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Genetic Analysis of a Plasmid-Encoded, Host Genotype-Specific Enhancement of Bacterial Fitness

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In the absence of antibiotics, carriage of pACYC184 reduces the competitive fitness of an *Escherichia coli* B genotype that was not previously selected for plasmid carriage, relative to that of an isogenic plasmid-free competitor. However, a host genotype propagated with the plasmid for 500 generations evolved an unexpected competitive advantage from plasmid carriage, relative to its own isogenic plasmid-free segregant. We manipulated the pACYC184 genome in order to identify the plasmid-encoded function that was required for the enhancement of the coevolved host genotype's competitive fitness. Inactivation of the plasmid-encoded tetracycline resistance gene, by deletion of either the promoter region or the entire gene, eliminated the beneficial effect of plasmid carriage for the coevolved host. This beneficial effect for the coevolved host was also manifest with pBR322, which contains a tetracycline resistance gene identical to that of pACYC184 but is otherwise heterologous.

Numerous studies have shown that plasmid carriage reduces the competitive fitness of bacteria in the absence of specific selective agents, such as antibiotics, for which the plasmid encodes some corresponding function, such as antibiotic resistance (22, 25, 27–29, 34, 38, 54, 55, 58, 59, 62, 64). This reduced competitive fitness contributes to the instability of plasmids in environments not subject to these selection pressures, because it means that plasmid-free segregants will tend to overgrow their plasmid-bearing counterparts (14, 32, 34, 36). Plasmid instability can be either a blessing or a nuisance, depending on the context. The tendency for bacteria carrying resistance plasmids to decline in frequency in the absence of antibiotic selection implies that the problematic spread of resistant bacteria may be halted, or at least slowed, by judicious use of antibiotics. On the other hand, plasmids are commonly used as expression vectors in biotechnological applications in which loss of plasmids from bacterial populations can be a costly problem.

Almost all of the studies demonstrating that plasmid carriage imposes a burden that reduces bacterial fitness have examined naive associations between bacteria and plasmids. That is, there is no evolutionary history of association between the particular plasmid and host genotypes that are used. Yet natural selection should favor bacterial and plasmid genomes that accommodate one another, for example, by changes in either the host or the plasmid genomes that reduce any deleterious side effects caused by plasmid carriage (39). Recent studies have in fact demonstrated that the detrimental effects of plasmid carriage can be reduced or even eliminated over the course of several hundred generations of association (8, 48, 49). In this paper, we analyze the genetic interactions that affect competitive fitness in one such changed association.

Plasmid pACYC184 confers resistance to two antibiotics, chloramphenicol and tetracycline. Bouma and Lenski (8) demonstrated that, in the absence of either antibiotic, carriage of this plasmid reduced the competitive fitness of a naive *Escherichia coli* B host relative to that of an isogenic plasmid-free strain. This host-plasmid association was then propagated for 500 generations in medium containing chloramphenicol, which prevented plasmid-free segregants from overgrowing their plasmid-bearing counterparts. At the end of this time, Bouma and Lenski (8) demonstrated that not only had the plasmid-bearing bacteria adapted to the experimental culture regimen, but moreover the plasmid actually increased the fitness of its host (relative to that of a plasmid-free segregant of the coevolved host genotype) even in the absence of antibiotic. "Thus, an association that was formerly mutualistic in the presence of antibiotic, but antagonistic in the absence of antibiotic, has evolved into an association that is mutualistic in both environments" (8). Bouma and Lenski (8) further showed that the genetic change responsible for this shift from burdensome to beneficial occurred in the host chromosome and not in the plasmid: plasmid-free segregants of the coevolved host that were retransformed with the ancestral plasmid were more competitive than these same plasmid-free segregants of the coevolved host.

Identifying a mutation in the bacterial chromosome responsible for this change in the effect of plasmid carriage on host fitness would be very difficult. However, these results also imply the existence of a plasmid-encoded function that enhances the competitive fitness of certain bacterial genotypes, but not others. The plasmid genome, being much smaller, is more amenable to a trial-and-error genetic analysis by deletion of particular regions or functions. Therefore, in this study, we sought to identify the functions encoded by pACYC184 responsible for its detrimental effect on the naive host genotype and those functions responsible for its beneficial effect on the host genotype that had a 500-generation history of association with the plasmid.

MATERIALS AND METHODS

Bacterial strains and plasmids. All of the bacterial genotypes used in this study were derived from a strain of *E. coli* B used in several other experimental studies of evolutionary processes (3, 11, 30, 31, 35, 37). Table 1 lists the four genotypes used in this study, as well as two intermediate genotypes used in their construction. JB11 and JB12 have no evolutionary history of association with pACYC184; they differ from one

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TABLE 1. Bacterial genotypes used in this study

Designation[a]	Relevant characteristic(s)	Reference(s)
JB11 [Ara$^-$ (0)]	*E. coli* B (Ara$^-$)	8, 30, 37
JB12 [Ara$^+$ (0)]	Spontaneous Ara$^+$ mutant of JB11	8, 30, 37
JB13	Transformant of JB11, using pACYC184	8
JB38	Derivative of JB13 propagated for 500 generations in DM supplemented with chloramphenicol	8
JB44 [Ara$^-$ (500)]	Spontaneous plasmid-free segregant of JB38	8
JB85 [Ara$^+$ (500)]	Spontaneous Ara$^+$ mutant of JB44	8

[a] Alternative designations (in brackets) are discussed in the text.

