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Plasmid macro-evolution: selection of deletions during adaptation in a nutrient-limited environment

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Abstract

Under conditions where plasmid-carriage is deleterious to the cell, evolutionary changes may be expected which result in an attenuation of the deleterious effect of the plasmid. During long-term growth in glucose-limited continuous culture, initiated with a single clone of *Escherichia coli* containing a derivative of the plasmid pBR322, a structural change arose in the plasmid and predominated in the plasmid-containing sector of the population. This variant possessed a 2.25 kb deletion encompassing the tetracycline resistance operon as well as a region of about 1.5 kb upstream from this operon. Competition experiments involving strains carrying the plasmid with the spontaneous deletion, and strains carrying plasmids with artificially constructed deletions, revealed that deletion of this region of the plasmid, involving loss of tetracycline resistance, resulted in an increment in fitness of between 10 and 20%. From the magnitude of the growth advantage, we conclude that the attenuation of the deleterious effect of the plasmid was mainly due to a reduction in the plasmid mediated interference in the metabolism of the cell caused by a deletion of the tetracycline resistance gene.

Introduction

There is now abundant evidence that the presence of a plasmid in a bacterial cell almost invariably depresses growth rate, in the absence of an environmental agent (such as an antibiotic) which specifically selects for the presence of the plasmid. Such growth-rate depression has been documented for R plasmids (Melling, Ellwood & Robinson, 1977; Engberg & Nordstrom, 1978; Godwin & Slater, 1979; Zund & Lebek, 1980; Wouters & van Andel, 1983) as well as for small non-conjugative ColEI-type plasmids (Inselburg, 1978; Jones *et al.*, 1980; Roth, Muller & Noack, 1980; Wouters *et al.*, 1980; Helling, Kinney & Adams, 1981; Noack *et al.*, 1981; Boe, Gerdes & Molin, 1987; Chiang & Bremer, 1988), though there are one or two notable exceptions (Lundquist & Levin, 1986; Wouters & van Andel, 1983). Furthermore, an increase in plasmid size has been observed to lead to a greater degree of growth rate depression (Weinberger & Helmstetter, 1979; Noack *et al.*, 1981; Warnes & Stephenson, 1986; Zund & Lebek, 1986) as well accelerated cell death (Cheah, Weigand & Stark, 1987).

Under conditions where plasmid-carriage is deleterious to the cell, evolutionary changes which result in an attenuation of the deleterious effect of the plasmids would be predicted from the application of classical predator-prey theory (Levin & Lenski, 1983). Under long-term laboratory culture, changes in both the bacterial chromosome (Bouma & Lenski, 1988; Modi & Adams, 1991) and the plasmid (Godwin & Slater, 1979; Modi & Adams, 1991) have been reported which have resulted in a decreased deleterious effect

of the plasmid. Evolutionary changes in the plasmid may involve base substitutions, but large deletions, which may reduce the maintenance cost of the plasmid and/or plasmid mediated interference substantially, will also be expected. Such macro-evolutionary changes have been observed in descendants of a gentamicin-resistance plasmid isolated from a nosocomial environment (Lee, Gerding & Cleary, 1984).

In this communication, we describe and analyze structural changes occurring in a derivative of pBR322 during long-term growth in glucose-limited chemostat culture. In particular, we show that, i) a spontaneous deletion occurred in the plasmid during growth under substrate-limiting conditions, ii) the strain carrying the plasmid with the deletion rapidly became predominant amongst the plasmid-containing strains in the population from which it was isolated, and iii) the deletion in the plasmid was responsible for most or all of the decrease in the deleterious effect of the plasmid.

Materials and methods

Growth media. Tryptone broth (TB) contained 5g NaCl and 10g tryptone (Difco) per liter. Tryptone agar 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}. Carbon source in the form of D-glucose was present at a concentration of 0.0125% w/v in chemostats and at 0.025% w/v in batch culture. The concentration of D-glucose gave population densities of approximately 1×10^8 organisms ml^{-1} when growth-limiting in chemostat culture. D-glucose (Sigma) and thiamine. 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 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 were used (Table 1). Strain JA122 is strain JA104 transformed with the plasmid pBR322Δ5, approximately 8.7 kb in size (Heffron, Kostriken, Morita & Parker, 1981) and its derivatives (Table 1). Strain RIM101 was a plasmid-containing ampicillin-resistant clone obtained from single colonies appearing on TA plates after plating samples from the chemostat bacterial population after approximately 416 generations of growth in glucose-limited chemostat culture. To aid in the interpretation of the competition experiments, the bacterial chromosomes and plasmid replicons are denoted by the symbols b_0, p_0 etc.

