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COEVOLUTION IN BACTERIAL-PLASMID POPULATIONS

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Abstract.—Evolutionary changes are described in plasmid-containing strains isolated after approximately 800 generations of growth in glucose-limited chemostat culture. The reproductive fitness increased dramatically over this period. Genetic changes associated with the increases in fitness were localized to both the bacterial and the plasmid chromosomes. In addition, some of the genetic changes on the bacterial and the plasmid chromosomes interact to minimize the deleterious effect of the plasmid. Thus, the changes observed may be considered coevolutionary. Reductions in the deleterious effects of the plasmid were shown to be associated with a decrease in plasmid copy number and an increase in the rate of segregational loss of the plasmid.

Key words.—Coevolution, continuous culture, *E. coli*, plasmid.

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It is now well known that bacteria often possess extrachromosomal circular molecules of DNA, or plasmids. Such plasmids, which may range in size from a few kilobases (kb) to hundreds of kb in size, may carry genes coding for a wide range of phenotypes, including resistances to antibiotics and heavy metals, the ability to metabolize exotic chemicals, as well as the ability to transmit themselves from one cell to another (conjugation). In a number of cases plasmids have been identified that endow the cell with no known phenotype (so-called cryptic plasmids). Plasmids, in contradistinction to their close relatives the bacteriophages, are unable to lead an independent existence outside of the bacterial cell. Thus, the evolutionary fates of plasmids are inseparably associated with those of their bacterial hosts.

In the absence of the appropriate selective agent, a number of studies have shown that antibiotic resistance plasmids, considered to be of recent origin (R-plasmids; Melling et al., 1977; Engberg and Nordstrom, 1975; Godwin and Slater, 1979; Zund and Lebek, 1980; Wouters and van Andel, 1979), as well as artifically constructed plasmids (Inselburg, 1978; Jones et al., 1980; Roth et al., 1980; Wouters et al., 1980; Helling et al., 1981; Noack et al., 1981; Lee and Edlin, 1985; Boe et al., 1987; Chiang and Bremer, 1988) have a strong negative effect on the growth and reproduction of the host, though there are notable exceptions (Wouters and van Andel, 1979). Such plasmids may therefore be considered to be parasites of the host bacterial cells harboring them. Application of classic host/parasite theory to evolution in bacterial-plasmid populations allows a number of predictions to be made, as discussed extensively by Levin and Lenski (1983). Among the evolutionary changes expected in the two chromosomes are a reduction of the deleterious effect of the plasmid(s), which can result from independent or coordinated changes in both the bacterial and plasmid chromosomes. Thus, Bouma and Lenski (1988), analyzing the evolution of a bacteria/plasmid association, showed that changes in the bacterial chromosome had occurred to modify the initial deleterious effect of the plasmid into one that was beneficial. Of particular interest is the possibility that *coevolutionary* changes (Futuyma and Slatkin, 1983) may occur in the bacterial chromosome and the plasmid; that is, evolutionary changes occurring in the bacterial chromosome in response to the presence of the plasmid, and evolutionary changes occurring in the plasmid in response to the cell environment specified by the bacterial chromosome.

In this communication we analyze changes that have occurred in two different plasmid-containing strains that were isolated after almost 800 generations of evolution in a glucose-limited environment (Helling et al., 1987). The population was inoculated with a single plasmid-containing clone. Shortly after generation 100, plasmid-free cells ap-

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peared in the population and increased in frequency rapidly. Until about generation 550, the frequency of plasmid-containing cells fluctuated, at frequencies varying between 0.1 and 0.6, until about generation 550. Thereafter, the frequency of plasmid-containing cells decreased significantly, although fluctuations in their frequency were still apparent. At the termination of the experiment, at generation 773, plasmid-containing cells constituted a small minority of the population. Similar fluctuations were observed by Helling et al. (1981) and were shown to be manifestations of adaptive genetic changes occurring in the bacterial-plasmid sector of the population. The two plasmid-containing clones isolated exhibited different colony morphologies as well as physiological differences (Helling et al., 1987). We show that i) the deleterious effect of the plasmid has been reduced during evolution; ii) evolutionary changes in the plasmid are primarily responsible for an attenuation of its deleterious effect; and iii) evolutionary changes in the plasmid and the bacterial chromosomes interact to maximize fitness.

Materials and Methods

Growth Media.—Tryptone broth (TB) contained 5 g NaCl and 10 g tryptone (Difco) per liter. Tryptone agar (TA) was TB plus 1.4% (w/v) agar. The minimal medium used was that of Davis and Mingioli (1950), lacking citrate and containing thiamine·HCl at 1 μg ml^{-1}. The carbon source in the form of D-glucose was present at a concentration of 0.025% w/v in batch cultures and at 0.0125% w/v in chemostats. This latter concentration of D-glucose which resulted in glucose being the limiting substrate, maintained population densities of approximately 1×10^8 organisms ml^{-1} in chemostat culture. D-glucose (Sigma) and thiamine·HCl (Sigma) were sterilized by filtration through 0.22 μm pore-size nitrocellulose membrane (Millipore GS) prior to use in the chemostat medium. After autoclaving the medium, ampicillin was added, when necessary, to a concentration of 50 μg ml^{-1} for solid or liquid medium.

