are transferred to start the next season.

Plasmids can be transferred from a donor cell into any recipient lacking that plasmid, creating a *transconjugant*. For example, N_1 and N_2 can transfer plasmids to each other, creating transconjugants N_{12}. Matings between donors with two plasmids and plasmid-free recipients yield one-third transconjugants with each single plasmid and one-third with both plasmids. (This is an arbitrary assumption about transfer of two plasmids; we varied the proportion of different transconjugants in a few simulations and found that it had negligible impact on our results.) Plasmid transfer occurs at rates equal to the product of the density of the donor and recipient populations and a rate parameter: γ for transfer to plasmid-free cells and γ_p for transfer to plasmid-bearing cells (the units of γ are ml cell^{-1} h^{-1}).

Vegetative segregation is a process by which cells lose plasmids. For cells carrying two plasmids, it occurs at a rate τ_p (h^{-1}) and yields equal numbers of segregants carrying each of the single plasmids. For bacteria carrying single plasmids, it occurs at a rate τ and results in plasmid-free cells.

The X plasmid is produced by recombination between plasmids 1 and 2 at a rate θ (h^{-1}); it can only occur in cells that carry both plasmids (N_{12}). We neglect recombination between other pairs of plasmids and between the chromosome and plasmids.

Implicit in the model is the assumption that the volume of the habitat is infinite, and genetic drift can therefore be ignored. With these definitions and assumptions, the rates of change in the densities of the different cell populations are given by the following differential equations (a dot ($\dot{}$) over a letter indicates differentiation with respect to time):

$$\dot{N} = \Psi_0 N - \gamma N(N_1 + N_2 + N_{12} + N_{1X} + N_{2X} + N_X) + \tau(N_1 + N_2 + N_X); \tag{2}$$

$$\dot{N}_1 = \Psi_1 N_1 + \gamma N(N_1 + N_{12}/3 + N_{1X}/3) - \tau N_1 + \tau_p(N_{12} + N_{1X})/2 - \gamma_p N_1(N_2 + N_X + N_{2X} + N_{1X}/2 + N_{12}/2); \tag{3}$$

$$\dot{N}_2 = \Psi_2 N_2 + \gamma N(N_2 + N_{12}/3 + N_{2X}/3) - \tau N_2 + \tau_p(N_{12} + N_{2X})/2 - \gamma_p N_2(N_1 + N_X + N_{1X} + N_{2X}/2 + N_{12}/2); \tag{4}$$

$$\dot{N}_{12} = \Psi_{12} N_{12} + \gamma N N_{12}/3 - \tau_p N_{12} - \theta N_{12} + \gamma_p N_1(N_2 + N_{12}/2 + N_{2X}/2) + \gamma_p N_2(N_1 + N_{12}/2 + N_{1X}/2); \tag{5}$$

$$\dot{N}_X = \Psi_X N_X + \gamma N(N_X + N_{1X}/3 + N_{2X}/3) - \tau N_X + \tau_p(N_{1X} + N_{2X})/2 + \theta N_{12} - \gamma_p N_X(N_1 + N_2 + N_{12} + N_{1X}/2 + N_{2X}/2); \tag{6}$$

$$\dot{N}_{1X} = \Psi_{1X} N_{1X} + \gamma N N_{1X}/3 - \tau_p N_{1X} + \gamma_p N_1(N_X + N_{1X}/2 + N_{2X}/2) + \gamma_p N_X(N_1 + N_{1X}/2 + N_{12}/2); \tag{7}$$

$$\dot{N}_{2X} = \Psi_{2X} N_{2X} + \gamma N N_{2X}/3 - \tau_p N_{2X} + \gamma_p N_2(N_X + N_{1X}/2 + N_{2X}/2) + \gamma_p N_X(N_2 + N_{2X}/2 + N_{12}/2); \tag{8}$$

$$\dot{r} = -\epsilon \Sigma(\Psi_i N_i). \tag{9}$$

For our analysis of the properties of these equations, we rely on numerical solutions employing the Euler method with a constant step size, $dt = 0.05$ h. Population growth continues until $r < 0.001$, at which time a new transfer cycle starts with a fraction δ of each population and R units of resource. To account for the decline in the rate of plasmid transfer with the cell growth rate (Levin et al. 1979), we let the transfer-rate parameters be functions of the resource concentration,

$$\gamma = \gamma_0 r/(K + r) \quad \text{and} \quad \gamma_p = \gamma_{p0} r/(K + r). \tag{10}$$

Rates of segregation also decline with resource concentration in this manner. The parameter values used in our simulations were within the ranges reported in the experimental portion of our study.

Simulation Results

In all runs, plasmid-free bacteria and bacteria carrying the plasmids 1 and 2 are sensitive to antibiotics in the medium and thus cannot grow and may suffer mortality. Types N_{12}, N_{1X}, N_{2X}, and N_X are resistant to all antibiotics and have the same growth rate. Because of segregation to the single-plasmid states, however, the N_{12} population has a disadvantage relative to those carrying the N_X plasmid, since some N_{12} cells lose one or the other plasmid and can no longer divide.

In figure 1, we present changes in the density of component populations over time for two different segregation rates, $\tau_p = 0.01$ and $\tau_p = 0.40$. In both cases, bacteria carrying just the stable X plasmid eventually dominate, with other cell types becoming rare minorities. The rate of ascent of cells carrying the X plasmid is approximately equal to the segregation rate, τ_p (fig. 2*A*). Changes in the fitness of the sensitive cells and the rate constant of plasmid transfer affect the rate of increase of cells carrying the X plasmid only slightly (fig. 2*B*). If, by contrast, neither plasmid transfers ($\gamma = \gamma_p = 0$), then the N_{12} population is not present and the X state cannot appear. But if even one of the two plasmids transfers, the N_{12} state is generated and X does evolve.

In figure 2*C*, we consider the contribution of the plasmid recombination rate until the time at which the stable X plasmid evolves. With high segregation rates, $\tau_p = 0.40$, and recombination rates in excess of 10^{-2}, a high frequency of cells carrying the stable plasmid can be anticipated after two transfers. Even with recombination rates of 10^{-8}, bacteria carrying the stable X plasmid dominate by the fifth transfer. We cannot, however, extend our model below a θ of about 10^{-8} per cell, since that would lead to initial densities of N_X less than one cell.

EXPERIMENTAL DESIGN AND METHODS

The theoretical results indicate that, if there is strong simultaneous selection favoring two genes carried on separate plasmids, (1) the rate of ascent of a population carrying a single plasmid with both genes would be equal to the rate at which the two-plasmid state decays by segregation, and (2) over a wide range, the rate at which the stable single plasmid is formed by recombination and the rates of