A plasmid-free derivative of RIM101 was initially isolated by assaying colonies for β-lactamase using the

Table 1. Bacterial strains.

Strain	Relevant characteristics	Replication designation *E.coli*	Plasmid
JA104	F$^-$ *thi* 1 *lac*Y 1 *ara*D139 *sup*E44 *hss*1 lysogenic for λ (Adams *et al.*, 1979)	b_0	
JA122 Original Strain	JA104 transformed with plasmid pBR322Δ5 (Heffron *et al.*, 1981); ampicillin and tetracycline resistant	b_0	p_0
RIM103	JA104 transformed with the plasmid isolated from RIM101; ampicillin resistant	b_0	p_1
RIM106	JA104 transformed with a derivative of plasmid pBR322Δ5 containing a 2.14 kb deletion (see Fig. 2); ampicillin resistant	b_0	p_2
RIM105	JA104 transformed with a derivative of plasmid pBR322Δ5 containing a 2.7kb deletion (see Fig. 2); ampicillin resistant	b_0	p_3
CF101	JA104 transformed with a derivative of plasmid pBR322Δ5 containing a 0.6kb deletion of the region defined by the unique *Hin*dIII and *Sal*I sites (see Fig. 2); ampicillin resistant	b_0	p_4
RIM101 Evolved strain	derivative of JA122 – evolved strain isolated after approximately 416 generations of growth in glucose-limited continuous culture; ampicillin resistant	b_1	p_1
RIM102	Plasmid-free derivative of RIM101 transformed with plasmid pBR322Δ5 (Heffron *et al.*, 1981); ampicillin and tetracycline resistant	b_1	p_0

iodometric assay described by Boyko and Ganschow (1982). Absence of the plasmid was confirmed by quantitative Southern analysis (Maniatis, Fritsch & Sambrook, 1982), probing with plasmid DNA, and analyzing the blots with a laser-scanning densitometer (LKB Ultroscan XL). Plasmid-containing strains were obtained by transformation with purified plasmid DNA (prepared as described below) using the standard $CaCl_2$ procedure. The presence of a plasmid in strains recovered from chemostat cultures was demonstrated by extracting the plasmid DNA by the alkaline lysis method and running the restriction endonuclease digested plasmid DNA on agarose gels stained with ethidium bromide (Maniatis, Fritsch & Sambrook, 1982).

Long-term continuous culture. Cultures were inoculated with a single clone of JA122 and maintained in an aerated culture vessel at 30°C in minimal medium at a dilution rate, $D \approx 0.20$, equivalent to a cell generation time of approximately 3.5 h (Kubitschek, 1970). Periodically an aliquot of the cell suspension was frozen in 40% glycerol and stored at –70 C for later analysis.

Procedure for competition experiments in continuous culture. Chemostat cultures were maintained at 30°C in a glucose-limited minimal salt chemostat medium (Helling, Kinney & Adams, 1981) in aerated culture vessels 120-195 ml in volume, at the same dilution rate ($D \approx 0.2$ h^{-1}), used in the long-term continuous culture. The bacterial population density was monitored by measuring the culture absorbance at 550nm in a Bausch and Lomb Spectronic 100 spectrophotometer. Two strains were first grown in batch mode for 14-18 h, in 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 cells from the batch cultures, of the two competing strains. 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 equilibrum (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, Vargas & Adams, 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 TA plus ampicillin (50 μg ml^{-1}) and TA plus ampicillin plus tetracycline (10 μg ml^{-1}) plates. An average of 200 colonies was replicated for each sample. Changes in the total frequency of plasmid-containing cells, were monitored from the ratio of the total number of colonies plated to the number of colonies appearing on TA plus tetracycline plates.