TABLE 2. Plasmids used in this study

Designation	Relevant characteristics[a]	Reference or source
pACYC184	Cm^r Tc^r; p15A Ori; 4,245 bp	8, 10
pBR322	Ap^r Tc^r; ColE1 Ori; 4,361 bp	7
pMP10	Cm^s Tc^r; pACYC184 with the 412-bp *Pvu*II-*Pvu*II fragment deleted (bp 104 to 515); 3,833 bp	This study
pMP11	Cm^s Tc^r; pACYC184 with the 1,323-bp *Ava*I-*Eco*RI fragment deleted (bp 2923 to 4245, *Eco*RI site regenerated); 2,922 bp	This study
pSCS1	Cm^r Tc^s; pACYC184 with the 1,392-bp *Hin*dIII-*Ava*I fragment deleted (bp 1529 to 2920, *Hin*dIII site regenerated); 2,853 bp	This study
pSCS12	Ap^r Tc^s; pBR322 with the 1,392-bp *Hin*dIII-*Ava*I fragment deleted (bp 35 to 1426, *Hin*dIII site regenerated); 2,969 bp	This study
pSCS13	Cm^r Tc^s; pACYC184 with the 95-bp *Xba*I-*Hin*dIII fragment deleted (bp 1428 to 1522, *Xba*I site regenerated); 4,150 bp	This study
pSCS14	Ap^r Tc^s; pBR322 with the 27-bp *Eco*RI-*Hin*dIII fragment deleted (bp 3 to 29); 4,334 bp	This study

[a] By convention, nucleotide 1 of pACYC184 is the G in GAATTC of the *Eco*RI site and nucleotide 1 of pBR322 is the first T in GAATTC of the *Eco*RI site.

another only in a mutation affecting L-(+)-arabinose utilization, which serves as a marker in the competition experiments described below. JB44 and JB85 were propagated for 500 generations (75 days) in association with pACYC184, but pACYC184 has been subsequently lost by spontaneous segregation (8); they also differ from one another only in the arabinose utilization marker. This marker is effectively neutral on both genetic backgrounds under the conditions used in the experiments reported here (8, 30, 37). For clarity, we will use Ara$^-$ (0) and Ara$^+$ (0) to designate JB11 and JB12, respectively, and we will use Ara$^-$ (500) and Ara$^+$ (500) to designate JB44 and JB85, respectively. The parenthetical notation thus denotes the number of generations of association between the bacterial chromosome and pACYC184.

All of the plasmids used in this study are derived from either pACYC184 (8, 10) or pBR322 (7), which encode identical class C tetracycline resistance determinants derived from pSC101 (1, 7, 10, 13, 47, 57, 61) but which are otherwise unrelated to one another. Both pACYC184 and pBR322 express tetracycline resistance constitutively. The origin of replication for pACYC184 derives from p15A (10, 57), whereas that for pBR322 is of the ColE1 type (7, 61). Also, pACYC184 confers resistance to chloramphenicol, whereas pBR322 confers resistance to ampicillin. Plasmids pACYC184 and pBR322 are of similar size and copy number, and neither is conjugative. Both pACYC184 (50, 57) and pBR322 (56, 61, 63) have been completely sequenced. All of the genetic manipulations performed in this study were based on restriction maps made from these published sequences, and all of our manipulations were consistent with the expected number and location of restriction sites.

Table 2 lists the plasmids derived from pACYC184 and pBR322 that were constructed (see below) and used in this study. Briefly, pMP10 and pMP11 are derived from pACYC184, and they are sensitive to chloramphenicol as the result of two large deletions impinging on that sequence; pSCS1 and pSCS12 are derived from pACYC184 and pBR322, respectively, and they are sensitive to tetracycline as the result of identical deletions of the entire *tet* gene; pSCS13 and pSCS14 are derived from pACYC184 and pBR322, respectively, and they are sensitive to tetracycline as the result of deletions in the *tet* promoter region that do not impinge on the sequence encoding the Tet protein.

Each of these plasmids was transformed into all four of the bacterial genotypes used in this study [Ara$^-$ (0), Ara$^+$ (0), Ara$^-$ (500), and Ara$^+$ (500)] by standard procedures (44). All plasmid-free and plasmid-bearing strains were stored as single-colony isolates in a glycerol suspension at −80°C in order to maintain isogenicity.

