The Maintenance of Plasmid-containing Organisms in Populations of *Escherichia coli*

By ROBERT B. HELLING,¹* THOMAS KINNEY¹
AND JULIAN ADAMS²

¹*Division of Biological Sciences, The University of Michigan, Ann Arbor, Michigan 48109, U.S.A.*

²*Division of Biological Sciences and Department of Human Genetics, The University of Michigan, Ann Arbor, Michigan 48109, U.S.A.*

(Received 19 February 1980; revised 10 October 1980)

Populations of *Escherichia coli* containing a small non-conjugative plasmid were grown in carbon-limited continuous culture. For all plasmids tested the presence of the plasmid lowered the growth rate of the host bacterium, and the proportion of plasmid-containing organisms in the total population declined initially. However, periodically, adaptive changes occurred in plasmid-containing organisms which increased their growth rate. This resulted in oscillations in the proportion of plasmid-containing organisms, and the delayed loss of the plasmid from the population.

INTRODUCTION

The prevalence of plasmids in micro-organisms suggests that they play a significant role in bacterial reproduction and evolution. Some selective advantages that plasmids may confer on host organisms can be inferred from the types of genes often associated with plasmids. It is generally supposed that plasmid-borne drug-resistance genes are responsible for the high frequency of drug-resistant bacteria in environments such as hospitals (Falkow, 1975; Davies & Smith, 1978). Possibly the same genes confer a selective advantage during growth in other habitats frequented by antibiotic-producing organisms, such as soil, although this has not been demonstrated conclusively. Some antibiotics are themselves products of plasmid-borne genes (Hopwood, 1978), as are various toxins and bacteriocins (Falkow, 1975). Bacteriocin production has been shown to confer a selective advantage on organisms grown in laboratory environments (Adams *et al.*, 1979), although few studies have attempted to delineate the role of any of those products in the growth and survival of the producing strain in nature (Smith & Huggins, 1976; Binns *et al.*, 1979). Some plasmids carry genes for unusual metabolic capabilities such as the ability to grow on hydrocarbons or aromatic compounds (Gunsalus *et al.*, 1975). Plasmids often confer the ability to transfer DNA from cell to cell. Furthermore, the transposable genetic elements present in many plasmids direct the transfer of DNA segments between plasmids and chromosomes and, in conjunction with transfer of DNA between cells, may increase the rate of evolution (Bennett & Richmond, 1978; Reanney, 1976).

There are also selective disadvantages associated with the maintenance of a plasmid in a population. Firstly, there is a maintenance cost to the cell due to the need to reproduce the plasmid, to synthesize RNA and protein from plasmid-borne genes and to carry out other functions determined by the plasmid. This cost can be calculated in terms of the additional energy expended and carbon sources required to maintain and replicate the plasmid and its

functions (Godwin & Slater, 1979). Secondly, the presence of a plasmid may have detrimental effects on a basic cellular structure or process; this may be termed plasmid-mediated interference. For example, a regulatory protein, coded for by a plasmid-borne gene, might inhibit transcription of a chromosomal gene whose product is essential for growth. Alternatively, a phage endopeptidase gene cloned into a plasmid might weaken the cell envelope or adversely affect active transport. It appears that many recombinant plasmids constructed *in vitro* inhibit the growth and survival of the host organism, presumably because of interference by the product of a cloned gene (Helling & Lomax, 1978). Interference may be defined as that fraction of the difference in growth rate between plasmid-free and plasmid-containing strains which cannot be attributed to maintenance cost.

We have compared plasmid-free and plasmid-containing organisms in order to determine the effect of plasmids on the reproduction of the host organism and their possible roles in bacterial evolution. The plasmids studied were derivatives of plasmid RSF2124 which contains the colicin-producing plasmid ColE1 and a transposon, Tn3, conferring resistance to ampicillin. Plasmid RSF2124 and its derivatives were chosen because they possess properties of both colicin-producing plasmids and drug-resistance plasmids. Previously, we reported that the outcome of competition between RSF2124-containing and plasmid-free organisms in glucose-limited continuous culture depended on the initial frequencies of the two strains (Adams *et al.*, 1979). When the RSF2124-containing organisms were present at a high frequency, sufficient colicin was produced to attenuate growth of the colicin-sensitive strain. At lower frequency, the advantage conferred by the plasmid, namely colicin production, was insufficient to compensate for the costs of maintaining the plasmid and so the plasmid-containing organisms were less competitive than the plasmid-free organisms. This paper analyses competition over longer periods between plasmid-free and plasmid-containing organisms which do not produce a colicin.

METHODS

Growth media. The minimal medium used was that of Davis & Mingioli (1950), lacking citrate and containing thiamin.HCl at $1\text{ }\mu\text{g ml}^{-1}$. Minor salts were added to the medium for some of the chemostat experiments (Adams & Hansche, 1974). Carbon sources were present at a concentration of 0.2% (w/v) in solid medium and 0.025% (w/v) in the liquid medium for the chemostat experiments. The latter concentration of D-glucose, maltose, L-arabinose or D-fructose gave population densities of approximately 1.0×10^8 organisms ml^{-1} when growth-limiting in chemostat culture. The carbon sources (Sigma) and thiamin.HCl (Sigma) were sterilized by filtration through 0.22 μm pore-size nitrocellulose membranes (Millipore GS) prior to use in the chemostat medium. After autoclaving the medium, filter-sterilized ampicillin (Sigma) was added, when necessary, to a concentration of $40\text{ }\mu\text{g ml}^{-1}$ for solid medium and $20\text{ }\mu\text{g ml}^{-1}$ for liquid medium. Viable counts were determined on tryptone agar (TA; Difco).