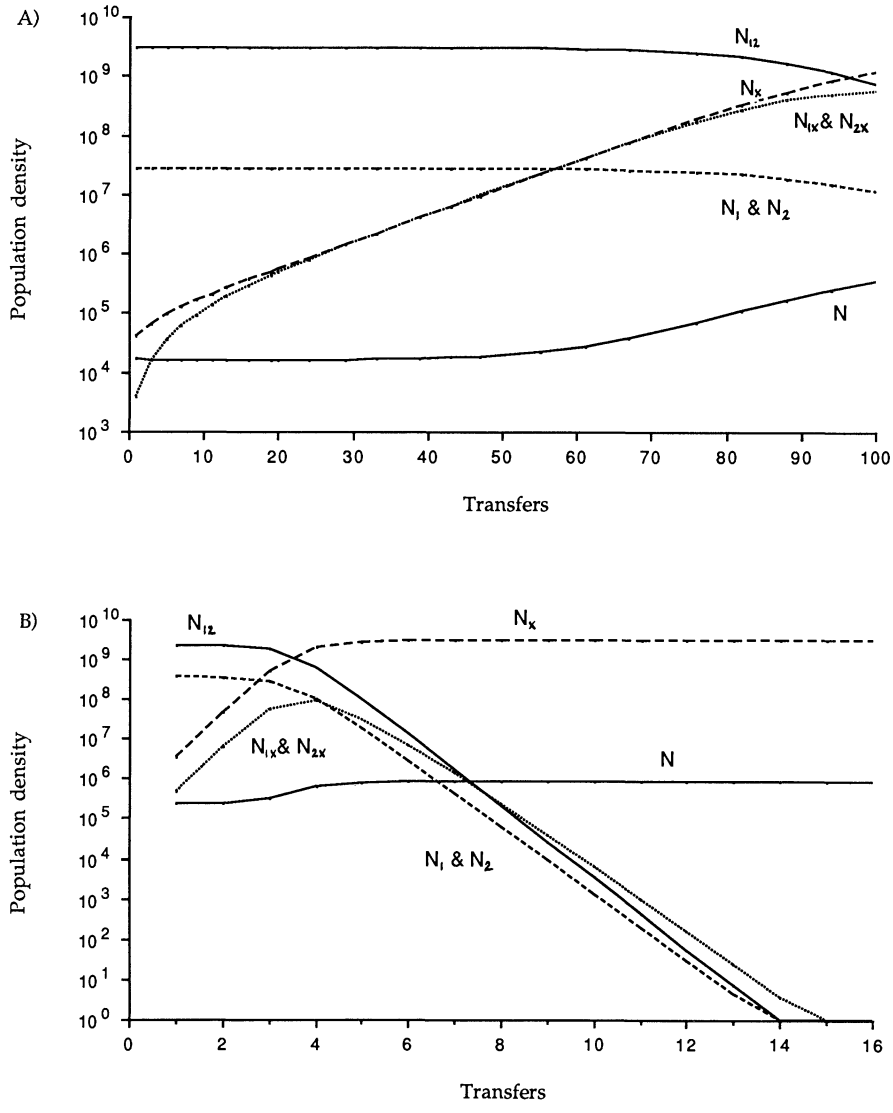

FIG. 1.—Simulation results. Population dynamics of the invasion of a strain carrying a multiple-resistance plasmid in media with two antibiotics present, showing densities of the component cell populations at the end of each transfer. *A*, Selection coefficients, $s_0 = s_1 = s_2 = 1.0$, $s_{12} = s_{1X} = s_{2X} = s_X = 0$; segregation rates, $\tau_p = 0.03\ h^{-1}$, $\tau = 10^{-3}\ h^{-1}$; conjugation rates, $\gamma = 10^{-9}$ and $\gamma_p = 10^{-10}$ ml cell^{-1} h^{-1}; recombination rate, $\theta = 10^{-6}\ h^{-1}$; maximum growth rate, $V = 1.6\ h^{-1}$; half-maximum concentration, $K = 4\ \mu g\ ml^{-1}$; conversion efficiency, $\epsilon = 5 \times 10^{-7}\ \mu g\ cell^{-1}\ h^{-1}$; resource concentration at the start of a transfer, $R = 1500\ \mu g\ ml^{-1}$; transfer fraction, $\delta = 2.5 \times 10^{-4}$. Initial densities, $N = 10^3$, $N_1 = N_2 = 10^5$, $N_{12} = N_{1X} = N_{2X} = N_X = 0$. *B*, Save for the segregation rate of the N_{12} cell line, $\tau_p = 0.40$, the parameters and initial cell densities were identical to those in view *A*.

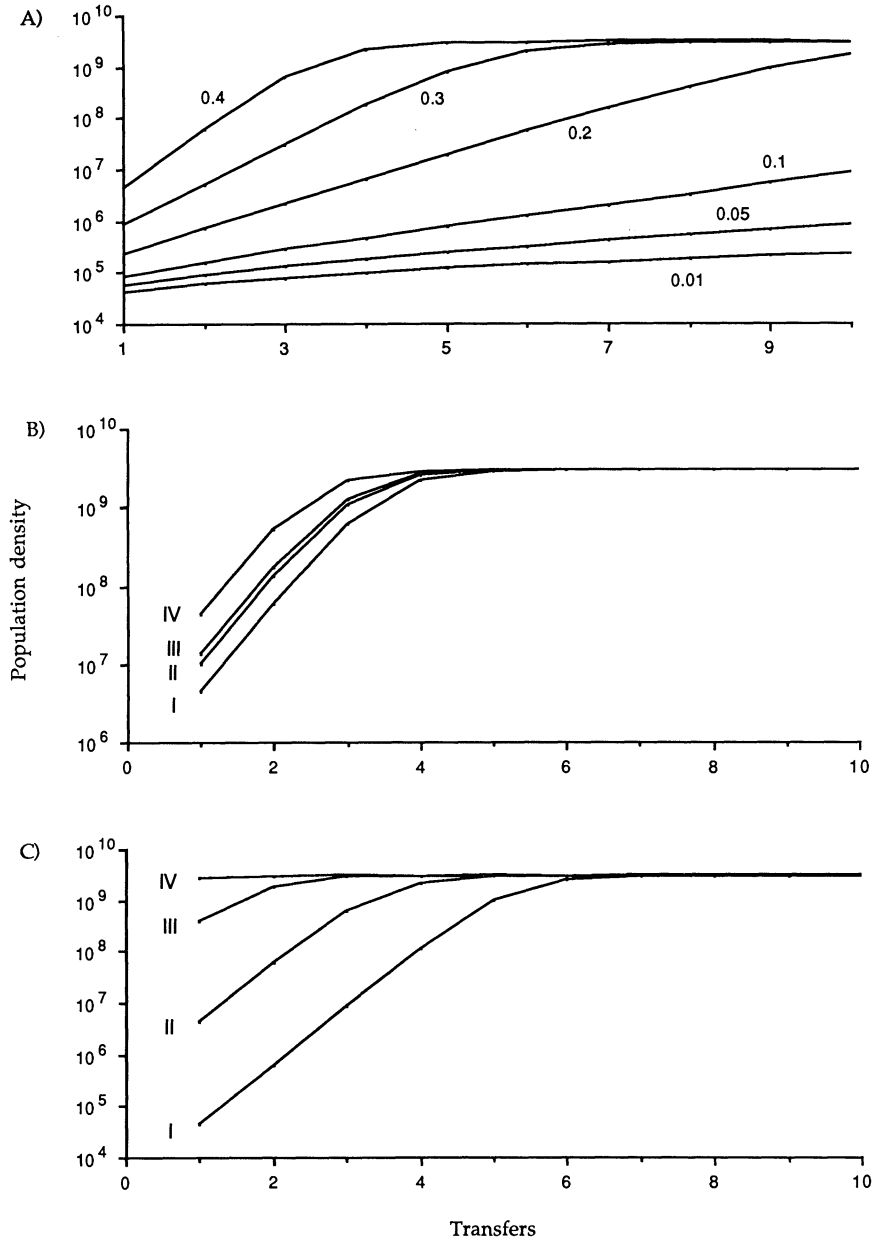

FIG. 2.—Simulation results. Invasion of cells carrying the X plasmid, with the density of all X-bearing cell lines plotted at the end of each transfer. The total cell density was 3×10^9 at the end of each transfer. Unless otherwise stated, the parameters and initial cell densities were identical to those in figure 1*B*. *A*, The effect of variation in the rate of segregation from the N_{12} population, τ_p; the value of τ_p used in a simulation is given next to the corresponding curve. *B*, Effect of variation in the intensity of selection against sensitive cells and variation in the rate of plasmid transfer. Case I, $s_0 = s_1 = s_2 = 1.0$ (identical to fig. 1*B*); case II, $s_0 = s_1 = s_2 = 2.0$; case III, $s_0 = s_1 = s_2 = 3.0$; case IV, $\gamma = 10^{-12}$, $\gamma_p = 10^{-13}$. *C*, Effect of variation in the recombination rate, θ. Case I, $\theta = 10^{-8}\,h^{-1}$; case II, $\theta = 10^{-6}\,h^{-1}$; case III, $\theta = 10^{-4}\,h^{-1}$; case IV, $\theta = 10^{-2}\,h^{-1}$.

plasmid transfer would have little effect on the time before bacteria with the stable plasmid dominate.