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 rates (μ) of the two strains, i and j, ($|\mu_i - \mu_j|$) were calculated by standard least square regression techniques after linearization of the frequencies with a logit transformation. The specific growth rates associated with the different genotypes were defined relative to a reference strain (strain 2) and are denoted relative fitnesses following the convention in population genetics. The standard errors associated with the relative fitnesses must be regarded as approximate as the errors associated with the data points are unavoidably autocorrelated.

Plasmid and probe preparation. Plasmid DNA was prepared by the alkaline lysis procedure described in Maniatis, Fritsch and Sambrook (1982). Plasmid DNAs used as probes were purified either by two cycles of CsCl-ethidium bromide density gradient centrifugation, or by using pZ523 spun columns for 5Prime-3Prime Inc., with the procedure described by the vendor. The purified DNA was also used for all transformations and restriction endonuclease digestions.

Plasmid deletion construction. Procedures for plasmid deletion constructions described by Ausubel *et al.* (1989) were used. Partial digestion of pBR322Δ5 plasmid DNA prepared from JA122, with *Eco*RV, produced linearized fragments of different lengths. The restriction endonuclease was heat inactivated (75 C for 10 min) to stop the reaction, and the plasmid

DNA then ethanol-precipitated and resuspended in sterile glass-distilled water to a final concentration of less than 0.1 μg/ml. The blunt-ended plasmid DNA fragments were ligated using ligation buffer containing ATP and T4 ligase (0.06-0.09 units/μl of reaction mixture) at room temperature for about 6 h. Partial digestions using restriction endonucleases and ligation reactions were checked by running the mixture on 0.7% agarose gels. The ligated plasmid DNA mixture was used to transform competent JA104 cells by the $CaCl_2$ procedure (Maniatis, Fritsch & Sambrook, 1982). To screen for plasmid deletions of interest, plasmid DNA was extracted from selected ampicillin resistant and tetracycline sensitive transformants as described above, and the resulting DNA samples were then analyzed by digestion with appropriate restriction endonucleases, using procedures as suggested by the manufacturer, and separation of the fragments by electrophoresis on 0.7% agarose 'submarine' gels (Maniatis, Fritsch & Sambrook, 1982).

Results

A population, initiated from a single clone of *E. coli* containing a derivative of the plasmid pBR322 was maintained for approximately 650 generations in continuous culture, under chemostat conditions. The cell generation time was 3.5 h, with growth limited by the concentration of glucose. Plasmid-containing cells constituted 100% of the population for approximately 100 generations, at which time plasmid-free cells increased rapidly in the population, until by generation 300 they constituted approximately 90% of the population. However, from generation 300 onwards, plasmid-containing cells never disappeared from the population completely but were maintained at frequencies varying between one and six percent. Some fluctuations in their frequency, indicative of periodic selection (Helling, Kinney & Adams, 1981), were observed between generations 200 and 600. During this time, differences in the frequencies of the two phenotypes, tetracycline-sensitive, ampicillin-resistance (tet^s, amp^r) and tetracycline resistance, ampicillin-resistance (tet^r, amp^r) became apparent. Figure 1 shows the frequency of tet^s, amp^r cells relative to the frequency of amp^r cells in the population. It can be seen that the frequency of tet^s, amp^r cells increased steadily from 5% to 99% in the plasmid-containing sector of the population between 380 and 560 generations.

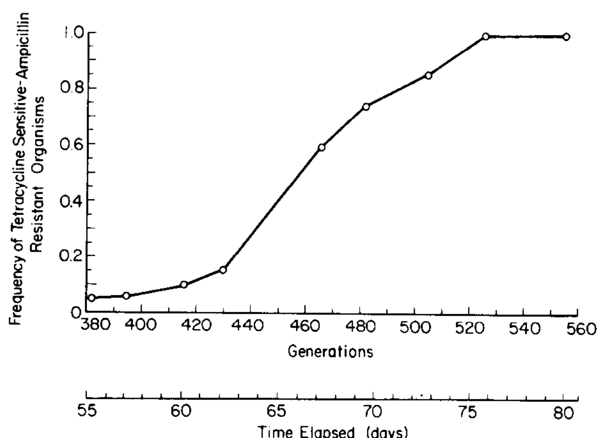

Fig. 1. Frequency of tetracycline-sensitive, ampicillin-resistant plasmid-containing organisms relative to the frequency of ampicillin-resistant organisms between generations 380 and 560.