Organisms and plasmids. *Escherichia coli* strain RH202 and its derivatives (Table 1) and plasmid RSF2124 and its derivatives (Table 2) were used. Strains RH253, RH269 and RH270 were plasmid-containing ampicillin-resistant strains obtained from single colonies appearing on TA plates after plating samples from various chemostat cultures (Table 1). Strain RH253 is a mutant adapted for glucose-limited continuous culture. It contains plasmid pRBH052 which is indistinguishable from plasmid pMIL17 of the parent strain RH251. Strain RH253 was used in the development of other strains used in the experiments described (see Tables 1 and 2). Plasmids RSF2124 and pRBH057 produce colicin E1 (Adams *et al.*, 1979). The other plasmids fail to produce colicin as a result of insertion of *Euglena* chloroplast DNA segments (pMIL17 and pMIL27; Adams *et al.*, 1979) or deletion (pRBH055) at the *EcoRI* endonuclease cleavage site in the colicin gene (Adams *et al.*, 1979; Hershfield *et al.*, 1974). The cloned *Euglena* DNA in pMIL17 contains a gene for 16S chloroplast ribosomal RNA, three transfer RNA genes and part of a 23S ribosomal RNA gene (El-Gewely *et al.*, 1981), and pMIL27 contains a chloroplast transfer RNA gene (El-Gewely *et al.*, 1981); the other genes in the cloned DNA of pMIL17 and pMIL27 are unknown. The plasmids with cloned foreign DNA were used because they are unable to produce colicin. Plasmid-containing strains were obtained by transformation with purified plasmid DNA using the standard CaCl_2 procedure (Adams *et al.*, 1979; El-Gewely & Helling, 1980). The ligation and cloning procedures have been described previously (Lomax *et al.*, 1977). The plasmid composition was verified by the characteristic band patterns of the plasmid DNA observed after cleavage by site-specific endonucleases and separation by agarose gel

Table 1. *Bacterial strains*

Strain	Genotype	Origin
RH202	F ⁻ <i>thi-1 lacY1 tonA21 supE44 hsdS</i>	Adams <i>et al.</i> (1979)
RH204	As RH202, but <i>araAh9</i>	Adams <i>et al.</i> (1979)
RH251	As RH202, containing plasmid pMIL17	Adams <i>et al.</i> (1979)
RH253	Contains plasmid pRBH052	Derivative of RH251 obtained at the 28th generation in the experiment of Fig. 2 (a)
RH254	As RH202, containing plasmid pRBH052	Transformation of pRBH052 from RH253 into RH202, selection for resistance to ampicillin
RH256	As RH202, containing plasmid pMIL27	Adams <i>et al.</i> (1979)
RH269	Contains plasmid pRBH053	Derivative of RH254 obtained after 18 generations growth in a glucose-limited chemostat
RH270	Contains plasmid pRBH054	Derivative of RH254 obtained after 20 generations growth in a fructose-limited chemostat
RH271	As RH202, containing plasmid pRBH053	Transformation of pRBH053 from RH269 into RH202, selection for resistance to ampicillin
RH273	As RH202, containing plasmid pRBH055	Transformation into RH202, selection for resistance to ampicillin (see Table 2)
RH275	As RH202, containing plasmid pRBH057	Transformation into RH202, selection for resistance to ampicillin (see Table 2)

Table 2. *Plasmids*

Plasmid	Genotype* (properties)	Origin
RSF2124	<i>amp⁺ col⁺ imm⁺</i>	So <i>et al.</i> (1975)
pMIL17	<i>amp⁺ col imm⁺</i> . RSF2124 plus Euglena chloroplast DNA fragments S, U and 2A inserted at the <i>EcoRI</i> site in the <i>col</i> gene	Lomax <i>et al.</i> (1977), Helling <i>et al.</i> (1979)
pMIL27	<i>amp⁺ col imm⁺</i> . RSF2124 plus Euglena chloroplast DNA fragment I inserted at the <i>EcoRI</i> site in the <i>col</i> gene	Lomax <i>et al.</i> (1977), Helling <i>et al.</i> (1979)
pRBH052	<i>amp⁺ col imm⁺</i> . RSF2124 plus Euglena chloroplast DNA fragments S, U and 2A inserted at the <i>EcoRI</i> site in the <i>col</i> gene. Probably identical to pMIL17	Obtained in RH253 as described in Table 1
pRBH053	<i>amp⁺ col imm⁺</i> . RSF2124 plus Euglena chloroplast DNA fragments S, U and 2A inserted at the <i>EcoRI</i> site in the <i>col</i> gene. Probably identical to pMIL17 and pRBH052	Obtained in RH269 as described in Table 1
pRBH054	<i>amp⁺ col imm⁺</i> . RSF2124 plus Euglena chloroplast DNA fragments S, U and 2A inserted at the <i>EcoRI</i> site in the <i>col</i> gene. Probably identical to pMIL17 and pRBH052	Obtained in RH270 as described in Table 1
pRBH055	<i>amp⁺ col imm⁺</i> . Lacks <i>EcoRI</i> site	Derivative of RSF2124 obtained by treating RSF2124 DNA with endonuclease <i>EcoRI</i> , and transforming RH202. The procedure selects for uncleaved (circular) DNA because the efficiency of transformation by non-circular DNA is very low (Helling & Lomax, 1978)
pRBH057	<i>amp⁺ col⁺ imm⁺</i> . Probably identical to RSF2124	As pRBH055, but treated DNA was ligated prior to transformation

* The *amp⁺* gene confers ampicillin resistance, the *col⁺* gene directs colicin E1 synthesis and the *imm⁺* gene confers resistance to colicin E1.

electrophoresis (El-Gewely & Helling, 1980). By this criterion, pRBH052, pRBH053 and pRBH054 were shown to be identical to pMIL17. Plasmid pRBH055 lacked the single *EcoRI* endonuclease cleavage site of the parent plasmid RSF2124, migrated slightly faster than RSF2124 in agarose gel electrophoresis and failed to confer ability to produce colicin to the host organism. These properties are consistent with deletion of the *EcoRI* site of RSF2124 (Hershfield *et al.*, 1974). The presence of a plasmid in strains recovered from chemostat cultures was demonstrated as described previously (El-Gewely & Helling, 1980).