Organisms and Plasmids.—*Escherichia coli* JA104 and its derivatives and plasmid pBR322Δ5 (Heffron et al., 1981) and its derivatives (Table 1) were used. Strains carrying the plasmid pBR322Δ5 are resistant to both ampicillin and tetracycline. CV101 and CV103 were plasmid-containing ampicillin-resistant and tetracycline-resistant strains obtained from single colonies on TA plates after plating samples from the chemostat bacterial population that had been maintained for approximately 773 generations of growth in glucose-limited chemostat culture. Plasmid-containing strains were obtained by transformation with purified plasmid DNA using the standard $CaCl_2$ procedure (Maniatis et al., 1982). The presence of a plasmid in strains recovered from chemostat cultures was demonstrated by extracting the plasmid DNA by the alkaline lysis method and resolving the restriction endonuclease digested DNA on agarose gels stained with ethidium bromide (Maniatis et al., 1982). Plasmid-free derivatives of JA122 and CV101 were initially isolated by assaying colonies for β-lactamase using the iodometric assay described by Boyko and Ganschow (1982). Plasmid-free derivatives of CV103 were initially isolated by replica-plating onto TA + tetracycline plates, and identification of tetracycline-sensitive colonies. This was necessary as plasmid-free derivatives of CV103 still possess a low level of ampicillin resistance. Absence of the plasmid was confirmed by quantitative Southern analysis (Maniatis et al., 1982), probing with plasmid DNA, and analyzing the resulting autoradiographs with a laser-scanning densitometer (LKB Ultroscan XL). Plasmid-free derivatives of CV103 contained below detectable levels of plasmid DNA (<0.04 plasmid copies/bacterial chromosome).

In the absence of an antibiotic resistance marker, an arabinose resistance marker was used to distinguish different strains in our assays of relative fitness. Arabinose-resistant derivatives were obtained by plating the appropriate strains on EMB (eosin-methylene blue) arabinose medium. L-Arabinose is toxic to strains containing the *araD*139 mutation. The arabinose non-utilizing but resistant mutants have second mutations in the *araA*, *araB* or *araC* genes (Boyer et al., 1962). All strains were stored at −70°C in 40% (w/v) glycerol.

Procedure for Competition Experiments

TABLE 1. Bacterial strains.

Strain	Relevant characteristics	Replicon designation	
		E. coli	Plasmid
JA104	F⁻ *thi*1 *lac*Y1 *ara*D 139 *sup*E44 *hss*1 lysogenic for λ (Adams et al., 1979)	b_0	
CV106	As JA104 but arabinose resistant	b_0	
JA122 original strain	JA104 transformed with plasmid pBR322Δ5 (Heffron et al., 1981); ampicillin and tetracycline resistant	b_0	p_0
CV107	As JA122 but arabinose resistant	b_0	p_0
JA234	JA104 transformed with plasmid from CV101	b_0	p_1
CV110	As JA234 but arabinose resistant	b_0	p_1
JA233	JA104 transformed with plasmid from CV103	b_0	p_2
CV109	As JA233 but arabinose resistant	b_0	p_2
RIM21	Spontaneous plasmid-free segregant of CV101	b_1	
CV101 evolved strain 1	Derivative of JA122-evolved strain isolated after approximately 773 generations of glucose-limited continuous culture (Helling et al., 1987); ampicillin and tetracycline resistant	b_1	p_1
CV105	As CV101 but arabinose resistant	b_1	p_1
RIM212	RIM21 transformed with plasmid from JA122	b_1	p_0
JA238	As RIM212 but arabinose resistant	b_1	p_0
RIM213	RIM21 transformed with plasmid from CV103	b_1	p_2
JA240	As RIM212 but arabinose resistant	b_1	p_2
CV103 evolved strain 2	As CV101 but independent isolate which forms small colonies (Helling et al., 1987)	b_2	p_2
RIM10	Spontaneous plasmid-free segregant of CV103; ampicillin resistant	b_2	

in Continuous Culture.—Chemostat cultures were maintained at 30°C in a glucose-limited minimal salt chemostat medium (Helling et al., 1981) in aerated culture vessels 120–195 ml in volume, at a constant dilution rate of 0.2 hr^{-1}, equivalent to a cell generation time of approximately 3.5 hours (Kubitschek, 1970). The bacterial population density was monitored by measuring the culture absorbance at 550 nm in a Bausch and Lomb Spectronic 100 spectrophotometer. Two strains were grown in batch mode for 14–18 hours, in a minimal medium containing glucose and supplemented with ampicillin (Sigma), when necessary. Typically, the competition experiments were initiated by inoculating the chemostat with an equal number of the two competing strains from the batch culture. To increase the time that data could be gathered, the initial frequencies for some competition experiments, such as between JA122 and CV103 where the fitness differences were large, were adjusted so that the superior strain was in the minority. Just before addition of the inoculum the outflow tube of the chemostat was clamped, the cultures were allowed to mix adequately, and the clamp was then removed. Data collection commenced when numerical equilibrium (as determined by culture absorbance at A_{550}) was attained. All competition experiments were terminated after 20–25 generations to avoid complications due to the occurrence of faster growing adaptive mutants (Helling et al., 1987).