Using experimental populations of *Escherichia coli*, we mimicked this theoretical scenario. Strains carrying two plasmids, each with an antibiotic-resistance gene not borne on the other (we call these *complementary* resistances), were maintained in serial-transfer culture in medium containing the two relevant antibiotics. We were able to vary segregation rate by performing experiments with compatible and with incompatible plasmid pairs. Compatible plasmids are unrelated molecules that can coexist in bacterial cells independently of one another, segregating at a low rate. In contrast, when incompatible plasmids share a single cell, they segregate at such a high rate that a bacterial clone cannot maintain both together (Novick and Hoppensteadt 1978; Novick 1987). In addition, by using hosts with and without homologous recombination (Rec^+ and Rec^-), we varied the rate at which the stable plasmid was formed. Finally, the different plasmids used varied in their rates of conjugation. Estimates of the parameters were made, and the observed rate of evolution of new plasmids was compared with that predicted by the model.

Methods

Bacteria and plasmids.—Two host strains were used in evolution experiments: CSH7, which is Rec^+, and DH1, which is Rec^-. Experiments with incompatible plasmids used derivatives of the plasmid R100-1*drd*. To construct genetically distinguishable variants of R100-1*drd*, different resistance genes were inserted into the plasmid in vivo using the transposons Tn5, which carries a kanamycin-resistance gene, and Tn3, which carries ampicillin resistance. We used a "mating out" procedure with strains DD1420 (carrying Tn5) and UB1731 (carrying Tn3) to insert the transposons (Morisato et al. 1983). We selected plasmids that acquired a transposon and also lost the chloramphenicol-resistance gene; chloramphenicol-sensitive versions were necessary because experiments required that plasmid pairs have complementary resistances. All R100 derivatives were incompatible with each other. The plasmids R390 and Sa, which are compatible with each other and with the R100 plasmids, were also used. The sources and relevant genetic markers of the bacteria and plasmids are presented in table 1. Figure 3 is a diagram of R100 and its derivatives.

Selective plating on media with various antibiotic combinations allowed us to distinguish four of the seven cell types described in the model: antibiotic-sensitive cells (N), cells resistant to antibiotics 1 (N_1) and 2 (N_2), and a fourth group, which included all cells resistant to both antibiotics (N_{12}, N_{1X}, N_{2X}, and N_X). Any other cell type carrying both resistance genes—for example, with a gene on the chromosome instead of a plasmid—would fall in this latter group. Various members of this latter group were distinguished by examining the transfer ability of the resistance genes (see below).

Culture and sampling media.—All experimental cultures were maintained in Luria broth (Miller 1972). The antibiotics ampicillin (Amp), chloramphenicol (Cam), kanamycin (Kan), and naladixic acid (Nal) were used at concentrations of 200, 50, 50, and 10 mg/liter, respectively. Tetrazolium lactose agar (Levin et al.

TABLE 1

BACTERIAL STRAINS AND PLASMIDS

Name	Relevant Phenotype*	Comment	Source
Bacteria			
CSH7	Lac$^-$	source of next two strains	Miller 1972
CSH7-1	Lac$^-$ Nal		
CSH7-2	Lac$^+$		
DH1	Rec$^-$ Nal		Maniatis et al. 1982
DD1420	Kan	source of Tn5 for plasmids	Hartl et al. 1983
UB1731	Amp	source of Tn3 for plasmids	Bennett & Richmond 1976
Plasmids			
R100	Cam; IncFII	source of next 3 plasmids	Nakaya et al. 1960; Iida 1980
R5A	Cam, Kan; IncFII	derivative of R100	
R5H	Kan; IncFII	derivative of R100	
R3	Amp; IncFII	derivative of R100	
R390	Amp, Cam; IncN		Coetzee et al. 1972
Sa	Cam, Kan; IncW		Hedges & Datta 1971

* Lac, Lactose fermentation; Rec$^-$ means recombination-deficient; all other strains are recombination-proficient. Nal, Resistant to naladixic acid; Amp, resistant to ampicillin; Cam, resistant to chloramphenicol; Kan, resistant to kanamycin; IncFII, plasmid-incompatibility group FII; plasmids in the same Inc group are incompatible with one another; those from different groups are compatible.

1977) with different combinations of antibiotics was used to estimate the densities of component populations. To maintain the serial-transfer populations, 0.25×10^{-4} of the preceding day's stationary culture was transferred to 10 ml of fresh medium. All experimental cultures were incubated at 37°C with shaking at approximately 150 revolutions per minute.

Plasmid characterization.—Plasmid DNA was extracted by the alkaline precipitation method described by Birnboim and Doly (1979). After the first ethanol precipitation, one phenol-chloroform extraction was carried out, followed by the addition of RNase and then a second phenol-chloroform extraction (Maniatis et al. 1982). In some cases, we simplified this method by omitting the phenol-chloroform extractions, using instead an ammonium acetate precipitation to purify the DNA (Crouse and Amorese 1987). Electrophoresis in 0.7% agarose gels and TAE buffer was used to characterize both the native and restriction-endonuclease-digested DNA (Maniatis et al. 1982). Restriction enzyme was obtained from New England Biolabs and International Biotechnologies, Inc., and used according to the companies' protocols.

Parameter Estimates

Growth rates.—Colony counts from samples of exponentially growing cultures were used to estimate growth rates in Luria broth with and without antibiotics. The slope of the natural logarithm of cell density against time (from linear regression) yielded the growth rate $V(1 - s_i)$.

Segregation rates.—Segregation rate from cells carrying two incompatible R100 plasmids was estimated by growing a heteroplasmid culture overnight in media containing antibiotics selecting for both plasmids. About 10^4 bacteria from