Plasmid construction. To construct pMP10, pACYC184 was cut with *Pvu*II, religated, and used to transform *E. coli*. Plasmid DNA was isolated from Cm^s Tc^r candidates and cut with *Pvu*II to confirm the deletion of a 412-bp fragment. To construct pMP11, pACYC184 was cut with *Ava*I and *Eco*RI, incubated with Klenow fragment and deoxyribonucleoside triphosphates, religated, and used to transform *E. coli*. Plasmid DNA was isolated from Cm^s Tc^r candidates and cut with *Eco*RI to confirm the deletion of a 1,323-bp fragment and the regeneration of the *Eco*RI site. To construct pSCS1 and pSCS12, respectively, pACYC184 and pBR322 were cut with *Ava*I and *Hin*dIII, incubated with Klenow fragment and deoxyribonucleoside triphosphates, religated, and used to transform *E. coli*. Plasmid DNA was isolated from Cm^r Tc^s and Ap^r Tc^s candidates, respectively, for pSCS1 and pSCS12 and cut with *Hin*dIII to confirm the deletion of a 1,392-bp fragment and the regeneration of the *Hin*dIII site. To construct pSCS13, pACYC184 was cut with *Xba*I and *Hin*dIII, incubated with Klenow fragment and deoxyribonucleoside triphosphates, religated, and used to transform *E. coli*. Plasmid DNA was isolated from Cm^r Tc^s candidates and cut with *Xba*I to confirm the regeneration of the *Xba*I site. To construct pSCS14, pBR322 was cut with *Eco*RI and *Hin*dIII, incubated with Klenow fragment and deoxyribonucleoside triphosphates, religated, and used to transform *E. coli*. Plasmid DNA was isolated from Ap^r Tc^s candidates and cut with *Eco*RI and *Hin*dIII to confirm the loss of those sites. These constructions are shown schematically in Fig. 1.

Media and culture conditions. The standard culture medium was Davis minimal (DM) medium supplemented with 25 μg of glucose per ml (9, 30). Bacterial cultures consisted of 10 ml of medium held in 50-ml Erlenmeyer flasks; cultures were incu-

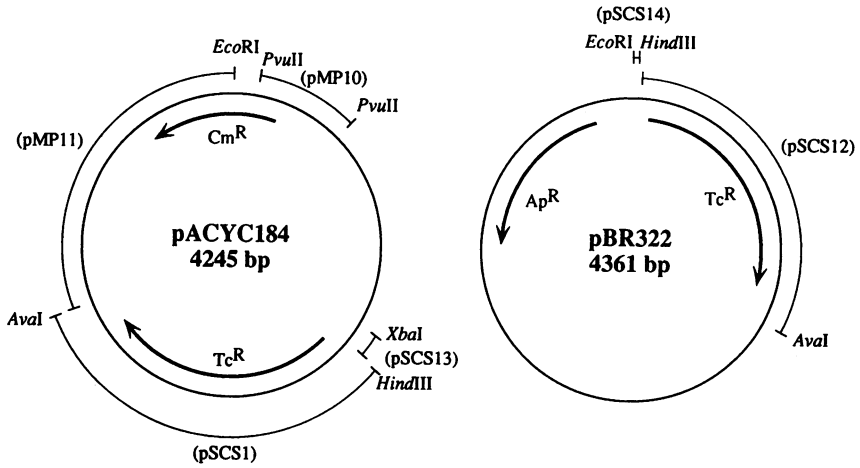

FIG. 1. Structures of plasmids used in this study. The parental plasmids, pACYC184 and pBR322, are represented by the unbroken circles. Concentric arcs bounded by restriction sites denote the extents of the deletions corresponding to the plasmids in parentheses. The open reading frames of the antibiotic resistance genes and their orientations are indicated by concentric arrows. Cm^R, chloramphenicol resistance gene; Tc^R, tetracycline resistance gene. Ap^R, ampicillin resistance gene.

bated with shaking (120 rpm) at 37°C. This medium and these conditions are identical to those used during the evolution of the bacterium-plasmid association from which Ara^- (500) and Ara^+ (500) are derived, except that chloramphenicol was present during the evolution experiment, and they are identical to those in which the consequences of that evolution for competitive fitness were measured (8).

Samples from the mixed competition cultures were spread on tetrazolium arabinose (TA) indicator agar (30, 40), which allows Ara^- and Ara^+ competitors to be distinguished by their red and white colonies, respectively. Subsamples of the colonies of putative plasmid-bearing genotypes were streaked onto medium supplemented with an appropriate antibiotic to confirm retention of the plasmid. Segregants never became a large fraction of the putative plasmid-bearing population during the competition experiments, which lasted 6 days. Any slight increase in the number of segregants tends to reduce the inferred difference in competitive fitness between a plasmid-bearing strain and its isogenic plasmid-free counterpart, and so inferences concerning the fitness effects of plasmid carriage are conservative.