Changes in the frequencies of the two competing strains were monitored by plating samples at appropriate dilutions on TA plates and replicating these onto the appropriate selective plates (TA plus arabinose [0.12%, w/v], TA plus ampicillin [50 μg ml^{-1}], TA plus arabinose plus ampicillin plates, or TA plus tetracycline [10 μg ml^{-1}]). Typically 200 colonies were replicated for each sample. In many competition experiments colony size on TA provided an independent estimate of frequency. The frequency of *ara*^{R} organisms in each sample was calculated as the ratio of the number of arabinose-resistant colonies to the total number of colonies replicated. Similarly, changes in the frequency of the plasmid were monitored by replicating the TA plates onto TA plus ampicillin or tetracycline. In experiments involving mixtures of plasmid containing arabinose-resistant and arabinose-sensitive strains, the frequency of arabinose-resistant organisms was estimated by also replicating the colonies appearing on TA onto TA plus arabinose and ampi-

cillin and scoring growth after 8–10 hours at 37°C. Thus, in all competition experiments involving plasmid-containing strains the frequencies of the two competing strains were corrected for the loss of plasmid. Experiments in which the frequency of the spontaneous plasmid-free derivatives increased above 6% of the total parent population were not considered in the data set. Control competition experiments were performed to estimate the selective effect of mutations to arabinose-resistance in glucose-limited chemostat culture. Under our conditions, the arabinose resistance phenotype exhibited a small selective disadvantage ($s = 0.035 \pm 0.009$). There was no evidence that the selective effect of the arabinose resistance phenotype was affected by the bacterial and plasmid genotypes in the competition experiments, described here. Relative fitnesses were corrected for the effect of the arabinose marker, where necessary. Each estimate of relative fitness is presented together with the standard error of the estimate and was obtained from a single competition experiment. For most pairs of strains, replicate competition experiments were carried out. We assume each experiment provides an unbiased, independent estimate of relative fitness.

The fitness of the strain predominating in the competition experiments is defined as unity. The selective coefficient of the less successful strain is defined as the absolute value of the slope of the least square regression of the change in the frequencies of the two strains over time (in cell generations) after linearization of the frequencies with a logit transformation. In all cases, the logit transformation resulted in an excellent fit of the data to the linear model, indicating no evidence for frequency-dependent selection. The number of cell generations was determined from $(D \times t)/\ln 2$, where D is the dilution rate (hr^{-1}) and t is the time (hr) since the beginning of the competition experiment (Kubitschek, 1970). The standard errors associated with the relative fitnesses must be regarded as approximate as the errors associated with the data points are unavoidably autocorrelated.

Occasionally, faster growing mutant derivatives of strain CV103, characterized by large colony morphology on tryptone agar (TA) plates, appeared during growth in batch culture. Therefore, in all competition experiments involving strain CV103 and its derivatives, samples were plated at the end of batch growth on tryptone agar (TA) plates to test for the presence of revertants with a large colony morphology. Competition experiments initiated using such cultures were aborted and were not included in the data set.

Determination of Plasmid Copy Number.—Direct estimates of plasmid copy number were obtained from quantitative Southern analysis using the Schleicher and Schuell, Minifold II "slot-blotting" apparatus. For continuous cultures, cells were obtained directly from the chemostats that had attained numerical equilibrium growing at a dilution rate of 0.2 hr^{-1}. For batch cultures, cells in mid-log phase were used. Approximately 10^7 cells grown in minimal medium at 30°C were pelleted in sterile tubes using a table-top micro-centrifuge (Fisher) for five minutes. The pellet was resuspended in 100 ml of 10 mM Tris·HCl, pH 7.0. The cells were lysed by incubating the suspension at 55°C for 90 minutes with occasional gentle vortexing, in 100 ml of lysis buffer, containing 0.05 M Tris-HCl, 1.0% Triton X-100, 1 mg/ml of lysozyme (Sigma), 1.0% proteinase K (Boehringer Mannheim Biochemicals), pH 8.0. The lysate was purified by two phenol/chloroform extractions. The aqueous layers from the two extractions were pooled, 0.1 volume (about 400 μl) of 3.0 M NaOH added and incubated for 0.5 hr at 65°C (to destroy RNA and denature the DNA), and then cooled to room temperature, and neutralized by adding 1.0 volume 2M ammonium acetate (pH 7.0). Various dilutions of the sample DNA, prepared as described above, were loaded on the Nytran (Schleicher and Schuell) membranes as suggested by the manufacturer. Pure DNA (as determined from the A_{260}/A_{280} ratio), used as quantitative standards, was resuspended in 400 ml of 10 mM Tris·HCl, pH 7.0, 1 mM EDTA, and then the NaOH and ammonium acetate were added as described above. The amount of DNA was estimated from the absorbance at 260 nm. Various dilutions of the DNA, used as internal standards for estimating the amount of DNA, prepared as described above, were loaded