Procedures for competition and selection experiments in continuous culture. Chemostat cultures were maintained at 37 °C with culture volumes fixed between 150 and 190 ml and constant dilution rates between 0.35 and 0.20 h⁻¹. The population density was monitored by measuring the culture absorbance at 550 nm in a Bausch and Lomb Spectronic 100 spectrophotometer. Two different techniques were used for initiating the competition experiments.

(1) The two strains were grown at the same dilution rates to numerical equilibrium in separate chemostats. One chemostat was then inoculated with organisms from the second. Generally, an inoculum of strain RH202 (lacking any plasmid) was added to a chemostat culture of a plasmid-containing strain to achieve a population containing a frequency of plasmid-free bacteria of 0.04 to 0.2. Just before addition of the inoculum the outflow tube of the chemostat was clamped off. After a brief time to allow the culture to mix adequately, the clamp was removed. This procedure was found necessary for most competition experiments in order to control precisely the initial frequencies of the competing strains.

(2) One strain was grown to numerical equilibrium in a chemostat and to start the competition experiment a few millilitres of an exponentially growing or early stationary phase batch culture of the second strain was added. This procedure was found to be satisfactory when the initial frequency of the second strain was required to be low (<0.04 of total population). Generally, the plasmid-containing strain was grown in the presence of ampicillin (20 µg ml⁻¹) to ensure that all organisms contained the plasmid at the start of the experiment. This was especially important for certain derived plasmids, such as pMIL27, that segregated plasmid-free organisms at a high rate. It was not necessary to remove ampicillin from the medium before the addition of the plasmid-free organisms, as most of it was degraded by the β-lactamase of the plasmid-containing organisms and the remainder was rapidly diluted out when medium flow was initiated.

Change in the frequency of plasmid-containing organisms was monitored by plating samples at appropriate dilutions on TA plates and replicating these on to TA plus ampicillin plates. An average of 500 colonies was replicated for each sample. The frequency of plasmid-containing organisms in each sample was calculated as the ratio of the number of ampicillin-resistant colonies to the total number of colonies replicated. Similarly, changes in the frequency of *ara*⁺ organisms were monitored by replicating the TA plates on to minimal plates with L-arabinose (0.2%, w/v) as the carbon source.

The number of cell generations was determined from $(D \times t)/\ln 2$, where D is the dilution rate (h⁻¹) and t is the time (h) since the beginning of the competition experiment (Kubitschek, 1970). Differences in the growth rate of two strains ($|\mu_i - \mu_j|$) were calculated by standard least squares regression techniques after linearization of the frequencies with a logit transformation. This procedure ignores the effect of segregational loss of the plasmid from plasmid-containing organisms (π) in altering the frequencies of the two strains. The error introduced by this simplification was vanishingly small for all cases as $|\mu_i - \mu_j| \gg \pi$. It is worth noting that while standard linear regression techniques allow the estimation of $|\mu_i - \mu_j|$, they do not allow calculation of the confidence interval for this difference as the errors associated with the data points are autocorrelated (Adams *et al.*, 1979). The specific growth rates associated with the different genotypes were defined relative to a reference genotype (genotype 2) and are denoted as relative fitnesses following the convention in population genetics.

Containment conditions. All experiments involving recombinant DNA molecules were carried out under P2-level conditions as specified in *Guidelines for Research Involving Recombinant DNA Molecules* [Federal Register 41; (131), 27913 (1976)] and as approved by the local biohazard committee.

RESULTS

Cyclic fluctuation in the frequency of plasmid-containing organisms grown in competition with plasmid-free organisms

When populations of *Escherichia coli* either with or without a non-colicin-producing plasmid were mixed and grown together in a glucose-limited chemostat, the frequency of the plasmid-containing strain initially declined. For example, *E. coli* strain RH273 (containing non-colicin-producing plasmid pRBH055) was grown in competition with the equivalent strain lacking the plasmid (strain RH202) with initial frequencies of 0.97 and 0.03, respectively. The non-colicin-producing plasmid-containing strain decreased in frequency for the first 7 generations before recovering unexpectedly during the following 8 generations (Fig. 1). The final sample taken indicated that a second phase of diminishing frequency had begun, although the mixed population in this case was not monitored further. The presence of the plasmid in the drug-resistant organisms was verified directly by plasmid isolation and characterization from organisms plated at several times during the cycle. Several

Fig. 1. Competition between plasmid-containing and plasmid-free strains of *E. coli* during growth in glucose-limited continuous culture at a dilution rate of 0.24 h^{-1} . The plasmid-containing strains RH275 and RH273 were mixed with the plasmid-free strain RH202 to initiate each experiment: ●, frequency of strain RH275, which produces colicin, in the total chemostat population (the initial organism concentrations were $102 \times 10^6 \text{ ml}^{-1}$ for strain RH275 and $2 \times 10^6 \text{ ml}^{-1}$ for strain RH202); ○, frequency of strain RH273, which does not produce colicin (the initial organism concentrations were $81.5 \times 10^6 \text{ ml}^{-1}$ for strain RH273 and $2.5 \times 10^6 \text{ ml}^{-1}$ for strain RH202).

ampicillin-resistant colonies arising from organisms plated at 15 generations were tested for colicin production; the results were negative, showing that the increase in frequency of plasmid-containing organisms during the interval between generations 8 and 15 did not result from inhibition of the colicin-sensitive plasmid-free strain by colicin in the culture.