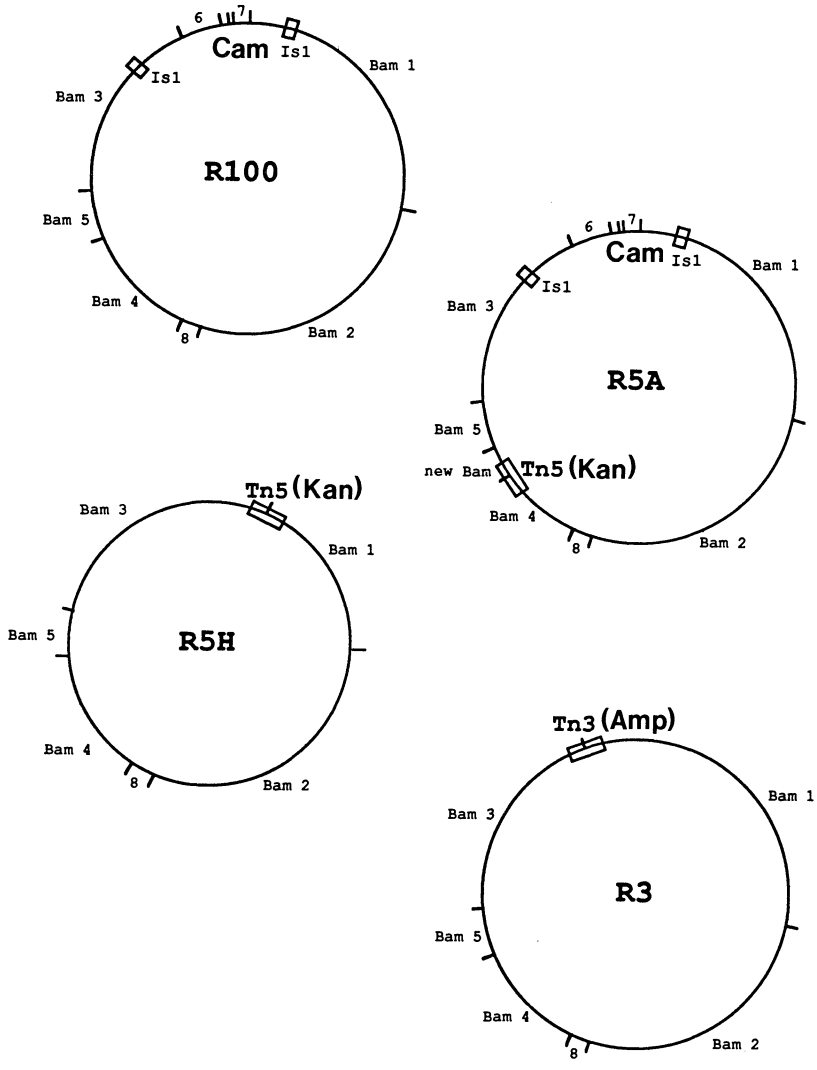

FIG. 3.—Diagrams of R100 plasmids used in the experimental studies, including *Bam*H1 restriction maps. The original R100 plasmid is 90 kilobases. The labels *Bam* 1 through *Bam* 8 refer to the largest through the smallest *Bam*H1 restriction fragments in R100 (there are two more small fragments between numbers 6 and 7). This numbering system is maintained in the three new plasmids, even though some fragment sizes change. R5H and R3 are missing fragments 6 and 7 and the chloramphenicol gene, with fragments 1 and 3 combined and then split by the *Bam*H1 site in the transposon. Maps are based on Iida 1980.

this culture were introduced into an antibiotic-free culture, and the total and heteroplasmid-cell densities were estimated at hourly intervals. At such low densities, plasmid transfer to segregants can be neglected, and the difference between the growth rate of the total cell population and the growth rate of the heteroplasmid-cell population provides an estimate of the segregation rate, τ_p (Uhlin and Nordstrom 1975). Since these experiments lasted only 6 h, or about 12 generations, fitness probably had no effect on the change in the relative abundance of heteroplasmid cells.

The segregation rate for cells with pairs of compatible plasmids was too low to determine using the same procedure. Instead, heteroplasmid cultures were maintained for 10 transfers (150 generations) without selection and were sampled at days 0, 5, and 10. At each sampling, 100 colonies carrying one of the resistance genes were screened for the loss of the other gene by transferring them to a plate containing a second antibiotic (a procedure called "patching"). Presumptive segregants were checked for other plasmid-borne markers (see table 1) to verify the loss of an entire plasmid. Since these experiments lasted more than 100 generations, it is probable that the relative abundance of segregants was affected by selection as well as by segregation (Lenski and Bouma 1987). We used our model to calculate a segregation rate or a fitness difference that accounted for the frequency of segregants by plugging in known values of conjugation parameters and varying segregation or growth-rate parameters to fit the results. For our present purposes, it does not matter which mechanism accounts for instability; we simply want an estimate of the magnitude of instability for comparison with the incompatible plasmids. Hence, we present only the estimate of segregation rate that would account for the observed instability.

Conjugation.—Estimates of conjugation parameters were made using a procedure described elsewhere (Levin et al. 1979). For plasmid-transfer experiments, it was necessary to have two distinguishable versions of CSH7. A lactose-fermenting strain and a lactose-negative, naladixic acid–resistant strain were isolated as spontaneous mutants (table 1). Unless stated otherwise, matings were done from cultures originating from a single cell.

Plasmid recombination.—A "three-point cross" between plasmids R5A and R3 was used to estimate the recombination rate θ among R100 derivatives; this was the only plasmid pair with resistance genes arranged to permit a three-point cross (see fig. 3). A heteroplasmid culture R5A-R3 was maintained for three transfers in broth and then plated onto various selective media, and patching was used to screen for different combinations of resistance to Amp, Cam, and Kan resistance. Because of rapid segregation, most cells had only a single plasmid; thus, it was possible to tally the frequency of unchanged plasmids with original resistance combinations, Cam-Kan or Amp, and recombinant plasmids with novel combinations, Amp-Kan, Amp-Cam, Kan, or Cam. For example, if R3 acquired the *kan* gene, then a cell would be resistant to Amp and Kan but sensitive to Cam; no other simple rearrangement could account for the appearance of this combination. Simulations of the model were then used to calculate the recombination rate, θ, by plugging in values of θ until finding the best fit to the experimentally observed frequency of recombinant plasmids.

A similar approach was used to examine recombination between pairs of compatible plasmids, but because of low segregation rates, a different procedure was needed to recover single plasmids out of a heteroplasmid strain. The two-plasmid strain was mated with a plasmid-free strain, producing three types of transconjugants: two with single plasmids and a third with both plasmids. The latter type, of *double transconjugants*, was relatively uncommon. Single-plasmid transconjugants were patched as described above to search for novel combinations of resistance genes.

Design of Evolution Experiments

Experiments with incompatible plasmids were begun by mixing equal volumes of overnight cultures of two different R100 variants in antibiotic-free media and allowed to grow to a stationary phase. Each culture was started from a colony that had originated from a single cell. A fraction 0.25×10^{-4} of these mated cultures was transferred into 10 ml of broth with antibiotics selecting for markers on both plasmids. These cultures were incubated for approximately 24 h (more than enough time to reach stationary phase), and the transfer process was repeated. Experiments with compatible plasmids were initiated with colonies of transconjugants carrying both plasmids (again, colonies were grown from a single cell). Serial-transfer populations were maintained in the same way as populations with incompatible plasmids.

Populations were monitored for changes by estimating total cell density and the density of cells carrying both resistance genes before each transfer. If the ratio of doubly resistant cells to total cells from plate counts exceeded 0.9, colonies were patched from plates with one antibiotic to plates with both antibiotics to obtain a more precise estimate.

EXPERIMENTAL RESULTS

Parameter Estimates

Estimates for growth-rate, segregation, and conjugation parameters are presented in table 2. In recombination experiments with incompatible plasmids in a Rec^+ host, 13 of 97 R3 plasmids acquired the *kan* gene, whereas 4 of 100 R5A plasmids lost it. A recombination rate of $10^{-2}\ h^{-1}$ for the exchange of the *kan* gene from R5A to R3 would approximately account for this frequency. We observed no R3 plasmids that acquired the *cam* gene and no R5A plasmids that lost it; therefore, the value of θ for exchange of the *cam* gene was less than about $10^{-3}\ h^{-1}$, the minimum value detectable according to our model. Identical experiments on a Rec^- host failed to yield any recombinants for either the *kan* or *cam* gene, demonstrating that the Rec^- strain was deficient in plasmid recombination, at least for exchange of the *kan* gene. No evidence for the exchange of single resistance genes between compatible plasmids was found.

Evolution in Populations of Incompatible Plasmids

Criteria for the evolution of stability and novel plasmids.—The ratio of doubly resistant cells to total cells was 0.2 or lower when a heteroplasmid culture was

TABLE 2

PARAMETER ESTIMATES FOR BACTERIA AND PLASMIDS USED IN EVOLUTION EXPERIMENTS

Strain or Plasmid	No Antibiotics	In Kanamycin	In Ampicillin	In Chloramphenicol	n*
	GROWTH RATES $V(1 - S_i)$, IN LURIA BROTH (h^{-1})				
CSH7		-4.2 ± 0.3	-1.0 ± 0.4	-0.07 ± 0.01	2
CSH7	1.7 ± 0.4				22
DH1	1.3 ± 0.2				4
	SEGREGATION RATES FROM SINGLE-PLASMID CELLS TO FORM PLASMID-FREE CELLS, τ (h^{-1})				
All plasmids	$<10^{-3}$				1 per plasmid
	SEGREGATION RATES FROM HETEROPLASMID CELLS TO FORM SINGLE-PLASMID CELLS, τ_p (h^{-1})				
R100 derivatives	0.40 ± 0.07				4
Sa-R390	$\approx 10^{-3}$				1
R5H-R390	$<10^{-3}$				1
Sa-R3	3×10^{-2}				1
	CONJUGATION RATE CONSTANTS INTO PLASMID-FREE CELLS, γ (ml $cell^{-1}$ h^{-1})				
R100, R3, R5A	$7.4 \pm 2.3 \times 10^{-9}$				9
R5H	$2.9 \pm 3.8 \times 10^{-10}$				4
Sa	$3.5 \pm 1.3 \times 10^{-11}$				5
R390	$1.0 \pm 0.9 \times 10^{-10}$				9
	CONJUGATION RATE CONSTANTS INTO PLASMID-BEARING CELLS, γ_p (ml $cell^{-1}$ h^{-1})				
R100, R3, R5A to cells with a different R100 derivative	$4.2 \pm 2.6 \times 10^{-10}$				4
R5H to R100-bearing cells	$1.9 \pm 1.6 \times 10^{-12}$				4

NOTE.—Parameter estimates are given as mean ± one standard deviation.
* n, Number of experiments.

grown without antibiotics. This is typical of a culture of incompatible plasmids and a sign of unstable inheritance: segregants formed so rapidly that they dominated the culture. In preliminary experiments, after 10 transfers in a medium selecting for both plasmids, the ratio of doubly resistant cells rose to about 1.0 and remained high even after transfer to an antibiotic-free medium. The cultures appeared to evolve stability.