on the Nytran (Schleicher and Schuell) membrane containing the sample DNAs. The membranes were hybridized with probe DNA (pure plasmid DNA isolated from JA122, described earlier) labeled by nick-translation with ^{32}P dCTP using the kit supplied by Boehringer Mannheim. The autoradiograms were scanned using the Ultroscan XL Laser densitometer and the amount of DNA homologous to plasmid DNA in various samples was determined. The plasmid copy number (number of plasmid copies per cell) was then calculated from $\hat{x}/\hat{y}\hat{z}$, where $\hat{x}$ is the estimated amount of plasmid DNA in the slot, as determined from the standards, $\hat{y}$ is the number of cells from which the plasmid DNA was obtained, and $\hat{z}$ is the amount of plasmid DNA/plasmid chromosome, estimated to be 9.862×10^{-9} ng.

Indirect Estimation of Plasmid Copy Number.—Relative plasmid copy number of the strain was estimated indirectly from the levels of ampicillin required to limit growth. Plasmid-containing cells were used to inoculate TB medium containing ampicillin at a final concentration of 50 μg/ml and the culture was grown at 30°C overnight. The overnight culture was used to inoculate TB containing ampicillin at concentrations varying between 0 to 7 mg/ml. A function, defined as,

$$f(A_{550}) = \frac{\exp\{\alpha + \beta x\}}{1 + \exp\{\alpha + \beta x\}} + \gamma$$

where x is the concentration of ampicillin (mg/ml), α and β are parameters of the logistic equation, and γ is a parameter representing displacement along the x axis, was fitted to the absorbance values (A_{550}) of the different cultures, after 16 hours of growth at 30°C in a roller drum (New Brunswick Scientific) in different ampicillin concentrations, using nonlinear regression employing numerical estimation of the partial derivative (Dixon et al., 1985). This equation was chosen as it gave an excellent fit to the data. The minimum inhibitory concentration for a strain is defined as the concentration of the antibiotic (ampicillin) that allows a maximum cell density of 0.15 absorbance units using the above growth conditions, as calculated from the least squares regression equation.

Determination of Plasmid Segregation Rate.—Segregation rate of the plasmid is defined here as the rate of loss of plasmid from the cell per cell per cell division. Thus, the change in the frequency of plasmid-containing cells is described by

$$p_t = p_0 e^{-vt} \approx p_0(1 - vt),$$

where p_0 is the frequency of plasmid-containing cells at time 0, p_t is the frequency of plasmid-containing cells at time t, and v is the plasmid segregation rate. The ratio of the total cell density (cells ml^{-1}) to the initial cell density was used to calculate the average number of generations during batch growth.

Plasmid-containing cells were used to inoculate minimal medium batch cultures as described earlier. Viable cell counts were determined by plating dilutions of the cell cultures onto TA plates. The rate of plasmid segregational loss was estimated by assaying the frequency of plasmid-free cells appearing after 14–16 hours of growth. Putative plasmid-free cells derived from JA122 and CV101 were identified using the iodometric assay of Boyko and Ganschow (1982) for β-lactamase. Putative plasmid-free cells derived from CV103 were identified by replica-plating colonies grown on TA plates, onto TA + tetracycline plates. Absence of a plasmid was confirmed by restreaking colonies and replica-plating onto TA + ampicillin + tetracycline plates. To verify the accuracy of this procedure, DNA preparations for a subsample of putative plasmid-free clones were resolved on agarose gels and asssayed for the presence of plasmid DNA using Southern hybridization as described above.

Estimates of the rate of segregational loss of the plasmid determined in this way are confounded with selective differences between plasmid-containing and plasmid-free strains (but see Noack et al., 1984; and Lenski and Bouma, 1987). However, this procedure allows *relative* changes in the rate of segregational loss to be identified, provided that the *differences* in the deleterious effects of the plasmids are not large.

Results

A population, inoculated with a single clone of a plasmid-containing strain of *E. coli* was maintained in long-term glucose-

TABLE 2. Reconstruction of changes.