By comparison, when strains of *E. coli* with or without a colicin-producing plasmid were grown in competition in a glucose-limited chemostat and the initial density of the plasmid-containing strain was high, the frequency of the plasmid-containing organism increased to over 0.99 and then remained constant (Adams *et al.*, 1979). For example, *E. coli* strain RH202 was grown in competition with *E. coli* strain RH275 (containing colicin-producing plasmid pRBH057) with initial frequencies of 0.02 and 0.98, respectively, under conditions identical to the other experiment shown in Fig. 1. In this experiment the frequency of *E. coli* strain RH275, as measured by growth on ampicillin plates, increased until plasmid-free organisms disappeared (<0.002) and remained at that high frequency for the rest of the experiment (Fig. 1).

Fluctuations were found in the frequency of all non-colicin-producing plasmid-containing organisms (with plasmids derived from plasmid RSF2124) in competition with plasmid-free *E. coli* (Fig. 2 and unpublished observations). In Fig. 2, the frequencies of the descendants of the initial plasmid-free strain RH204 and of the plasmid-free descendants of the initial plasmid-bearing strains are plotted separately (see next section). Typically, the frequency of the ampicillin-resistant organisms, that is those strains containing plasmids, dropped below 0.002 after one or two fluctuations. In occasional experiments the plasmid-containing organisms persisted at much higher levels (e.g. frequencies of 0.2 to 0.4) for as long as the experiment continued.

No obvious differences in colony morphology or growth factor requirements were observed between the original plasmid-containing organisms and descendants obtained after 10 or more generations of growth in chemostat culture. Nevertheless, the descendants were

Fig. 2. Competition between plasmid-containing and plasmid-free strains of *E. coli* during growth in glucose-limited continuous culture. (a) Competition between the plasmid-free strain RH204 (●) (initial organism concentration $1 \times 10^6 \text{ ml}^{-1}$) and the non-colicin-producing plasmid-containing (pMIL17) strain RH251 (○) (initial organism concentration $77 \times 10^6 \text{ ml}^{-1}$). Plasmid-free descendants obtained from the plasmid-containing strain (□), detected as ampicillin-sensitive *ara*⁺ organisms. (b) Competition between the plasmid-free strain RH204 (●) (initial organism concentration $1.4 \times 10^6 \text{ ml}^{-1}$) and the non-colicin-producing plasmid-containing (pMIL27) strain RH256 (○) (initial organism concentration $79 \times 10^6 \text{ ml}^{-1}$). Plasmid-free descendants obtained from the plasmid-containing strain (□).

genetically different because, unlike the parent strain, they displaced the plasmid-free strain RH202 when grown in mixed culture. For example, strain RH253 (containing plasmid pRBH052) was isolated at the 28th generation from the mixed population described in Fig. 2(a). When grown in competition with strain RH202 under glucose limitation, the frequency of RH253 always increased during the first 5 to 10 generations (not shown) in contrast to the decrease in frequency of its parent, RH251, under the same conditions of competition with RH204 (as shown in Fig. 2a). Therefore we concluded that the fluctuations reflected a replacement of the original plasmid-containing strain by mutant descendants which were better adapted for growth under the conditions used.

Similar experiments to examine competition between plasmid-containing and plasmid-free strains were undertaken using different growth-limiting substrates, namely, fructose, maltose, arabinose or glycerol. In every case the frequency of plasmid-containing organisms declined, increased and declined again suggesting replacement of the initial strains by adaptive variants (as illustrated for glucose-limited cultures in Fig. 2).

The mutations in the plasmid-containing organisms which resulted in the appearance of a more competitive plasmid-containing organism were, in some cases, specific to the nature of the growth-limiting factor. For example, Fig. 3 shows the results of competition experiments between the plasmid-free *E. coli* strain RH202 and the plasmid-containing *E. coli* strain RH270. Strain RH270 was derived from strain RH254 (containing plasmid pRBH052) after

Fig. 3. Specificity of improved fitness of a strain selected during growth in fructose-limited continuous culture. The upper curve (●) shows the outcome of competition between plasmid-containing strain RH270 (previously selected for rapid growth in fructose-limited culture) and plasmid-free strain RH202 during fructose-limited continuous culture. The lower curve (○) shows the outcome of competition between the same strains when limited by glucose. For each experiment, each strain was grown in a chemostat inoculated with 5 ml of an overnight culture in the same medium. The chemostat cultures were grown as batch cultures until the population densities were between $0.6 \times 10^8 \text{ ml}^{-1}$ and $1.0 \times 10^8 \text{ ml}^{-1}$; at this time medium flow was initiated to give a dilution rate of 0.20 h^{-1} . After 15 min, the strains were mixed to initiate each experiment. The initial organism concentrations were $5.3 \times 10^7 \text{ ml}^{-1}$ for strain RH270 and $2.9 \times 10^7 \text{ ml}^{-1}$ for strain RH202 in the fructose-limited population, and $6.9 \times 10^7 \text{ ml}^{-1}$ for strain RH270 and $2.7 \times 10^7 \text{ ml}^{-1}$ for strain RH202 in the glucose-limited population.