Analysis of fitness effects by competition experiments. From the freezer, bacterial strains were streaked onto agar plates (supplemented with an appropriate antibiotic in the case of plasmid-bearing strains) for single colonies. Colonies were inoculated into Luria-Bertani (44) medium (again, with an appropriate antibiotic in the case of plasmid-bearing strains) and incubated overnight. These Luria-Bertani cultures were then diluted $1:10^4$ in DM, but without supplemental antibiotic for any of the strains. The DM cultures were incubated for 24 h (during which time the bacteria exhausted the available glucose and entered stationary phase) prior to the actual start of the competition experiment. These conditioning procedures ensured that two strains were comparably acclimated to the conditions that would hold during their subsequent competition.

At time zero of the competition experiment, a 1:1 volumetric mixture of the two comparably acclimated competitors (one of which would be Ara^- and the other Ara^+) was diluted 1:100 into 10 ml of fresh DM. A sample of this mixed competition culture was immediately taken, appropriately diluted, and spread onto TA agar. The competition culture was then incubated for 24 h, during which time the mixed population exhausted the glucose and entered stationary phase. Another sample was taken and spread onto TA agar, and then the mixed population was diluted 1:100 into fresh DM. This procedure was continued until the competition experiment had lasted 6 days, during which time the mixed population underwent ~40 cell doublings ($6 \times \log_2 100$).

From each competition experiment, we calculated a selection rate constant, s, as the slope of the following regression model (17, 32): $\log_e R(t) = \log_e R(0) + st$, where R is the ratio of the abundances of the two competing genotypes, t is time in days, and s has units of day^{-1}. The selection rate constant is a measure of the difference between two competing strains' realized Malthusian parameters (i.e., rates of population growth averaged over the entire growth cycle) during the competition experiment (37). If there is no difference in fitness between the competing strains, then one expects that $s = 0$. In this paper, we express the selection rate constant such that s is negative if plasmid carriage reduces host fitness and positive if plasmid carriage improves host fitness relative to that of an isogenic plasmid-free competitor. No statistical significance is attached to any single estimate of the selection rate constant. Instead, statistical inferences are based on the reproducibility of estimates of the selection rate constant (32, 33). Figure 2 shows the dynamics of one competition experiment.

RESULTS

Carriage of pACYC184 reduces the fitness of the naive host genotype but enhances the fitness of the coevolved host genotype. Table 3 summarizes the selection rate constants associated with carriage of pACYC184. Carriage of this plasmid significantly reduces the fitness of the naive host genotype relative to that of its plasmid-free counterpart (Table 3, rows 1 to 3). However, carriage of this same plasmid significantly increases the fitness of the coevolved host genotype relative to that of its own isogenic plasmid-free competitor (Table 3, rows 4 to 6). The experiments summarized in Table 3 were all performed after those reported by Bouma and Lenski (8), and they confirm their key results.

Chloramphenicol resistance encoded by pACYC184 is costly to both naive and coevolved host genotypes. *E. coli* carrying

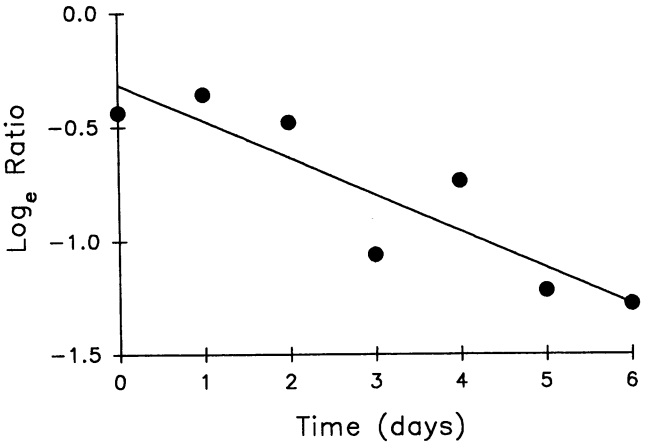

FIG. 2. Dynamics of a competition experiment between Ara$^+$ (0)/pACYC184 and Ara$^-$ (0). The selection rate constant, s, is estimated by regressing the $\log_e$-transformed ratio of plasmid-bearing to plasmid-free cell densities against time; $s = -0.161$ day^{-1} in the experiment that is illustrated. Between 3 and 18 replicate experiments were performed for each pair of competitors.

either pMP10 or pMP11 is sensitive to chloramphenicol as the result of large deletions in the pACYC184 genome. In contrast to pACYC184, carriage of either of these plasmids imposes no discernible burden on the naive host genotype (Table 4, rows 1 and 2). A two-tailed t test comparing the combined selection rate constants for these two plasmids (Table 4, row 3) with those estimated for pACYC184 on the same naive host background (Table 3, row 3) is significant ($t = 3.904$, 40 df, $P < 0.001$). Chloramphenicol resistance is evidently responsible for most of the reduced competitive fitness associated with carriage of pACYC184.