Strain 1		Strain 2	Fitness of strain 2 relative to a value of 1 for strain 1 ± SE‡
CV101 $b_1 + p_1$	⊗	CV107 $b_0 + p_0$	0.453 ± 0.094†
CV103 $b_2 + p_2$	⊗	CV105 $b_1 + p_1$	0.820 ± 0.012†
CV103 $b_2 + p_2$	⊗	CV105 $b_1 + p_1$	0.805 ± 0.050†
CV103 $b_2 + p_2$	⊗	CV107 $b_0 + p_0$	0.066 ± 0.082†

† Fitnesses corrected for the effect of the arabinose marker.
‡ Standard error of the estimate.

limited continuous culture. At the termination of the experiment after approximately 773 generations, the population was shown to be polymorphic (Helling et al., 1987), with two majority and two minority types. The minority strains, designated CV101 and CV103, contained plasmids, which were shown on the basis of restriction mapping to be derived from the plasmid in the original strain used to inoculate the chemostat (JA122). A variety of physiological assays (Helling et al., 1987) indicated that all four strains were genetically different from each other and from the strain used to inoculate the chemostat (JA122).

Strains Isolated after Extensive Growth under Glucose Limitation Exhibit Higher Fitnesses in that Environment.—The physiological assays reported by Helling et al. (1987) suggest that the strains isolated after almost 800 generations of growth under glucose-limitation have adapted significantly to that environment. To confirm these increases in relative fitness, the strains were placed in direct competition with each other in the same glucose-limited environment, one of the strains being marked with resistance to arabinose. Table 2 shows the results of these experiments. The selective coefficients are corrected for the effect of the arabinose marker (see Materials and Methods). To aid in the interpretation of these and the other experiments described below, the bacterial chromosomes of JA122, CV101, and CV103 are denoted by b_0, b_1, and b_2, respectively, whereas the plasmid chromosomes of the same three strains are denoted as p_0, p_1, and p_2. The results indicate significant increases in the level of adaptation, with CV103 showing a significantly higher level of adaptation than CV101. Comparison of the pairwise relative fitness differences among the three strains does not indicate a simple additive or multiplicative relationshp between the fitness increments. Thus, the difference in fitness of JA122 and CV101 is 0.547, and the difference in fitnesses of CV101 and CV103 is 0.18–0.195. Yet, the difference in fitnesses between JA122 and CV103 is 0.943, a value significantly larger than that expected if fitness effects were either additive or multiplicative, suggesting an interaction between the strains beyond a simple competition for a limiting nutrient.

Genetic adaptation to growth under glucose-limitation could have occurred as a result of one or more of the following: i) evolutionary changes in the bacterial chromosome; ii) evolutionary changes in the plasmid chromosome, resulting in a reduction of the deleterious effect associated with the presence of the plasmid; iii) coevolutionary changes in both the plasmid and the bacterial chromosomes, such that the interaction of the two resulted in fitness increases. To identify and distinguish among these three categories, strains were constructed carrying different combinations of bacterial and plasmid chromosomes, and competition experiments were carried out between them.

Adaptive Changes Occurring in the Bacterial Chromosome.—To identify adaptive changes that may have occurred in the bacterial chromosome, the two "evolved" strains, CV101 ($b_1 + p_1$), and CV103 ($b_2 + p_2$) were cured of their plasmids, and placed in pairwise competition with themselves and with JA104, the plasmid-free strain used to construct JA122. Where necessary, one strain of each pair was marked with resistance to arabinose to distinguish them. Previous results had shown that this arabinose phenotype is weakly selected under our conditions (see Materials and Methods section)

TABLE 3. Adaptation in the bacterial chromosome.

Strain 1		Strain 2	Fitness of strain 2 relative to a value of 1 for strain 1 ± SE‡
RIM21 b_1	⊗	CV106 b_0	0.356 ± 0.055†
RIM21 b_1	⊗	CV106 b_0	0.454 ± 0.066†
RIM10 b_2	⊗	RIM21 b_1	0.794 ± 0.018
RIM10 b_2	⊗	RIM21 b_1	0.878 ± 0.026
RIM10 b_2	⊗	RIM21 b_1	0.844 ± 0.019
RIM10 b_2	⊗	JA104 b_0	0.000 ± 0.067
RIM10 b_2	⊗	JA104 b_0	0.178 ± 0.094

† Fitnesses corrected for the effect of the arabinose marker.
‡ Standard error of the estimate.

and the results of the experiments, shown in Table 3, were corrected where noted for the effect of this marker. As may be expected, the results show that dramatic genetic changes have occurred in the bacterial chromosomes of both CV101 and CV103 resulting in increases in fitness in both strains. For example, the fitness of strain JA104 (b_0) is not significantly different from zero, relative to the fitness of the plasmid-free derivative (b_2) of the second "evolved" strain.

Adaptation Reduces the Deleterious Effect of the Plasmid.—A multitude of studies has indicated that the presence of a wide variety of plasmids is generally deleterious to the growth of its bacterial host, though there are some notable exceptions (e.g., Wouters and van Andel, 1979; Bouma and Lenski, 1988). For pBR322 in particular, previous results (Jones et al., 1980; Wouters et al., 1980; Noack et al., 1981; Lee and Edlin, 1985) have shown a strong selective disadvantage associated with the presence of this plasmid. The first section of Table 4 shows a similar result for the plasmid (pBR322Δ5), before long-term growth under glucose-limitation. The selective coefficient associated with this plasmid was 0.123–0.162. It would thus be expected that evolutionary changes would tend to reduce the selective disadvantage associated with the plasmid, either through changes in the bacterial chromosome, or through changes in the plasmid chromosome, or both.