20 generations of growth in a fructose-limited chemostat (not shown). In mixed culture under fructose-limited conditions *E. coli* strain RH270 competed successfully, replacing strain RH202 (Fig. 3, upper curve). However, when a mixed population of the same organisms was grown in glucose-limited culture, strain RH202 grew faster, replacing the plasmid-containing strain during the first 5 generations (Fig. 3, lower curve). Therefore, RH270 is a mutant of RH254 adapted specifically for successful growth and competition under fructose-limited growth conditions. Other adaptive mutants of plasmid-containing strains have been isolated which have a competitive advantage irrespective of whether the substrate is fructose or glucose (unpublished results).

Appearance of plasmid-free organisms originating from plasmid-containing organisms

The preceding experiments were initiated by mixing plasmid-containing and plasmid-free bacteria. Therefore, some or all of the plasmid-free organisms increasing in frequency initially were expected to be descendants of the plasmid-free bacteria in the inoculum. However, the plasmid-free organisms appearing subsequently could have been derived from plasmid-containing organisms, arising, for example, by improper segregation of the plasmid or failure in plasmid replication. To determine the source(s) of the plasmid-free organisms, the plasmid-free strain added in low frequency at the beginning of the experiment was marked with an *araAh9* mutation. Therefore, plasmid-free organisms derived from the plasmid-free inoculum should be *araAh9* in contrast to those derived from the initial plasmid-containing organisms which should be *ara*⁺. The *araAh9* mutation was selectively neutral under our experimental conditions (Adams *et al.*, 1979) and so the possibility of selecting a revertant from *araAh9* to *ara*⁺ was unlikely.

Figure 2(a) shows that all detectable plasmid-free organisms during the first cycle of fluctuation in frequencies were descendants of organisms in the plasmid-free inoculum. During the second cycle these *araAh9* organisms disappeared (frequency <0.002) and were replaced by plasmid-free organisms that were *ara*⁺ and therefore derived from the plasmid-bearing inoculum.

Figure 2(b) shows the results of an analogous experiment involving a different plasmid (pMIL27). During the first cycle, the plasmid-free organisms stemmed from both initial strains, whereas after the first cycle plasmid-free organisms were all descendants of plasmid-bearing organisms.

Thus, the results shown in Fig. 2 are consistent and show that the plasmid-free organisms increasing in frequency after the first cycle were exclusively *ara*⁺ and were therefore derived from plasmid-containing organisms. None of the strains used in these experiments carried a conjugative plasmid, so we did not expect transfer of a plasmid from organism to organism. If plasmid transfer had occurred, any *araAh9* plasmid-free organism that received a plasmid should have concomitantly acquired resistance to ampicillin. As expected, all ampicillin-resistant colonies tested were found to be *ara*⁺, so plasmid transfer was not a factor in these experiments.

Organisms with increased fitness result from chromosomal mutations

The results shown in Figs 1 and 2 show that there was a stage during the competition experiment when the plasmid-free strain was decreasing in frequency (after its initial increase phase) and the plasmid-containing strain was increasing (after its initial phase of uncompetitive growth). This indicates that a mutation had occurred in a plasmid-containing organism which enabled it to outgrow the plasmid-free strain despite the cost of maintaining the plasmid. If this mutation were located on the plasmid, then it might be possible to transfer the new selective advantage to a different strain by transformation with the mutated, successful plasmid.

The results of an experiment to test this hypothesis are shown in Fig. 4. Figure 4(a) describes the result of competition between the plasmid-free strain RH202 and the plasmid-containing strain RH254. The latter is a transformant of RH202 containing the plasmid from a strain (RH253) adapted for rapid growth in glucose-limited chemostat culture (Table 1). If the increased competitiveness of RH253 had resulted from a mutated plasmid, then the transformant RH254, containing the same plasmid, should also outgrow the plasmid-free strain. However, strain RH254 was found to be uncompetitive, being rapidly replaced by strain RH202 during the first 8 generations of competition (Fig. 4a).

Mutants of strain RH254 adapted for more rapid growth appeared later (generations 8 to 25, Fig. 4a). The result of competition between one such mutant (RH269) and strain RH202 is shown in Fig. 4(b). As expected, the adapted strain was more competitive and displaced RH202.

The plasmid from strain RH269 was transferred into RH202 by transformation. The transformant, strain RH271, was expected to have a growth advantage over RH202 if the adaptive mutation of RH269 was located on the plasmid. On the other hand, if the adaptation to rapid growth resulted from a chromosomal change in RH269, the transformant should be uncompetitive with the plasmid-free parent strain. The results of an experiment in which strain RH271 competed with strain RH202 showed clearly that the presence of the plasmid from the adapted strain RH269 reduced the competitiveness of the host organism rather than increasing it (Fig. 4c). Therefore, the increased fitness of RH269 cannot be attributed to its plasmid. Results similar to those shown in Fig. 4(a, b) were found in experiments involving plasmids from other strains selected for faster growth during glucose limitation. We therefore attribute the increased fitness of plasmid-containing cells to a chromosomal mutation.