For a quantitative consideration of the evolutionary change, we initiated 10 replicates, 5 on a Rec^+ host and 5 on Rec^-, of each of four different plasmid pairs and recorded the number of transfers necessary before the culture became stable. The pairs were R100-R5H (selected in Cam and Kan), R100-R3 (Amp and Cam), R5H-R3 (Amp and Kan), and R5A-R3 (Amp and Kan as well as Amp and Cam). In these experiments, we adopted a simple criterion for stability: a fraction of doubly resistant cells above 0.9 (90%).

We were interested in the evolution of plasmids with two resistance genes, not just the evolution of stability. We therefore tested the evolved cultures for transfer

TABLE 3

TIME TO EVOLUTION OF STABLE STRAINS IN CULTURES STARTING WITH PAIRS OF INCOMPATIBLE PLASMIDS

PLASMIDS	HOST PHENOTYPE	ANTIBIOTICS USED	TIME TO STABILITY (NO. OF TRANSFERS)		
			Mean	SD	*n*
R100-R3	Rec^+	Amp Cam	8.4	2.1	5
R100-R3	Rec^-	Amp Cam	8.0	1.4	4
R100-R5H	Rec^+	Kan Cam	2.4	0.5	5
R100-R5H	Rec^-	Kan Cam	3.6	1.2	5
R5H-R3	Rec^+	Amp Kan	2.6	0.5	5
R5H-R3	Rec^-	Amp Kan	3.8	1.2	5
R5A-R3	Rec^+	Amp Kan	2.2	0.4	5
R5A-R3	Rec^-	Amp Kan	3.8	0.4	5

NOTE.—Antibiotics used were those in which the evolution experiment was carried out. SD, Standard deviation. R100-R3 pairs evolved significantly more slowly than the others; there were no other differences in rate. When the last three pairs were combined, Rec^+ hosts evolved stability significantly faster than Rec^- hosts. *n*, Number of transfers; one transfer equals 15 generations. All statistical comparisons were based on the Mann-Whitney *U*-test (Sokal and Rohlf 1973).

ability of the resistance markers, comparing them with the original cultures that had not been subject to selection with antibiotics. We mixed 0.1 ml taken directly from the experimental cultures with an equivalent density of a plasmid-free strain for 30 min in 10 ml of medium without antibiotics. Transconjugant colonies were screened to determine how many carried one or the other or both resistance markers, thus providing an estimate for the frequency of cotransfer of the two resistance markers.

Finally, molecular procedures were used to substantiate the cotransfer studies by determining whether novel plasmid molecules had evolved.

Rate of evolution.—Table 3 documents variation in the time that it took stability to evolve. There was a slight effect of host strain, with Rec^- hosts taking longer to evolve stability than Rec^+ hosts, and a moderate effect of the pair of plasmids used to initiate the culture. In figure 4, we show the changes in the proportion of cells carrying double resistance, illustrating the rapid increase in stability.

As a control, we maintained several heteroplasmid cultures for up to eight serial transfers without antibiotic selection. In no case was there any change in the frequency of doubly resistant cells; hence, there was no indication of change in the stability of the resistance genes. Another control was run using cultures with only a single resistance gene in a medium with two antibiotics. These cultures never grew.

Most newly evolved strains derived from the R5A-R3 pair lost the resistance gene that was not subject to selection while evolving stability of the two selected genes. That is, most cultures selected in Amp-Kan lost Cam resistance at the same time that they evolved the stability of the Amp and Kan genes, and likewise, those selected in Amp-Cam lost the Kan gene. There were exceptions: stable, triply resistant (Amp-Cam-Kan) strains appeared in six replicates. In all six cases, the triple-resistance pattern appeared simultaneously with a doubly resistant strain in the same experiment. It appears that a polymorphism evolved, with two different

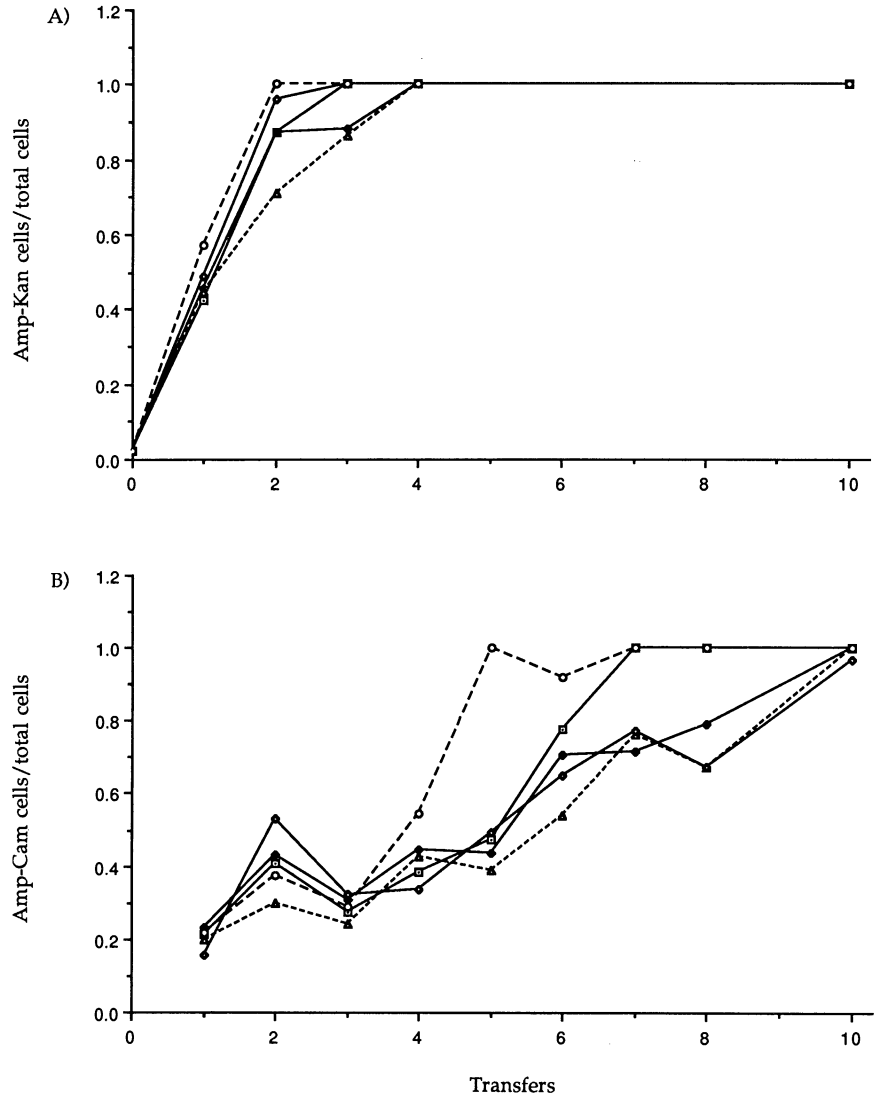

FIG. 4.—Experimental population studies with incompatible plasmids. Changes in the population of doubly resistant cells as a fraction of total cell density during selection with two antibiotics. *A*, Rise of Amp-Kan-resistant cells in experiments starting with plasmid-pair R5H-R3. *B*, Rise of Amp-Cam-resistant cells in the R100-R3 experiments. All doubly resistant cells are included in the calculation of these ratios (N_X, N_{1X}, N_{2X}, and N_{12}), but the latter three cell types always had density less than 0.20 of the total. For aesthetic reasons, when the estimated frequency of doubly resistant cells exceeded the estimated total density, the ratio was rounded off to 1.0. Symbols indicate the five replicates of the experiment.