Carriage of either pMP10 or pMP11 is beneficial to the coevolved host genotype (Table 4, rows 4 and 5), as was carriage of pACYC184. Thus, the chloramphenicol resistance function is not responsible for the beneficial effect of pACYC184 on the coevolved host genotype. A two-tailed t test comparing the combined selection rate constants for these two

TABLE 3. Effects of pACYC184 carriage on the competitive fitness of naive and coevolved *E. coli* genotypes in antibiotic-free medium

Host genotype and competing strains	Selection rate constant[a]		
	Mean (n)	SD	t
Naive			
1. Ara$^+$ (0)/pACYC184 vs Ara$^-$ (0)	−0.108 (12)	0.084	−4.455***
2. Ara$^-$ (0)/pACYC184 vs Ara$^+$ (0)	−0.101 (18)	0.099	−4.313***
3. Rows 1 and 2 combined	−0.103 (30)	0.092	−6.155***
Coevolved			
4. Ara$^+$ (500)/pACYC184 vs Ara$^-$ (500)	0.050 (12)	0.104	1.658
5. Ara$^-$ (500)/pACYC184 vs Ara$^+$ (500)	0.124 (18)	0.083	6.338***
6. Rows 4 and 5 combined	0.094 (30)	0.097	5.287***

[a] Selection rate constant has units of day^{-1} and is expressed such that a negative value indicates a cost to the host of plasmid carriage whereas a positive value indicates a benefit of plasmid carriage. Each sample mean is based on n replicate assays. t, t statistic. Significance is based on results of a two-tailed t test: ***, $P < 0.001$.

TABLE 4. Effects of carriage of pMP10 and pMP11 on the competitive fitness of naive and coevolved *E. coli* genotypes in antibiotic-free medium

Host genotype and competing strains[a]	Selection rate constant[b]		
	Mean (n)	SD	t
Naive			
1. Ara$^-$ (0)/pMP10 vs Ara$^+$ (0)	0.031 (6)	0.074	1.030
2. Ara$^-$ (0)/pMP11 vs Ara$^+$ (0)	0.015 (6)	0.133	0.282
3. Rows 1 and 2 combined	0.023 (12)	0.103	0.781
Coevolved			
4. Ara$^-$ (500)/pMP10 vs Ara$^+$ (500)	0.169 (6)	0.030	14.108***
5. Ara$^-$ (500)/pMP11 vs Ara$^+$ (500)	0.216 (6)	0.043	12.284***
6. Rows 4 and 5 combined	0.193 (12)	0.043	15.569***

[a] pMP10 and pMP11 are sensitive to chloramphenicol, unlike pACYC184, from which they are derived (see Table 2).
[b] See Table 3, footnote *a*. ***, $P < 0.001$.

plasmids (Table 4, row 6) with those estimated for pACYC184 on the same coevolved host background (Table 3, row 6) is again significant ($t = 3.363$, 40 df, $P = 0.002$), indicating that the chloramphenicol resistance function is detrimental to the fitness of the coevolved host genotype, just as it was to the naive host genotype. The magnitude of the reductions in fitness caused by the chloramphenicol resistance function are similar for the naive (0.13 day^{-1} [difference between Table 3, row 3, and Table 4, row 3]) and coevolved (0.10 day^{-1} [difference between Table 3, row 6, and Table 4, row 6]) host genotypes.

Tetracycline resistance encoded by pACYC184 has no effect on the naive host genotype, but its expression is required for the beneficial effect of plasmid carriage on the coevolved host genotype. Cells carrying either pSCS1 or pSCS13 are sensitive to tetracycline as the result of deletions of the entire *tet* coding region or only the corresponding promoter region, respectively. Carriage of either of these plasmids is deleterious to the naive host genotype (Table 5, rows 1 to 6). A two-tailed t test indicates that the fitness effects of pSCS1 and pSCS13 combined (Table 5, row 7) and of pACYC184 (Table 3, row 3) for the naive host are statistically indistinguishable ($t = 0.187$, 58 df, $P = 0.852$). Tetracycline resistance is evidently not responsible for the burden that pACYC184 imposes on the naive host genotype, but neither does it provide any discernible competitive advantage to that genotype.