Fig. 4. The increased fitness of plasmid-containing cells did not result from a change in the plasmid. Plasmid-containing strains were mixed with the plasmid-free strain RH202 to initiate each experiment. (a) Frequency of strain RH254 in glucose-limited continuous culture at a dilution rate of 0.26 h^{-1} . Strain RH254 is a transformant of RH202 containing the plasmid (pRBH052) from a strain (RH253) adapted for rapid growth in glucose-limited continuous culture. The initial organism concentrations were $8.1 \times 10^7 \text{ ml}^{-1}$ for strain RH254 and $3.0 \times 10^7 \text{ ml}^{-1}$ for strain RH202. (b) Frequency of plasmid-containing strain RH269, a derivative of RH254 adapted for rapid growth in glucose-limited continuous culture. The initial organism concentrations were $10 \times 10^7 \text{ ml}^{-1}$ for strain RH269 and $1.8 \times 10^7 \text{ ml}^{-1}$ for strain RH202. The dilution rate was 0.25 h^{-1} . (c) Frequency of strain RH271, a transformant of RH202 containing the plasmid (pRBH053) from RH269. The initial organism concentrations were $6.4 \times 10^7 \text{ ml}^{-1}$ for strain RH271 and $1.9 \times 10^7 \text{ ml}^{-1}$ for strain RH202. The dilution rate was 0.26 h^{-1} .

A model to explain oscillations in the frequency of plasmid-containing organisms

Our results suggest that immediately after initiation of a chemostat population, the frequency of plasmid-containing organisms decreases, principally because they have a lower growth rate than bacteria lacking the plasmid but also because an occasional organism fails to receive a plasmid from its parent. However, spontaneous mutations occur which cause an improved growth rate. These mutations are expected to appear more frequently among the plasmid-containing bacteria than among plasmid-free organisms because plasmid-containing organisms constitute the majority of the initial chemostat population. The plasmid-containing mutants grow faster than even the existing plasmid-free bacteria, and so the total frequency of plasmid-containing organisms increases. Eventually derivatives of the faster-growing plasmid-containing mutants appear which no longer contain a plasmid. These organisms have a still greater growth rate because they do not have to maintain a plasmid, and so, in turn, they displace the faster-growing plasmid-containing organisms. Although new mutations conferring better growth recur, the cost of plasmid maintenance and the irreversibility of plasmid loss dictate the eventual disappearance of plasmid-containing organisms.

A model of the changes that occur in such populations during the first cycle can be described by the following system of differential equations:

$$\frac{dn_1}{dt} = n_1(\mu_1 - D) - n_1 \pi \quad (1)$$

$$\frac{dn_2}{dt} = n_1 \pi + n_2(\mu_2 - D) \quad (2)$$

$$\frac{dn_3}{dt} = n_3(\mu_3 - D) - n_3 \pi \quad (3)$$

$$\frac{dn_4}{dt} = n_3 \pi + n_4(\mu_4 - D) \quad (4)$$

Fig. 5. Predicted and observed frequencies of plasmid-containing organisms in competition with a plasmid-free strain during glucose-limited growth in continuous culture. Plasmid-containing strains were mixed with strain RH202 at a total population density of $8 \times 10^7 \text{ ml}^{-1}$ to initiate each experiment. See Table 3 for the strains, initial frequencies and estimates of other parameters used. Observed frequencies of plasmid-containing strains (●). Predicted results (— and shaded area).

Table 3. *Estimates of the parameters used to generate the curves shown in Fig. 5*

The plasmid-free strain was RH202 in every case. μ_i denotes the specific growth rate of strain i ; Amp^r, ampicillin-resistant (plasmid-containing); Amp^s, ampicillin-sensitive; Ad⁻, no adaptive mutation; Ad⁺, adaptive mutation.

Fig.	Plasmid-containing strain	Dilution rate (h ⁻¹)	Estimates of relative fitness (μ_i/μ_2) of the strains				Rate of plasmid loss per generation	10 ³ × Initial frequency	
			1	2	3	4		Amp ^s Ad ⁻	Amp ^r Ad ⁺
			Amp ^r Ad ⁻	Amp ^s Ad ⁻	Amp ^r Ad ⁺	Amp ^s Ad ⁺			
5 (a)	RH251	0.247	0.380	1.000	1.285	1.445	5×10^{-5}	20	0.10–0.25
5 (b)	RH273	0.237	0.426	1.000	1.745	2.480	3×10^{-6}	70	0.01–0.03
5 (c)	RH256	0.273	0.525	1.000	1.149	1.342	1.75×10^{-3}	5	0.75–1.25

where n_i represents the concentration of the i th genotype, μ_i is the growth rate of the i th genotype, D is the dilution rate of the culture and π is the rate of loss of the plasmid (through faulty segregation or failure to replicate). Genotypes 1 (Amp^rAd⁻) and 3 (Amp^rAd⁺) denote plasmid-containing bacteria without and with the adaptive (growth rate advantage) mutation, respectively, and genotypes 2 (Amp^sAd⁻) and 4 (Amp^sAd⁺) denote the corresponding plasmid-free bacteria. These equations assume that (i) the dilution rate is constant throughout the experiment, (ii) the rate of plasmid loss is constant and independent of the genotype of the bacterial cell, and (iii) there is no interaction between the genotypes beyond the competition for the growth-limiting substrate.

In continuous culture systems, the growth of the organisms is assumed to be a function of the concentration of the growth-limiting substrate, the maximum specific growth rate and the saturation constant (Powell, 1958). Consequently, the growth rates of the competing strains in continuous culture will change with the frequency of the two types (Powell, 1958). However, to fit equations 1 to 4 to the observed population changes, it was assumed that the specific growth rates of the four genotypes do not change appreciably over the range of fluctuations in the frequency of individual genotypes observed. This assumption is common to other estimates of competitive differences in continuous culture (Moser, 1958; Francis & Hansche, 1972; Godwin & Slater, 1979).