TABLE 4

TRANSFER OF RESISTANCE MARKERS FROM STABLE STRAINS EVOLVED FROM INCOMPATIBLE PLASMID PAIRS

PLASMIDS	HOST PHENOTYPE	ANTIBIOTICS USED	FREQUENCY OF ISOLATES TRANSFERRING EACH COMBINATION OF MARKERS*			
			(1) Both as a Unit	(2) One	(3) None	(4) Both Separately
R100-R3	Rec^+	Amp Cam	1	1	4	0
R100-R3	Rec^-	Amp Cam	1	4	1	0
R100-R5H	Rec^+	Kan Cam	4	1	1	0
R100-R5H	Rec^-	Kan Cam	2	5	0	0
R5H-R3	Rec^+	Amp Kan	6	0	0	0
R5H-R3	Rec^-	Amp Kan	2	5	0	0
R5A-R3	Rec^+	Amp Kan	8	0	0	0
R5A-R3	Rec^-	Amp Kan	2	5	0	0
R5A-R3	Rec^+	Amp Cam	1	2	0	1
R5A-R3	Rec^-	Amp Cam	3	0	0	0
All	Rec^+	various	20	4	5	1
All	Rec^-	various	10	19	1	0

NOTE.—The four patterns of transfer and cotransfer are described in the text. The frequency is the number of independently evolved replicates that showed a particular pattern. When all plasmid pairs are combined, there is a statistically significant effect of host type, with type 1 being more common on Rec^+ hosts and type 2 more common on Rec^- hosts (based on a χ^2 test).

* Numbers in parentheses indicate the numbers of the transfer patterns in the text.

stable types arising at the same time and gaining dominance over the unstable forms.

Transfer ability of evolved strains.—Newly evolved strains that stably carried two resistance markers showed four different patterns of transfer of the markers to plasmid-free strains. The incidence of each outcome for various plasmid-host combinations is presented in table 4.

1. The majority that evolved on Rec^+ hosts cotransferred completely. Of 56 transconjugant colonies screened from each experiment (28 for each marker), all colonies that acquired one resistance gene also acquired the other. This was quite different from nonselected cultures carrying two plasmids, from which only about 10% of transconjugant colonies carried both resistance genes.

2. In Rec^- hosts, it was more common for one marker to transfer but not the other. The *kan* gene repeatedly lost the ability to transfer, whereas *amp* or *cam* genes failed to transfer in only a few cases.

In both types 1 and 2, transfer rates of plasmids in evolved strains were similar to those for parental plasmids.

3. Neither marker transferred (minimum detectable γ about 10^{-15} ml $cell^{-1}$ h^{-1}). Most examples of this came when R100 and R3 were the parent plasmids.

4. Of the six triply resistant strains (*amp cam kan*) that evolved from R5A-R3 parents, one transferred all three markers but did not cotransfer them. The others transferred all three markers as a unit (as in type 1 above) or transferred one marker but not the other two (as in type 2).

Restriction digests.—Because the original R100 variants could be distinguished on agarose gels using *Bam*H1 digests (fig. 5), this enzyme alone could be used to

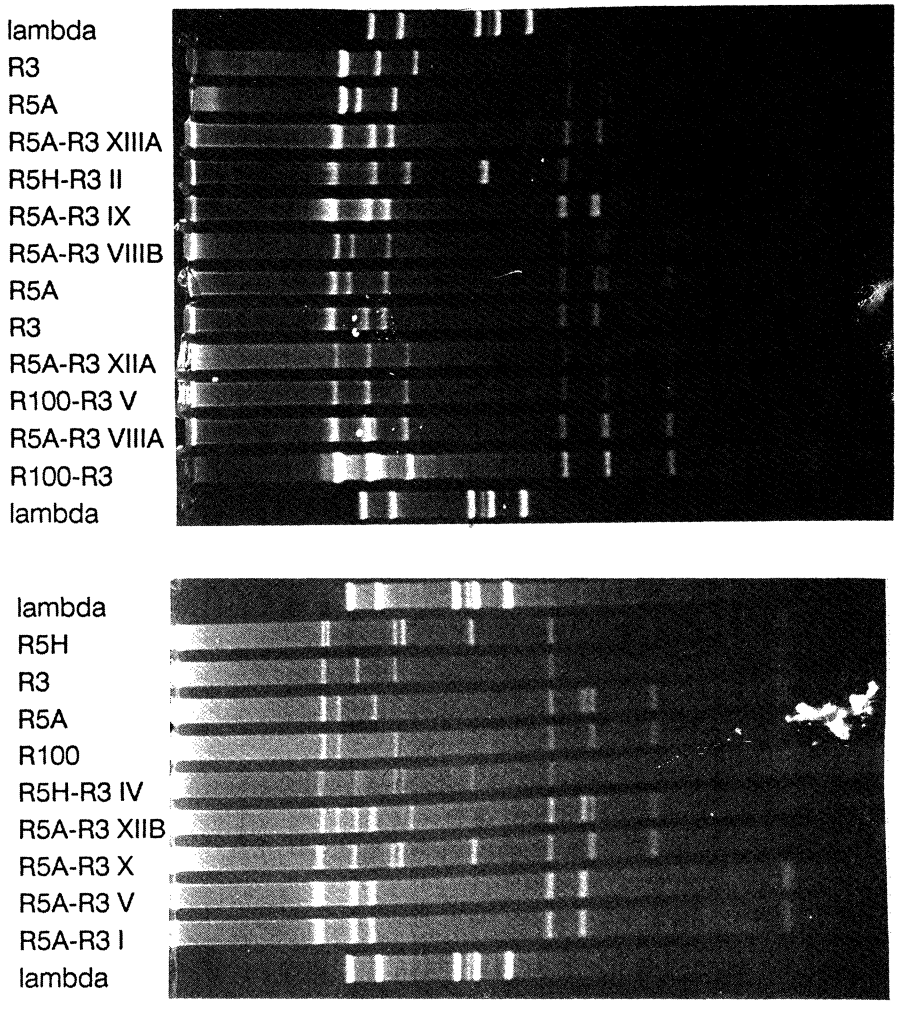

FIG. 5.—*Bam*H1 restriction fragments of plasmid DNA: parental R100 variants, 15 different evolved plasmids, and λ DNA. The evolved plasmids are named for the parental plasmids from which they were derived, with a roman numeral designating the experiment number. Loading wells are on the left. The first band of R100 is a doublet, so that, for example, the third heaviest band appears to be the second band from the left.

assess molecular changes in the evolved plasmids. In 21 of 22 stable strains that transferred only one of the two resistance markers (type 2 above), digests of plasmid DNA were indistinguishable from one of the parental plasmids. In each case, the matching parent was the one indicated by transfer results. This suggests that one resistance gene was on an unchanged plasmid and the other was on the chromosome. Since this result was more common on Rec^- than on Rec^+ hosts (table 3), we conjecture that transposition of Tn5 was the usual mechanism for transfer to the chromosome, since transposition functions are Rec-independent

(Kleckner et al. 1978; Kleckner 1981). Tn3 moved to the chromosome in only one case. There was one exception to this pattern: a new strain that transferred only one marker but showed new restriction fragments. This is one of several exceptional cases for which we do not yet have explanations.