By contrast with pACYC184, neither pSCS1 nor pSCS13 confers any benefit on the coevolved host genotype. Instead, carriage of either pSCS1 or pSCS13 (Table 5, rows 8 to 13) imposes a significant reduction in competitive fitness, whereas pACYC184 conferred a significant benefit (Table 3, row 6). Therefore, possession and actual expression of the tetracycline resistance gene are required for the beneficial effect of pACYC184 that is specific to the coevolved host genotype. However, a two-tailed t test comparing the selection rate constants (associated with pSCS1 and pSCS13 combined) on the naive (Table 5, row 7) and coevolved (Table 5, row 14) host genotypes is significant ($t = 3.607$, 58 df, $P < 0.001$). This result indicates that the tetracycline resistance function cannot account for all of the change in the fitness effect of pACYC184 on the naive versus coevolved host genotypes. Nonetheless, the tetracycline resistance function is responsible for ~70% of the host's adaptation to plasmid carriage (difference between

TABLE 5. Effects of carriage of pSCS1 and pSCS13 on the competitive fitness of naive and coevolved *E. coli* genotypes in antibiotic-free medium

Host genotype and competing strains[a]	Selection rate constant[b]		
	Mean (*n*)	SD	*t*
Naive			
1. Ara^+ (0)/pSCS1 vs Ara^- (0)	−0.126 (3)	0.058	−3.761
2. Ara^- (0)/pSCS1 vs Ara^+ (0)	−0.109 (9)	0.125	−2.636
3. Rows 1 and 2 combined	−0.114 (12)	0.109	−3.595**
4. Ara^+ (0)/pSCS13 vs Ara^- (0)	−0.097 (12)	0.029	−11.675***
5. Ara^- (0)/pSCS13 vs Ara^+ (0)	−0.072 (6)	0.036	−4.872**
6. Rows 4 and 5 combined	−0.089 (18)	0.033	−11.506***
7. Rows 3 and 6 combined	−0.099 (30)	0.073	−7.408***
Coevolved			
8. Ara^+ (500)/pSCS1 vs Ara^- (500)	−0.060 (3)	0.043	−2.419
9. Ara^- (500)/pSCS1 vs Ara^+ (500)	−0.057 (9)	0.050	−3.375**
10. Rows 8 and 9 combined	−0.058 (12)	0.047	−4.259**
11. Ara^+ (500)/pSCS13 vs Ara^- (500)	−0.043 (12)	0.024	−6.290***
12. Ara^- (500)/pSCS13 vs Ara^+ (500)	−0.022 (6)	0.031	−1.730
13. Rows 11 and 12 combined	−0.036 (18)	0.028	−5.569***
14. Rows 10 and 13 combined	−0.045 (30)	0.037	−6.582***

[a] pSCS1 and pSCS13 are sensitive to tetracycline, unlike pACYC184, from which they are derived (see Table 2).
[b] See Table 3, footnote *a*. **, $0.001 < P < 0.01$; ***, $P < 0.001$.

TABLE 6. Effects of pBR322, pSCS12, and pSCS14 on the competitive fitness of naive and coevolved *E. coli* genotypes in antibiotic-free medium

Host genotype and competing strains[a]	Selection rate constant[b]		
	Mean (*n*)	SD	*t*
Naive			
1. Ara^+ (0)/pBR322 vs Ara^- (0)	−0.050 (6)	0.157	−0.777
2. Ara^- (0)/pBR322 vs Ara^+ (0)	−0.064 (6)	0.032	−4.870**
3. Rows 1 and 2 combined	−0.057 (12)	0.108	−1.815
4. Ara^+ (0)/pSCS12 vs Ara^- (0)	−0.100 (6)	0.090	−2.723*
5. Ara^- (0)/pSCS12 vs Ara^+ (0)	−0.047 (6)	0.012	−9.460***
6. Rows 4 and 5 combined	−0.074 (12)	0.067	−3.799**
7. Ara^+ (0)/pSCS14 vs Ara^- (0)	−0.139 (6)	0.113	−3.000*
8. Ara^- (0)/pSCS14 vs Ara^+ (0)	−0.083 (6)	0.045	−4.541**
9. Rows 7 and 8 combined	−0.111 (12)	0.087	−4.405**
10. Rows 6 and 9 combined	−0.092 (24)	0.079	−5.761***
Coevolved			
11. Ara^+ (500)/pBR322 vs Ara^- (500)	0.109 (6)	0.023	11.859***
12. Ara^- (500)/pBR322 vs Ara^+ (500)	0.058 (6)	0.023	6.304**
13. Rows 11 and 12 combined	0.084 (12)	0.034	8.434***
14. Ara^+ (500)/pSCS12 vs Ara^- (500)	−0.025 (6)	0.058	−1.034
15. Ara^- (500)/pSCS12 vs Ara^+ (500)	−0.044 (6)	0.025	−4.347**
16. Rows 14 and 15 combined	−0.034 (12)	0.044	−2.693*
17. Ara^+ (500)/pSCS14 vs Ara^- (500)	−0.133 (6)	0.039	−8.392***
18. Ara^- (500)/pSCS14 vs Ara^+ (500)	−0.117 (6)	0.027	−10.734***
19. Rows 17 and 18 combined	−0.125 (12)	0.033	−13.277***
20. Rows 16 and 19 combined	−0.080 (24)	0.060	−6.518***

[a] Plasmid pBR322 encodes a tetracycline resistance function identical to that of pACYC184, but they are otherwise heterologous; pSCS12 and pSCS14 are sensitive to tetracycline, unlike pBR322, from which they are derived (see Table 2).
[b] See Table 3, footnote *a*. *, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$; ***, $P < 0.001$.