Figure 5 shows the cyclic changes in the frequency of plasmid-containing organisms in three separate experiments, and the changes predicted by the model. The initial frequency of plasmid-free bacteria was determined directly as the frequency of ampicillin-sensitive organisms at the beginning of the experiment. The frequency of plasmid loss per generation and the frequency of adaptive mutants of plasmid-containing organisms (Amp^rAd^+) were chosen to obtain a reasonable fit of the data to the predictions of the model. In each case a range is shown for the frequency of Amp^rAd^+ organisms to show effects of this parameter on the dynamics of the population. The close fit of the model to the observed dynamics of the populations in each of the cases further supports our conclusions concerning the genetic changes occurring in these populations. A detailed examination of the influence of these and other variables will be presented elsewhere.

In every case shown in Fig. 5, it was necessary to assume the adaptive mutant(s) to be present in the population at the start of the experiment, but at low frequency. Generation of the adaptive mutant by mutation during the course of the experiments did not produce acceptable correspondence with the observed data. Thus, we conclude that our populations were polymorphic for adaptive mutants at the initiation of the experiments. This result is in accord with, and explains the results of, other workers (Andrews & Hegeman, 1976) who observed rapid and unpredictable changes occurring in the initial few generations of chemostat populations.

DISCUSSION

Early studies on the long-term growth of bacteria in continuous culture showed that the population may undergo periodic genetic change (Novick & Szilard, 1950; Atwood *et al.*, 1951). Each change observed by these workers represented an increased level of adaptation and originated from a mutation conferring increased fitness. These changes occurred repeatedly and the frequency of the changes was a function of the number and characteristics of the mutations produced continuously in the chemostat and also on the type of environment (Novick, 1958).

Our results demonstrate another, but related, facet of population dynamics, i.e. the fitness mutations could also appear within selectively disadvantaged organisms. These organisms were disadvantaged because they bore the costs of plasmid maintenance and interference, but the advantage conferred by some mutations was more than sufficient to overcome these disadvantages. As a result, a series of cyclic increases and decreases occurred in the frequency of plasmid-containing organisms when in competition with plasmid-free organisms, manifesting a sequence of reversals in the magnitude of the relative fitnesses of these two cell types. These oscillations significantly prolonged the period the population was polymorphic for plasmid-containing cells.

The results of these experiments showed that two evolutionary events were involved: (i) adaptive mutations occurred in the chromosome of plasmid-containing bacteria and (ii) the plasmid was lost from the plasmid-containing bacteria. The number of cycles occurring before disappearance of the plasmid depends on the frequency of plasmid-bearing bacteria at the time of appearance of an adaptive mutation. The fluctuations may terminate after one cycle, two cycles or longer (Figs 1 to 4 and unpublished results).

An important assumption, implicit in the conclusions drawn from these experiments, is that the role of the plasmid is purely negative and serves only to mark the initial population of organisms. Expressed in another way, we have assumed that the probability that an adaptive mutation may occur in a plasmid-containing bacterium is exactly equal to the frequency of plasmid-containing bacteria in the population. However, genetic change may not be random, but may occur more frequently in plasmid-bearing organisms. Some plasmids have been shown to increase the rate of occurrence of mutation (Fowler *et al.*, 1979). Furthermore, plasmids often carry transposable elements mediating genetic changes such as gene addition,

deletion and inversion (Reanney, 1976). In some cases such changes might be selectively advantageous. In our experiments, we did not detect change in the plasmid. Godwin & Slater (1979) have studied competition between strains with and without a large plasmid conferring multiple drug resistance over longer periods of time. In most of their experiments, change in the structure of the plasmid occurred. Loss of the plasmid was almost never observed, despite the superiority of plasmid-free organisms to organisms containing the altered plasmids. It seems likely that maintenance of the plasmid-containing organisms in their experiments resulted from the recurrence of fitness mutations within the plasmid-containing sector of the population. By this rationale, then, the net effect was a dynamic equilibrium with both new 'improved' plasmid organisms and new cured derivatives continually appearing in the culture.

It is not difficult to understand the survival in an appropriate environment of a plasmid conferring ability to utilize a novel substrate, ability to produce a material inhibitory to other organisms, or resistance to a toxic agent. Plasmid-containing organisms may also survive in a fluctuating environment where periodically they have an advantage, even though disadvantaged under the usual conditions. However, simple fluctuation of the environment is not a sufficient condition for the maintenance of a polymorphism (Haldane & Jayakar, 1963). Even without direct selection, plasmid-containing bacteria can survive as a small minority by attachment to a substrate (such as the intestinal lining or the wall of a culture vessel) from which they are not easily dislodged. Our results demonstrate a way in which plasmid-containing organisms can survive in competition with other organisms even though the presence of the plasmid appears to be a liability. We believe such indirect selection to be important in nature.

This work was supported in part by U.S. Public Health Service Grant GM25565 and Department of Energy contract EY-78-C-02-2828.