In contrast, in 26 of 28 evolved strains in which both markers cotransferred, the *Bam*H1 digests differed from digests of either of the parental plasmids. In the majority of cases, the new pattern was a simple composite of the parentals. One example of this is R5A-R3 I in figure 5, a strain that evolved from parent plasmids R5A and R3. It shares four *Bam*H1 fragments with both parents, a single fragment with just R3, and another fragment with just R5A. Identical composite patterns often appeared in independent isolates evolved from the same parents. On gels of unrestricted DNA, all evolved plasmids appeared to be the same size as the parents. There were two examples countering this pattern: strains that cotransferred the two resistance genes but showed restriction patterns identical to those of one parent.

We believe that plasmids showing composite restriction patterns arose by an exchange of homologous regions of DNA carrying the resistance genes, for four reasons. (1) They were approximately the same size as parental plasmids and thus not a fusion of the two. (2) They appeared far less frequently on Rec^-, suggesting that host recombination functions were involved. A few plasmids showing composite restriction patterns did not arise on Rec^- hosts, but some homologous recombination can occur among plasmids even in the absence of the *recA* gene (Laban and Cohen 1981). (3) Since both Tn3 and Tn5 have a single *Bam*H1 site (Heffron et al. 1979; Jorgensen et al. 1979), a transposition between plasmids would yield a novel restriction fragment. (4) New plasmids from R5A-R3 parents usually lost the nonselected gene (see above), further demonstration that they could not have been simple fusions.

But a few of the novel restriction patterns included fragments not found in either parent. For example, the sixth largest fragment in R5A-R3 X cannot be found in either R5A or R3 (fig. 5). All patterns with new fragments appeared in only one experiment. We suggest that these were created by transposition between plasmids, but other mechanisms may have been involved.

Strains that failed to transfer any resistance (type 3) did have plasmid DNA. Most showed *Bam*H1 patterns identical to those of one parent, R3. We do not yet understand what happened in these cases. Finally, the single strain with the type-4 mating pattern (transfer of three markers but not cotransfer) showed composite restriction fragments such as those typical for the type-1 mating pattern. This may represent a breakdown in plasmid incompatibility, allowing the separate plasmids to coexist, but further data are needed.

Evolution in Populations of Compatible Plasmids

Unlike incompatible plasmids, compatible plasmids are stably maintained in a bacterial clone, which does not permit the evolution of stability to be used as a criterion for plasmid evolution. Instead, we relied on a cotransfer test to determine whether any change in the plasmids occurred during double-antibiotic selection. In matings from strains carrying two resistance markers to plasmid-free

TABLE 5

COTRANSFER OF TWO COMPATIBLE PLASMIDS BEFORE AND AFTER SELECTION

Plasmid Pairs in Donor Strains	Before Selection	After Selection
R5H-R390	0.06 ± 0.04	0.10 ± 0.03
Sa-R390	0.06 ± 0.03	0.16 ± 0.14
Sa-R3	0.12 ± 0.06	0.16 ± 0.09*

NOTE.—Cells carrying pairs of compatible plasmids were mated with a plasmid-free strain (see the text). We express cotransfer as the fraction of transconjugants that carried both resistance markers, where one marker started on each plasmid. Each entry is the mean ± standard deviation of five independent experiments with 50 colonies screened for each. There were no statistically significant differences caused by selection (comparisons based on a χ^2 test). Experiments lasted 10 transfers, or 150 generations.

* Only four replicates.

strains, the proportion of transconjugants that carried both markers was tallied. If a new plasmid carrying both resistance genes appeared and rose in frequency, then the two markers would cotransfer at a rate higher than that of the original strain.

In no case was there evidence for an increase in the frequency of cotransfer in the selected cultures (table 5) or for any other change in transfer patterns or rates. Since there was no genetic evidence for plasmid evolution in strains with compatible plasmids, we did not analyze restriction digests of these plasmids.

DISCUSSION

Our theoretical analysis predicts that intense selection favoring genes borne on separate plasmids results in the evolution of single plasmids carrying those selected genes. The pace of evolution would be governed primarily by the rate at which the separate plasmids segregate, with recombination, conjugation, and fitness playing minor roles. Our experiments were designed to test this hypothesis and to uncover other chicanery that real bacteria and plasmids may resort to when confronted with these selective conditions.

In experimental cultures with antibiotic-mediated selection favoring resistance genes borne on incompatible plasmids, a population with those genes in a stable state achieved dominance within 150 generations. The rate of evolution was roughly equal to the segregation rate of the plasmids; other parameters had relatively little effect on the time until the stable state was achieved. There was a 10-fold or greater difference in the rate of plasmid recombination between Rec^- and Rec^+ hosts, a 100-fold difference in conjugation rate among plasmids, and a large difference in the toxicity of the antibiotics, with kanamycin strongly bacteriocidal and chloramphenicol only bacteriostatic (preventing cell division). Nevertheless, the rate of evolution of the stable state differed by no more than about 3-fold in all experiments with incompatible plasmids.

We recognize that control of the various parameters was not complete. For example, since there was only one plasmid with a reduced conjugation rate, experiments with pairs of slow plasmids were not possible. In addition, it was necessary to vary some of the parameters simultaneously; changing antibiotics required changing resistance genes and transposons. Finally, the model did not consider detoxification of antibiotics, which we know from independent work weakens the toxic effect of ampicillin and chloramphenicol but not kanamycin (Lenski and Hattingh 1986). Any or all of these factors may have contributed to the evolution rate observed in R100-R3 pairs, which was about one-third that in R100-R5H or R100-R5A pairs. Our main point is not understanding this variation, but explaining how little variation there was. Despite simultaneous changes in conjugation, recombination, and fitness parameters, there was still rapid evolution of new strains.

The results of our experiments with compatible plasmids provide confirmation for a conclusion about the importance of segregation. Antibiotic treatment, conjugation rates, resistance genes, and transposons used in experiments with compatible plasmids were comparable to those used in experiments with incompatible plasmids. But compatible plasmids did not evolve; the two resistance genes were located on separate plasmids at the end of experiments, as they were at the beginning. We conclude that because the compatible plasmids coexisted stably, new arrangements of the resistance genes had no selective advantage.

The lack of evolution in compatible plasmids has an alternative explanation. Pairs of compatible plasmids recombine with one another at lower rates than do pairs of incompatible plasmids; therefore, one could argue that the evolution of multiple-resistance units from compatible plasmids was prevented by the lack of genetic variability, not by the lack of selective pressure. We believe that we can reject this alternative for two reasons. First, some of the experiments with compatible plasmids involved the transposon Tn5, and we know that Tn5 was capable of moving to the chromosome. We never, however, observed the evolution of a strain with Tn5 on the chromosome from a strain carrying two compatible plasmids; this must have been due to the lack of a segregation cost. The second reason is that, in recent experiments involving a completely different selective regimen, we have observed stable co-integrates between these same pairs of compatible plasmids, suggesting that the multiple-resistance units can be generated.

The multiple-resistance plasmids that evolved from incompatible parents could have been generated from separate plasmids by various molecular mechanisms: exchange of homologous regions of DNA via "legitimate" recombination, transposition ("illegitimate" recombination), or fusion of the two plasmids, which could result from either homologous recombination or transposition (Shapiro 1979). Each of these mechanisms could lead to a variety of different plasmid molecules, all coding for the same antibiotic-resistant phenotype. Multiple-resistance plasmids that evolved from the same parental plasmids and under the same selection regimen were sometimes different molecules. Whether these different forms varied in stability, transmissibility, or fitness remains to be studied.

In addition, we found that stable inheritance of the resistance markers some-

times occurred via transposition of one gene to the chromosome. We had not anticipated this outcome, and our mathematical model did not expressly consider the possibility. Reconsider the model, though: the N_X cell state could be viewed as any cell type that stably carries the two resistance genes. The dynamics of the N_X population would be only slightly different if one marker were on the chromosome, if two markers were on the same plasmid, or if the two plasmids remained separate but evolved compatibility. Our model has no power to predict how a stable state evolves, only how fast it increases in frequency once it appears. As it happened, multiple-resistance plasmids were the most frequent mechanism creating the stable state, at least when bacteria had normal recombination proficiency.