Table 5, row 14, and Table 3, row 6, divided by difference between Table 3, row 3, and Table 3, row 6).

The beneficial effect of tetracycline resistance for the coevolved genotype is manifest when the identical gene is encoded by an otherwise heterologous plasmid. Plasmids pBR322 and pACYC184 encode identical tetracycline resistance genes, but they are otherwise heterologous. We used pBR322 to determine whether the beneficial effect of the tetracycline resistance function in pACYC184 on the coevolved (but not the naive) host genotype would be manifest in a heterologous plasmid construct. As with pACYC184, carriage of pBR322 tends to reduce the naive host genotype's fitness relative to that of its isogenic plasmid-free competitor (Table 6, rows 1 to 3). As with derivatives of pACYC184, this detrimental effect of plasmid carriage for the naive host genotype is still manifest if either the entire *tet* gene (Table 6, rows 4 to 6) or only the corresponding promoter region (Table 6, rows 7 to 9) has been deleted from pBR322. And as with derivatives of pACYC184, the cost of plasmid carriage is statistically indistinguishable whether (Table 6, row 3) or not (Table 6, row 10) the *tet* gene is present and expressed ($t = 1.128$, 34 *df*, $P = 0.267$).

More importantly, carriage of pBR322 provides a significant benefit to the host genotype that had evolved for 500 generations in association with pACYC184 (Table 6, rows 11 to 13), as does carriage of pACYC184 itself (Table 3, rows 4 to 6). The change between the naive and coevolved genotypes in the fitness effect of plasmid carriage may be slightly less for pBR322 (0.14 [difference between Table 6, row 3, and Table 6, row 13]) than for pACYC184 (0.20 [difference between Table 3, row 3, and Table 3, row 6]), but the changes clearly go in the same direction. And as was the case for pACYC184, the beneficial effect of pBR322 for the coevolved host genotype disappears, such that plasmid carriage becomes a significant burden, when either the entire *tet* gene (Table 6, rows 14 to 16) or its promoter region (Table 6, rows 17 to 19) is deleted. The effects of pSCS12 and pSCS14, which do not confer tetracycline resistance, are indistinguishable for the naive (Table 6, row 10) and coevolved (Table 6, row 20) host genotypes ($t = 0.637$, 46 *df*, $P = 0.527$). Expression of the tetracycline resistance function is evidently beneficial to the coevolved host genotype (but not to its naive ancestor) whether it is present in pACYC184 or in a heterologous plasmid construct such as pBR322.

DISCUSSION

In an earlier study (8), we sought to address the following general question: could the fitness cost associated with plasmid carriage (including the expression of plasmid-encoded prod-

TABLE 7. Effects of expression of tetracycline resistance on the competitive fitness of the naive and coevolved hosts in antibiotic-free medium

Plasmid[a]	Tetracycline resistance	Host genotype[b]	
		Naive	Coevolved
pACYC184	Tc^r	−	+
pSCS1	Tc^s	−	−
pSCS13	Tc^s	−	−
pBR322	Tc^r	−	+
pSCS12	Tc^s	−	−
pSCS14	Tc^s	−	−

[a] Both pACYC184 and pBR322 express tetracycline resistance (Tc^r), but this function was eliminated from the other plasmids by deleting either the entire *tet* gene or its promoter region (Tc^s).

[b] The symbols + and − indicate that plasmid carriage is beneficial or deleterious, respectively, to a host.

ucts) be reduced or eliminated by allowing host-plasmid associations to coevolve? Any such change would tend to stabilize a host-plasmid association by slowing the spread of plasmid-free segregants in environments where plasmid-encoded functions, such as resistance to antibiotics, were not under positive selection. In fact, after 500 generations of evolution of the association between *E. coli* B and pACYC184, not only was the cost of plasmid carriage reduced, but pACYC184 was actually beneficial to the coevolved host genotype (8). The mutations responsible for this change had occurred in the bacterial chromosome, because the coevolved host genotype experienced the same benefit when a segregant was retransformed with the original plasmid (8). However, it stands to reason that particular plasmid-encoded functions must be responsible both for the detrimental effect of plasmid carriage experienced by the naive host and for the beneficial effect conferred by the plasmid on the coevolved host.