Table 4 shows the results of competition experiments between the original strain, the two evolved strains, and their corresponding plasmid-free derivatives. As expected the selective coefficients associated with the evolved plasmid p_2 are significantly smaller than those associated with the original plasmid p_0 ($\alpha = 0.05$; Mann-Whitney nonparametric test, e.g., Conover, 1971). The results shown in Table 4 do not permit us to draw any conclusion regarding the relative deleterious effect of the evolved plasmid p_1. Therefore, the comparative deleterious effects of the two plasmids p_0 and p_1 were determined directly from competition experiments between them in two different bacterial genomic backgrounds, b_0, and b_1. The results in Table 5 clearly show that plasmid p_0 is more deleterious than plasmid p_1, irrespective of the bacterial genomic background. The selective coefficients are not significantly different for the backgrounds b_0 and b_1, arguing that changes in the plasmid are mainly responsible for the reduction in the deleterious effect of the plasmid, and provide no evidence for an interaction, or coevolutionary changes between the the bacterial chromosome b_1 and the plasmid chromosome p_1.

Coevolutionary Changes in the Bacterial and Plasmid Chromosomes.—Table 6 shows the selective effect of the plasmid p_2 in two different bacterial genomic backgrounds b_0 and b_1, together with the results from Table 5 for comparison purposes. In these two

TABLE 4. Adaptation reduces the deleterious effect of the plasmid.

Strain 1		Strain 2	Fitness of strain 2 relative to a value of 1 for strain 1 ± SE‡
JA104 b_0	⊗	JA122 $b_0 + p_0$	0.838 ± 0.039
JA104 b_0	⊗	JA122 $b_0 + p_0$	0.843 ± 0.051
JA104 b_0	⊗	JA122 $b_0 + p_0$	0.877 ± 0.025
RIM21 b_1	⊗	CV105 $b_1 + p_1$	0.900 ± 0.021
RIM10 b_2	⊗	CV103 $b_2 + p_2$	0.926 ± 0.021
RIM10 b_2	⊗	CV103 $b_2 + p_2$	0.887 ± 0.047
RIM10 b_2	⊗	CV103 $b_2 + p_2$	0.944 ± 0.024

‡ Standard error of the estimate.

TABLE 5. Analysis of the effect of the plasmid p_1.

Strain 1		Strain 2	Fitness of strain 2 relative to a value of 1 for strain 1 ± SE‡
JA234 $b_0 + p_1$	⊗	CV107 $b_0 + p_0$	0.965 ± 0.006†
CV110 $b_0 + p_1$	⊗	JA122 $b_0 + p_0$	0.895 ± 0.022†
CV105 $b_1 + p_1$	⊗	RIM212 $b_1 + p_0$	0.897 ± 0.028†
CV105 $b_1 + p_1$	⊗	RIM212 $b_1 + p_0$	0.917 ± 0.007†

† Fitnesses corrected for the effect of the arabinose marker.
‡ Standard error of the estimate.

"foreign" backgrounds, the relative deleterious effects of the three plasmids can be ordered as

$$p_1 < p_0 < p_2,$$

such that the plasmid p_2 is the most deleterious, and p_1 the least deleterious. The pattern of inequality is preserved in both genetic backgrounds, and contrasts with the results of Tables 4 and 5, which can be summarized in the same way as,

$$p_2 \leq p_1 < p_0.$$

Thus, the deleterious effect of plasmid p_2 is significantly less when assayed in the genomic background in which it has evolved, than in a "foreign" genomic background.

Plasmid Stability and Copy Number.— Multi-copy plasmids generally are lost from cultures at low rates, due to the imperfect segregation of plasmids into the daughter cells at cell division (Summers and Sherratt, 1984). Consequently, stability of such plasmids would be expected to be influenced by copy number. Furthermore, under conditions where the plasmid has a negative effect on growth, it is reasonable to expect that a reduction in copy number would be accompanied by a reduction in the deleterious effect associated with the plasmid. Accordingly, plasmid copy number and the rate of segregation loss were measured for the original strain and the two evolved strains, CV101 ($b_1 + p_1$) and CV103 ($b_2 + p_2$). The results shown in Table 7 indicate that the rate of segregational loss has increased significantly in both "evolved" strains, and that the strain exhibiting the highest fitness in the chemostat, CV103 ($b_2 + p_2$) possesses a significantly higher segregation rate than the other "evolved" strain, CV101 ($b_1 + p_1$). These estimates of segregation rate are biased upwards, due to selection against plasmid-containing cells, as detailed in the Materials and Methods section. However, the differences between JA122 ($b_0 + p_0$) and the "evolved" strains, and between the two evolved strains, CV101 ($b_1 + p_1$) and CV103 ($b_2 + p_2$), will be underestimated, because of the reduced deleterious effect of the plas-

TABLE 6. Analysis of the effect of the plasmid p_2 in different chromosome backgrounds.