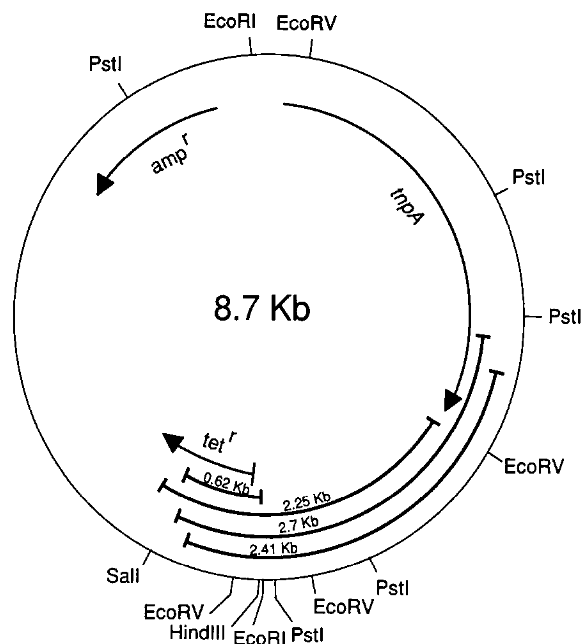

Fig. 2. Restriction map of the plasmid isolated from evolved strain RIM101, as well as three artificial plasmid deletion constructs, showing restriction sites for enzymes, *Eco*RI, *Eco*RV, *Hin*dIII, *Pst*I and *Sal*I. 2.25 Kb deletion - naturally occurring deletion. 0.6, 2.4, and 2.7 Kb deletions - artificially constructed deletions. The position and extent of the *tet*^{r}, *amp*^{r} and *tnpA* genes are shown by the arrows. The arrows also indicate the direction of transcription with the head of the arrow indicating the 3' end of the coding region. Details of the construction of the artificial deletions are given in the Materials and methods section.

To determine the basis for the loss of tetracycline resistance, plasmid DNA was prepared from RIM101, a tet^s, amp^r clone, isolated after about 416 generations from this chemostat, and a restriction map constructed. This map (see Fig. 2), shows that the plasmid carried by RIM101 had suffered a 2.25 kb deletion, approximately one quarter of the plasmid, a region encompassing most of the tetracycline resistance operon, as well as a region upstream from the tetracycline gene of approximately 1.5 kb. Plasmid DNA extracted from various other tet^s, amp^r strains from chemostat samples between generation 382 and 505 also showed the same restriction endonuclease digestion pattern. Thus, it can be concluded that the difference in the frequency between tet^r, amp^r and tet^s, amp^r isolates can be explained by the presence of this naturally occurring deletion plasmid in the population. The increase in the frequency of the deletion further suggested that the spontaneous deletion in the plasmid could decrease the deleterious effect of the plasmid in the host, such that cells carrying the deleted plasmid would increase in frequency relative to cells carrying the unmodified plasmid.

The deleterious effects of the unmodified plasmid and the plasmid carrying the naturally occurring deletion were compared by placing cells carrying these plasmids in direct competition in the same glucose-limited chemostat environment in which the naturally occurring deletion plasmid arose. To aid in the interpretation of these, and subsequent competition experiments, the various bacterial and plasmid chromosomes are denoted using the symbols b_0, p_0 etc. (see Table 1). The results shown in Table 2 express the results in terms of relative fitnesses, where a fitness of greater (or less than) than one denotes a competitive superiority (inferiority) for that strain, relative to the reference strain, which by convention is assigned a fitness of one. In both bacterial chromosome backgrounds, cells carrying the plasmid with the spontaneous deletion p_l possess a large competitive advantage over cells carrying the original plasmid p_0. It is noteworthy that the fitness increment which can be attributed to the plasmid carrying the naturally occurring deletion, is significantly greater in the bacterial chromosome background b_l in which it evolved than in the original background b_0. This indicates that the bacterial and plasmid chromosomes have co-evolved to maximize fitness.

These results do not distinguish between a decrease in the deleterious effect of the plasmid, p_l, caused by the gross deletion and a decrease in the deleterious effect due to structural changes within the plasmid, independent of the deletion. To determine the role of the deletion in the fitness increments observed, strains were constructed containing artificially induced dele-

Table 2. Analysis of the effect of change in plasmid p_l in different bacterial chromosome backgrounds.