REFERENCES

- ADAMS, J. & HANSCH, P. E. (1974). Population studies in micro-organisms. I. Evolution of diploidy in *Saccharomyces cerevisiae*. *Genetics* **76**, 327-338.
- ADAMS, J., KINNEY, T., THOMPSON, S., RUBIN, L. & HELLING, R. B. (1979). Frequency-dependent selection for plasmid-containing cells of *Escherichia coli*. *Genetics* **91**, 627-637.
- ANDREWS, K. J. & HEGEMAN, G. D. (1976). Selective disadvantage of non-functional protein synthesis in *Escherichia coli*. *Journal of Molecular Evolution* **8**, 317-328.
- ATWOOD, K. C., SCHNEIDER, L. K. & RYAN, R. J. (1951). Selective mechanisms in bacteria. *Cold Spring Harbor Symposia on Quantitative Biology* **16**, 345-355.
- BENNETT, P. M. & RICHMOND, M. H. (1978). Plasmids and their possible influence on bacterial evolution. *The Bacteria*, vol. 6, *Bacterial Diversity*, pp. 1-69. Edited by L. N. Ornston & J. R. Sokatch. New York: Academic Press.
- BINNS, M. M., DAVIES, D. L. & HARDY, K. G. (1979). Cloned fragments of the plasmid ColV.I-K94 specifying virulence and serum resistance. *Nature, London* **279**, 778-781.
- DAVIES, J. & SMITH, D. I. (1978). Plasmid-determined resistance to antimicrobial agents. *Annual Review of Microbiology* **32**, 469-518.
- DAVIS, B. D. & MINGIOLI, E. S. (1950). Mutants of *Escherichia coli* requiring methionine or vitamin B12. *Journal of Bacteriology* **60**, 17-28.
- EL-GEWELY, M. R. & HELLING, R. B. (1980). Preparative separation of DNA-ethidium bromide complexes by zonal density gradient centrifugation. *Analytical Biochemistry* **102**, 423-428.
- EL-GEWELY, M. R., LOMAX, M. I., LAU, E. T., HELLING, R. B., FARMERIE, W. & BARNETT, W. E. (1981). A map of specific cleavage sites and tRNA genes in the chloroplast genome of *Euglena gracilis* *bacillaris*. *Molecular and General Genetics* (in the Press).
- FALKOW, S. (1975). *Infectious Multiple Drug Resistance*. London: Pion.
- FOWLER, R. G., MCGINTY, L. & MORTELMANS, K. E. (1979). Spontaneous mutational specificity of drug resistance plasmid pKM101 in *Escherichia coli*. *Journal of Bacteriology* **140**, 929-937.
- FRANCIS, J. C. & HANSCH, P. E. (1972). Directed evolution of metabolic pathways in microbial populations. I. Modification of the acid phosphatase pH optimum in *Saccharomyces cerevisiae*. *Genetics* **70**, 59-73.
- GODWIN, D. & SLATER, J. H. (1979). The influence of the growth environment on the stability of a drug resistance plasmid in *Escherichia coli* K12. *Journal of General Microbiology* **111**, 201-210.
- GUNSALUS, I. C., HERMAN, M., TOSCANO, JR. W. A., KATZ, D. & GARG, G. K. (1975). Plasmids and metabolic diversity. *Microbiology - 1974*, pp. 207-212. Edited by D. Schlessinger. Washington, D.C.: American Society for Microbiology.

- HALDANE, J. B. S. & JAYAKAR, S. D. (1963). Polymorphism due to selection of varying direction. *Journal of Genetics* **58**, 237–242.
- HELLING, R. B. & LOMAX, M. I. (1978). The molecular cloning of genes – general procedures. *Genetic Engineering*, pp. 1–30. Edited by A. M. Chakrabarty. West Palm Beach, Florida, U.S.A.: CRC Press.
- HELLING, R. B., EL-GEWELY, M. R., LOMAX, M. I., BAUMGARTNER, J. E., SCHWARTZBACH, S. D. & BARNETT, W. E. (1979). Organization of the chloroplast ribosomal RNA genes of *Euglena gracilis bacillaris*. *Molecular and General Genetics* **174**, 1–10.
- HERSHFIELD, V., BOYER, H. W., YANOFSKY, C., LOVETT, M. A. & HELINSKI, D. R. (1974). Plasmid ColEI as a molecular vehicle for cloning and amplification of DNA. *Proceedings of the National Academy of Sciences of the United States of America* **71**, 3455–3459.
- HOPWOOD, D. A. (1978). Extrachromosomally determined antibiotic production. *Annual Review of Microbiology* **32**, 273–292.
- KUBITSCHKE, H. E. (1970). *Introduction to Research with Continuous Cultures*. Englewood Cliffs, New Jersey: Prentice-Hall.
- LOMAX, M. I., HELLING, R. B., HECKER, L. I., SCHWARTZBACH, S. D. & BARNETT, W. E. (1977). Cloned ribosomal RNA genes from chloroplasts of *Euglena gracilis*. *Science* **196**, 202–205.
- MOSER, H. (1958). *The Dynamics of Bacterial Populations Maintained in the Chemostat*. Publication 614. Washington, D.C.: Carnegie Institute of Washington.
- NOVICK, A. (1958). Some chemical bases for evolution of microorganisms. *Perspectives in Marine Biology*, pp. 533–546. Edited by A. A. Buzzati-Traverso. Berkeley: University of California Press.
- NOVICK, A. & SZILARD, L. (1950). Experiments with the chemostat on spontaneous mutations of bacteria. *Proceedings of the National Academy of Sciences of the United States of America* **36**, 708–719.
- POWELL, E. O. (1958). Criteria for the growth of contaminants and mutants in continuous culture. *Journal of General Microbiology* **18**, 259–268.
- REANNEY, D. (1976). Extrachromosomal agents as possible agents of adaptation and development. *Bacteriological Reviews* **40**, 552–590.
- SMITH, H. W. & HUGGINS, M. B. (1976). Further observations on the association of the colicin V plasmid of *Escherichia coli* with pathogenicity and with survival in the alimentary tract. *Journal of General Microbiology* **92**, 335–350.
- SO, M., GILL, R. & FALKOW, S. (1975). The generation of a ColEI-Ap' cloning vehicle which allows detection of inserted DNA. *Molecular and General Genetics* **142**, 239–249.