Strain 1		Strain 2	Fitness of strain 2 relative to a value of 1 from strain 1 ± SE‡
In background b_0			
CV109 $b_0 + p_2$	⊗	JA122 $b_0 + p_0$	1.206 ± 0.090†
CV109 $b_0 + p_2$	⊗	JA234 $b_0 + p_1$	1.550 ± 0.058†
JA233 $b_0 + p_2$	⊗	CV110 $b_0 + p_1$	1.360 ± 0.044†
JA234 $b_0 + p_1$	⊗	CV107 $b_0 + p_0$	0.965 ± 0.006†
CV110 $b_0 + p_1$	⊗	JA122 $b_0 + p_0$	0.895 ± 0.022†
In background b_1			
JA240 $b_1 + p_2$	⊗	RIM212 $b_1 + p_0$	1.198 ± 0.026†
RIM213 $b_1 + p_2$	⊗	JA238 $b_1 + p_0$	1.111 ± 0.013†
RIM213 $b_1 + p_2$	⊗	CV105 $b_1 + p_1$	1.088 ± 0.016†
RIM213 $b_1 + p_2$	⊗	CV105 $b_1 + p_1$	1.092 ± 0.007†
CV105 $b_1 + p_1$	⊗	RIM212 $b_1 + p_0$	0.897 ± 0.025†
CV105 $b_1 + p_1$	⊗	RIM212 $b_1 + p_0$	0.917 ± 0.006†

† Fitnesses corrected for the effect of the arabinose marker.
‡ Standard error of the estimate.

TABLE 7. Rate of segregational loss of the plasmid.†

Strain	Designation	Segregation rate ± SD‡
JA122	$b_0 + p_0$	$5.1 \times 10^{-4} \pm 0.8 \times 10^{-4}$
CV101	$b_1 + p_1$	$2.7 \times 10^{-2} \pm 0.9 \times 10^{-2}$
CV103	$b_2 + p_2$	$7.2 \times 10^{-2} \pm 1.2 \times 10^{-2}$

† Per cell per cell division.
‡ Standard deviation.

TABLE 8. Plasmid copy number.

Strain	Designation	Copy number ± SEM†	M.I.C. ± SEM‡
JA122	$b_0 + p_0$	152 ± 17	1.91 ± 0.17
CV101	$b_1 + p_1$	160 ± 17	1.69 ± 0.03
CV103	$b_2 + p_2$	40 ± 7	0.54 ± 0.06

† Average value obtained from six different sample preparations from two separate experiments. Cells were grown in glucose-limited chemostat culture at a dilution rate of 0.2 hr^{-1}.
‡ Minimum Inhibitory Concentration of ampicillin (mg/ml). See Materials and Methods for further details. M.I.C.'s are indirect estimates of plasmid copy number. Each figure represents an average of six different experiments ± the standard error of the mean.

mids. Thus, the increase in segregation rate, inferred from the data of Table 7, is conservative.

Table 8 presents two independent estimates of plasmid copy number in the same three strains. Direct plasmid copy number estimates were obtained by quantitative DNA blot hybridization using "slot-blotting" while an indirect measure of copy number was obtained by estimating the minimum inhibitory concentration (M.I.C.'s) of ampicillin. Both sets of estimates are consistent and show that plasmid copy number is reduced significantly in CV103 ($b_2 + p_2$), but that there is no significant reduction in plasmid copy number in CV101 ($b_1 + p_1$). Comparison of the results in Tables 7 and 8, however, suggests that the increase in segregation rates observed cannot completely be explained by a reduction in copy number; CV101, which exhibits a significantly higher segregation rate compared to JA122, does not show a corresponding reduction in copy number.

DISCUSSION

Evolution of a host-parasite relationship may result in adaptive changes in one or both of the component members. Thus, in the context of bacterial-plasmid association, five possible evolutionary responses (or combinations thereof) are possible.

1) Adaptive genetic changes in the bacterial chromosome that are independent of the presence of the plasmid.
2) Adaptive genetic changes in the bacterial chromosome that ameliorate the deleterious effect of the plasmid, but which may or may not be specific to a particular plasmid. Such changes have been reported by Bouma and Lenski (1988).
3) Adaptive genetic changes in the plasmid that do not result in an amelioration of the deleterious effect of the plasmid, but which render the plasmid competitively superior in relation to other plasmids within the cell. Such changes would have evolutionary significance only for conjugative plasmids, or plasmids that are nonconjugative, but nevertheless able to be moblized by conjugative plasmids.
4) Adaptive genetic changes in the plasmid that result in a general (that is not necessarily specific to the particular host) amelioration of the deleterious effect of the plasmid. Godwin and Slater (1979) have described the attenuation of the deleterious effect of a plasmid that was apparently due to changes in the plasmid itself.
5) Adaptive genetic changes in both the bacterial chromosome and the plasmid, which are specific to each other.