Strain 1		Strain 2	Fitness of strain 2 relative to a value of 1 for strain 1 ± SEM
In background b_l			
RIM102 $b_l + p_0$	⊗	RIM101 $b_l + p_l$	1.87 ± 0.32
RIM102 $b_l + p_0$	⊗	RIM101 $b_l + p_l$	1.51 ± 0.19
In background b_0			
JA122 $b_0 + p_0$	⊗	RIM103 $b_0 + p_l$	1.17 ± 0.04
JA122 $b_0 + p_0$	⊗	RIM103 $b_0 + p_l$	1.25 ± 0.05

Table 3. Reconstruction of plasmid change.

Strain 1		Strain 2	Fitness of strain 2 relative to a value of 1 for strain 1 ± SEM
Plasmid construct 1-2.4 kb deletion			
JA122 $b_0 + p_0$	⊗	RIM106 $b_0 + p_2$	1.22 ± 0.03
JA122 $b_0 + p_0$	⊗	RIM106 $b_0 + p_2$	1.11 ± 0.01
JA122 $b_0 + p_0$	⊗	RIM106 $b_0 + p_2$	1.41 ± 0.06
Plasmid construct 2-2.7 kb deletion			
JA122 $b_0 + p_0$	⊗	RIM105 $b_0 + p_3$	1.13 ± 0.01
JA122 $b_0 + p_0$	⊗	RIM105 $b_0 + p_3$	1.08 ± 0.01
JA122 $b_0 + p_0$	⊗	RIM105 $b_0 + p_3$	1.08 ± 0.01
Plasmid construct 3-0.6 kb deletion			
JA122 $b_0 + p_0$	⊗	CF101 $b_0 + p_4$	1.13 ± 0.03
JA122 $b_0 + p_0$	⊗	CF101 $b_0 + p_4$	1.08 ± 0.01
Effect of plasmid pBR322Δ5 (p_0)*			
JA122 $b_0 + p_0$	⊗	JA104 b_0	1.19 ± 0.05
JA122 $b_0 + p_0$	⊗	JA104 b_0	1.17 ± 0.06
JA122 $b_0 + p_0$	⊗	JA104 b_0	1.14 ± 0.03

* Data from Modi and Adams (1991).

tions, and placed in competition with strains that were isogenic except for the deletions. Plasmid deletions were induced using the procedures described in the Materials and methods section, and were screened for deletions that were approximately the same size and location as the naturally occurring deletion in the plasmid, p_1. In addition, a plasmid was isolated with a much smaller (0.6 kb) deletion of the region defined by the unique *Sal*I and *Hin*dIII sites. Deletion of this region of the plasmid, removes approximately 40% of the coding sequence of the tetracycline reistance protein, plus 57 nucleotides 5' to the initiation of transcription (Peden, 1983). Insertion of DNA into either the *Sal*I or the *Hin*dIII site causes loss of tetracycline resistance (Peden, 1983). As expected, cells carrying any one of these three plasmids are tet^s, amp^r. The restriction maps of these plasmids (denoted p_2, p_3 and p_4) are shown in Figure 2 together with that of the naturally occurring plasmid, p_1.

The results of the competition experiments, presented in Table 3, show that in all three cases, deletion of a section of the plasmid chromosome produced significant fitness increments of between 10% and 25%. The increments in fitness which can be ascribed to the naturally occurring deletion and the three artificially constructed deletions are not significantly different, and are unrelated to the size of the deleted region. Deletion and inactivation of a portion of the tetracycline resistance coding locus, resulted in a fitness increment equivalent to those for deletions three to four times larger. We therefore conclude that the 2.25 kb spontaneous deletion in the plasmid accounted for a great majority of the decrease in the deleterious effect of the evolved plasmid, p_1, and the consequent predominance of cells carrying this plasmid in the plasmid bearing sector of the population. In addition, the results strongly implicate the tetracycline resistance locus as the principal factor determining the selective increments which can be ascribed to the plasmid deletions.