We believe that the scenario considered here, multiple-resistance plasmids derived from genes on separate plasmids, is realistic. Bacteria from natural sources typically carry more than one plasmid and sometimes as many as eight (Macrina et al. 1978; Jamieson et al. 1979; Caugant et al. 1981). Plasmids often have host ranges that include bacteria of different genera and habitats, giving these infectiously transmitted replicons the opportunity to acquire resistance genes from many sources. It seems reasonable that there are bacterial populations with many different plasmids carrying different resistance genes. Intensive antibiotic-mediated selection is common, and multiple antibiotics are often used for treatment or prophylaxis.

If our scenario is plausible for natural populations, then it ought to lead to predictions about the evolution of multiple-resistance plasmids. One significant prediction is that such evolution would not usually be limited by the availability of genetic variation in the plasmid population. In our experiments, even when the rate of formation of the stable, multiple-resistance state was too low to measure, stability rapidly evolved. Likewise, in the model, a 10^6-fold change in the rate of plasmid recombination had little effect on the time before a multiple-resistance plasmid dominated the culture. Of course, rates of rearrangement must be greater than zero; variants must appear.

Another prediction is that the evolution of multiple resistance would be most rapid if resistance genes were initially on incompatible plasmids. This might be testable from retrospective studies of the evolution of new plasmids; DNA sequence analysis might reveal where different resistance genes on a single plasmid originated. We cannot exclude, though, the possibility that evolution sometimes occurs from compatible plasmids, since our results indicate that pairs of compatible plasmids are not absolutely stable. Segregants overgrew the heteroplasmid cells in cultures of compatible pairs at rates of about 1% per generation. Whether this instability was caused by selection against heteroplasmid cells or by segregation remains unclear. Techniques to separate the two effects are available (Lenski and Bouma 1987), and we plan to establish which mechanism was at work. If selection causes plasmid instability, there may be no advantage to the multiple-resistance state; this requires further modeling. Indeed, our failure to observe evolution from compatible plasmids may be because the instability observed was due to selection.

Of course, we do not claim that our scenario for the evolution of multiple resistance is the only one possible. Thus far, we have considered only simultane-

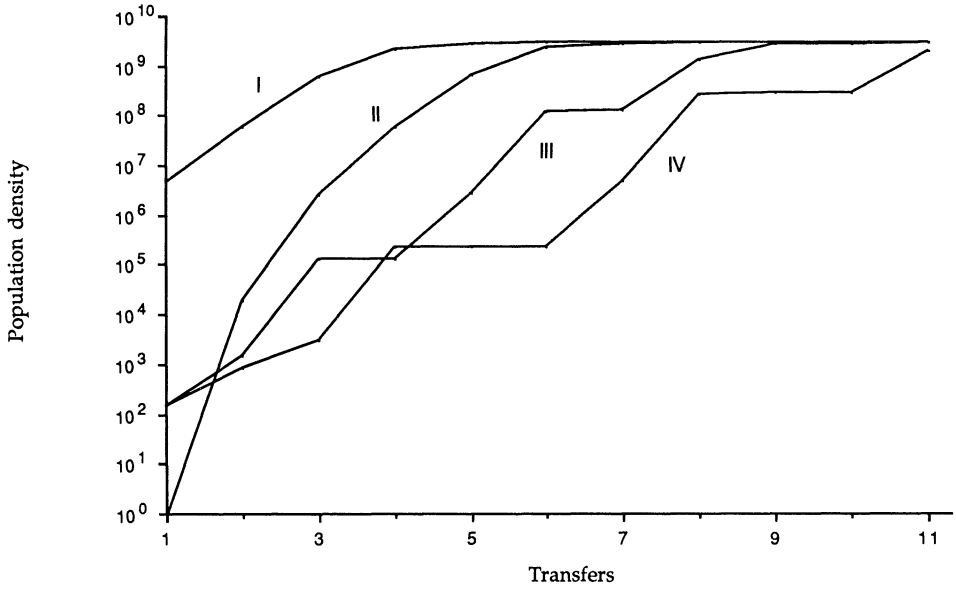

FIG. 6.—Simulation results. The effect of variation in selection regimen on the evolution of the X plasmid, plotted as in figure 2. Unless otherwise stated, the parameters and initial cell densities were identical to those in figure 1*B*. Case I, Continuous selection for double resistance, with parameter values identical to those in figure 1*B*. Case II, Selection for the 1 and 2 plasmid reversed every transfer, with $s_1 = 1$ then 0, $s_2 = 0$ then 1, etc. Case III, The same as case II, but with a respite of one transfer without selection between the transfers favoring alternate plasmids. Case IV, The same as case III, but with a two-transfer respite between transfers with selection.

ous selection by two antibiotics, but from our theoretical analysis we can show that sequential selection with different antibiotics also leads to rapid evolution. This is illustrated in figure 6, where we present the results of simulations with alternating selection for genes on opposite plasmids and periods without selection. At this juncture, we have not performed experiments analogous to these simulations.

In addition, there may be alternative scenarios for the evolution of multiple resistance in which a plasmid's capacity for horizontal transfer is important. In the scenario described here, infectious transfer of the plasmid played a small role, necessary only to produce cells carrying two distinct plasmids, but unstable inheritance was the driving selective force. Consider another scenario in which one bacterial strain has one resistance gene on its chromosome and a second one on a transmissible plasmid. If there were a second strain better adapted to the local habitat in all ways but lacking those resistance genes, then selection would favor a plasmid that acquired the chromosomal resistance gene. This plasmid could transfer both resistances to the second bacterial population. Indeed, the capacity for infectious transfer may be the reason that certain genes, like those for antibiotic resistance, are borne on plasmids in the first place.

Some studies of resistance to more than one antibiotic in gram-negative enteric

bacteria in humans and farm animals appear inconsistent with the selection regimens considered above. In these studies, treatment with a single antibiotic led to the ascent of multiple-resistance plasmids, which were not detected before antibiotic treatment (Moller et al. 1977; Levy 1978; review in Levy 1986). While suggesting the possibility of mechanisms for the evolution of multiple-resistance plasmids not considered in our study, we believe this evidence to be insufficient, because the invasion of multiple-resistance plasmids subsequent to treatment was not excluded. On the basis of our analyses, we conjecture that either simultaneous or sequential selection with several antibiotics is necessary for the evolution of multiple-resistance plasmids.

SUMMARY

We used a mathematical model and experiments with laboratory populations of *Escherichia coli* to examine conditions for the evolution of plasmids conferring resistance to multiple antibiotics. In our model and its experimental analogue, two different resistance genes are initially carried on separate plasmids, and recombination between the plasmids can generate a third element that carries both resistances. The environment is such that only cells carrying the two resistance genes can replicate. Our simulations predict that under these conditions, populations carrying a single plasmid with both resistance genes increase in frequency at a rate approximately equal to that at which the two-plasmid state decays by vegetative segregation. When the segregation rate is high, the pace of evolution is nearly independent of the rates of recombination and plasmid transfer or of whether the antibiotic is bacteriostatic or bacteriocidal.

In the main, our experimental results are consistent with these predictions. When the complementary genes were borne on incompatible plasmids and segregation occurred at a high rate (0.4 h^{-1}), stable inheritance of the resistance genes evolved in less than 150 generations. In the majority of cases, the stable state was achieved by the evolution of a single transmissible plasmid carrying both resistance genes. Variation in rates of conjugation, recombination, and fitness had little or no effect on the rate at which the stable state evolved. When the resistance genes were borne on compatible plasmids and segregation rates were low (0.01 h^{-1} or lower), there was no evidence for an evolutionary change of the plasmids, although genetic variants were probably generated.

Not all of the experimental results were anticipated from our theoretical analysis. In some cases, stable inheritance of the complementary resistance genes was obtained by the movement of one gene to the chromosome and the persistence of the other gene on an unaltered plasmid. In a few cases, we could not determine the molecular basis of evolution.

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