This last category can be considered to be a *coevolutionary* response of the bacterial host and the plasmid parasite and presupposes that the changes in each component member will be dependent on, and will occur in response to, changes in the other. However, a broader definition of *coevolution* does not require that the adaptive responses in the two components are in any way specific, and may simply involve a combination of the second and fourth evolutionary responses listed above. For example, evolution towards attenuation of the effect of the parasite on the host may result in attenuation of the parasitic effect for all hosts, not only those in which the parasite evolved. These broad and narrow definitions of coevolution are analogous to the concepts of general and specific combining ability, used in plant breeding (e.g., Allard, 1960). Futuyma and Slatkin (1983) have proposed similar definitions of coevolution that focus on the process of adaptation rath-

er than on the resulting adaptive phenotypes.

The results presented here provide examples of evolutionary changes in bacterial-plasmid populations that include several of the responses listed above, and which can be termed coevolutionary in both the general and the restrictive sense. Long-term adaptation to a glucose-limited chemostat environment results in changes in both the bacterial and plasmid chromosomes. In one case, the bacterial and plasmid chromosomes have evolved so as to minimize the deleterious effects of the plasmid *in the genomic environment in which it had evolved.*

Under conditions where plasmid-carriage is deleterious to the cell, selection for an increased rate of reproduction in the presence of the plasmid is obviously an important evolutionary strategy (Levin and Lenski, 1983), and has been reported previously (Helling et al., 1981; Bouma and Lenski, 1988). Our results show a major increase in the fitness of the bacterial chromosome, either in the presence or the absence of a plasmid (response 2, listed above), as well as a reduction in the deleterious effect of the plasmid p_1 relative to the original plasmid p_0 (response 4, listed above). Results from the competition experiments clearly show that changes in the plasmid have reduced the deleterious effect of the plasmid (Tables 4 and 5), and also implicate changes in the bacterial chromosome (Table 6).

Changes that result in an attenuation of the deleterious effect of the plasmid would also be expected and are predicted by theoretical models (Levin and Lenski, 1983). Mutations have been described in both the host chromosome and the plasmid, which result in changes in copy number (e.g., Scott, 1984; Kües and Stahl, 1989). A lower copy number may reduce the energy load placed on the cell for plasmid DNA replication, transcription and translation, as well as the level of plasmid-mediated interference (Helling et al., 1981). The data in Table 8 show that CV103 has a significantly lower copy number than the original strain, JA122, though there is no apparent reduction in copy number for CV101. It is significant in this regard that the values for plasmid copy number under continuous culture conditions (see Table 8) are extremely high. This observation is consistent with the results of others who have shown that copy number of ColE1 type plasmids (such as pBR322) can increase drastically under conditions where growth rate is decreased (Chao and Bremer, 1986*a*, 1986*b*; Taxis du Poet et al., 1987). A higher plasmid copy number should allow a proportionately greater opportunity for a reduction in the deleterious effect of the plasmid, by a reduction in copy number. A reduction in the copy number is consistent with the increases in the rate of segregational loss observed (Table 7). Since pBR322 does not possess a *par* or *par*-like locus, segregational loss is determined by random diffusion of plasmid copies into the daughter cells at cell division. Thus, a reduction in copy number will result in a higher rate of segregational loss (Summers and Sherratt, 1984). The results in Tables 7 and 8 do not, however, show a perfect correspondence between a reduction in copy number and an increase in the rate of segregational loss, and this may be explained by changes in the relative levels of circular plasmid oligomers. Thus, a plasmid that forms high levels of circular oligomers will be expected to have, *ceteris paribus,* a higher level of segregational loss relative to a plasmid that exists exclusively in the monomeric form, even though the physical copy number is identical (Summers and Sherratt, 1984). Copy number as measured by quantitative DNA-DNA hybridization estimates the number of physical copies of the plasmid present in the cell without regard to the organization of the plasmid DNA into circular oligomers. The levels of circular oligomeric forms have been shown to be determined by both genetic elements in both the host chromosome and the plasmid (James et al., 1983), and in this regard we have noted variations between JA122, CV101 and CV103 (Al-Qadi, Helling, and Adams, unpubl. data).

Specific interactions between the bacterial and plasmid chromosomes, reducing the deleterious effect of the plasmid, involve the bacterial chromosomes b_2 and the plasmid p_2, are shown in Table 6 and can be considered an example of coevolutionary changes in the narrow sense. From our knowledge of host and plasmid factors affecting copy number (e.g., Scott, 1984), it is

not difficult to understand how interactions of this sort may occur. We believe that such interactions have been important factors in the evolution of associations between naturally occurring plasmids and their hosts.

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