Discussion

A substantial amount of evidence has now accumulated suggesting that the bacterial genome can undergo major changes in structure at a surprisingly high frequency. For example, in *Salmonella typhimurium* duplications can occur at a frequency of as high as 1×10^{-3} and can include large amounts of the genome (Anderson & Roth, 1977). At the same time, these duplications are quite unstable, being lost a high frequency under nonselective conditions (Roth & Schmid, 1981). A number of studies have now shown that duplications are often selected in populations adapting to conditions of nutrient limitation (e.g., Horiuchi, Horiuchi & Novick, 1963; Rigby, Burleigh & Hartley, 1974; Sonti & Roth, 1989), as well as to conditions which favor activity of a specific enzyme (e.g., Tisty, Albertini & Miller, 1974; Edlund & Normark, 1981; Jessop & Clugston, 1985). It has been suggested that the lability of the bacterial genome in this respect may be an important evolutionary strategy by which bacteria adapt to changing environmental conditions (Anderson & Roth, 1977).

Much less is known however, concerning the frequency and evolutionary significance of the obverse of gene duplications, namely gene deletions. Although comparison of sequences from related species suggests a role for deletions in generating evolutionary change (e.g., Hwu *et al.*, 1986) we are aware of only one study that has documented the selection of a deletion during adaptation to nutrient limited conditions (Miller *et al.*, 1984). The results presented in this paper show that deletion of a substantial portion of the plasmid chromosome may increase fitness significantly by attenuating the deleterious effect of the plasmid. The deletion encompassed most of the tetracycline operon as well as 1.5 kb upstream of this region, and rendered cells carrying this plasmid tetracycline sensitive. In earlier studies, Godwin and Slater (1979) and Noack *et al.* (1981) had reported the predominance over time of tetracycline sensitive plasmid derivatives, but in neither case was the molecular basis of the tetracycline sensitivity identified.

The deleterious effect associated with the carriage of plasmid DNA can be ascribed to either the maintenance cost of the plasmid and/or plasmid-mediated interference. The maintenance cost can be defined as the energy cost to the cell incurred by the replication and transcription of plasmid DNA, and the translation of plasmid-encoded proteins (Godwin & Slater, 1979; Helling, Kinney & Adams, 1981), whereas plasmid-mediated interference can be defined as the

cost due to detrimental effects on the basic cellular structure or process due to presence of the plasmid (Helling, Kinney & Adams, 1981; Boe, Gerdes & Molin, 1987). The magnitude of the selective effect associated with a deletion of approximately one quarter of the plasmid suggests that plasmid-mediated interference, rather than maintenance cost, is the major cause of the selective reduction associated with the deletion. The selective disadvantage associated with the plasmid pBR322Δ5 (p_0) has been determined to be 0.14-0.19 under the same conditions (see Table 3). Thus, if the selective disadvantage associated with plasmid carriage were solely due to simple energetic considerations, the difference in the relative fitness of the two plasmids would be expected to be 0.04-0.05 ($1/4 \times 0.14$-0.19), whereas, the values calculated from the competition experiments are approximately four-fold larger. The similarity of the selective increments for the naturally occurring deletion, and the much smaller deletion covering the first 40% of the coding region of the tetracycline resistance gene, strongly implicate this region as the principal factor reducing the deleterious effect of the plasmid. Our results do not allow us to distinguish between an increment in fitness caused by the mere *presence* of the tetracycline resistance locus, or by its *expression*. However, two independent sources of information strongly implicate expression as being responsible for the deleterious effect. i) The tetracycline operon is constitutively expressed in pBR322 (Tait & Boyer, 1978), and overproduction of the tetA protein has been shown to result in cell death and loss of membrane potential (Eckert & Beck, 1989). Furthermore, under glucose-limited continuous culture conditions, plasmid copy number is greatly elevated (Modi & Adams, 1991; see also Chao & Bremer, 1986a, b; Taxis du Poet, 1987) which would result in overproduction of the tetA protein. ii) Expression, but not the presence of the tetracycline resistance operon has been shown to be selectively deleterious by Lee and Edlin (1985) and Nguyen *et al.* (1989). The magnitude of the selective differences that we observed are on the same order of magnitude as those seen by Lee and Edlin (1985) for chemostat culture, though they are significantly larger than those determined by Nguyen *et al.* (1989) who assayed selective differences only under batch culture conditions.

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