When we say that carriage of pACYC184 reduces the competitive fitness of the naive host genotype, it is important to recognize that this applies to pACYC184 in its entirety and not necessarily to every component of that plasmid. For example, pACYC184 might encode two functions, one of which imposes a large cost to the naive host, whereas the other confers a smaller benefit (such that the overall effect is some net cost of plasmid carriage). In that case, the host might benefit from plasmid carriage if it could evolve tolerance of the detrimental plasmid-encoded function, thereby allowing the effect of the existing beneficial function to become apparent. Indeed, several other studies have demonstrated unexpected enhancements of bacterial fitness associated with possession of certain accessory genetic elements including transposons (4, 5, 24) and temperate phage (16, 19, 20, 42; see also reference 2), although none of these other studies observed the evolutionary transition whereby possession of an element went from being costly to beneficial. Alternatively, a plasmid-encoded function that was neutral or even detrimental to a naive bacterial host might benefit a host that had evolved in association with the plasmid.

To address these alternative hypotheses, we manipulated the pACYC184 genome in order to identify the particular plasmid-encoded functions that were responsible for the costs and benefits of plasmid carriage for the naive and coevolved hosts, respectively. The chloramphenicol resistance function was costly to both the naive and coevolved host genotypes, to similar degrees. By contrast, the tetracycline resistance function was selectively neutral (or nearly so) for the naive host genotype, but this function was responsible for the beneficial effect of pACYC184 on the coevolved host genotype (Table 7). Inactivation of the tetracycline resistance gene, by deletion of either the promoter region or the structural gene itself, eliminated the benefit of plasmid carriage for the coevolved host genotype.

The beneficial effect of this tetracycline resistance gene for the coevolved host was also manifest in a heterologous plasmid construct. Plasmid pBR322 encodes the same class C tetracycline resistance gene as does pACYC184, and the two plasmids are similar in overall size and copy number; however, the origin of replication of pBR322 differs from that of pACYC184 (ColE1 versus p15A, respectively), and pBR322 confers resistance to ampicillin but not to chloramphenicol. When pBR322 was transformed into the naive and coevolved host genotypes, it too was costly to the former and beneficial to the latter. Moreover, when either the tetracycline resistance gene or its promoter region was deleted from pBR322, the beneficial effect of plasmid carriage for the coevolved host genotype was eliminated, as was observed for pACYC184 and its derivatives (Table 7).

These results do not support the hypothesis that the host evolved tolerance of some costly function encoded by the plasmid, thereby allowing some existing beneficial function to become evident. Instead, our results support the alternative hypothesis that the coevolved host genotype acquired some new benefit from the expression of a plasmid-encoded function.

The mechanism of tetracycline resistance encoded by pACYC184 is active efflux of tetracycline by the Tet protein, which is associated with the inner membrane of the bacterial cell envelope (26, 46). Several previous studies have shown that overexpression of the tetracycline resistance efflux mechanism may impose a large selective disadvantage on *E. coli* cells in medium that does not contain this antibiotic (18, 29, 49, 51, 52, 54). It is therefore all the more surprising that the tetracycline resistance function is responsible for the beneficial effect of pACYC184 on the coevolved host genotype. However, these other studies have used different media (rich versus minimal), different *E. coli* host strains (K-12 versus B), and/or different tetracycline resistance determinants (Tn*10* derived versus pSC101 derived). For example, fully induced expression of the Tn*10*-derived *tet* function integrated into a multicopy plasmid is effectively lethal (52, 54), so that it is impossible to isolate constitutive mutants defective for the TetR repressor unless they also have down mutations in the *tet* promoter (51). In contrast, the pSC101-derived *tet* function is expressed constitutively in pACYC184 and without any discernible effect on the fitness of the naive host genotype in this study.

Whatever the explanation for the innocuous effect of the pACYC184-encoded tetracycline resistance function on the fitness of the naive host genotype, an even more interesting question concerns this function's beneficial effect on the coevolved host genotype. We see two distinct hypotheses, and there may be other possible explanations as well. According to one hypothesis, the coevolved host may have usurped the efflux mechanism of tetracycline resistance for some other function. For example, the coevolved host might use the Tet protein to export a toxic metabolite that would otherwise accumulate intracellularly to a level that inhibited growth. Several studies have demonstrated unexpected phenotypic effects associated with expression of tetracycline resistance (6, 15, 23, 43), which may indicate that this function has relatively low substrate specificity (15). Alternatively, the coevolved host may have become dependent in some way on the presence of the membrane-bound Tet protein. For example, the host might

have adapted to the experimental environment by a change in some other membrane-bound protein, the beneficial effect of which depends on the Tet protein because of biophysical interactions between the two membrane-bound proteins. Determining which (if either) of these hypotheses is correct and how the coevolved and naive host genotypes differ from one another awaits further study. However, we have clearly established that it is the expression of tetracycline resistance that confers the unexpected benefit of plasmid carriage on the host genotype that evolved in association with pACYC184 for 500 generations.

The finding that bacteria can adapt genetically to the burdensome effects of plasmid carriage has potentially serious implications for the persistence of antibiotic-resistant bacteria in nature, which is an important issue for public health (12, 21, 26, 53, 60). Much, if not most, clinically important resistance to antibiotics is the result of plasmid-encoded genes (12, 21, 41). As we have shown experimentally, bacterial adaptation to plasmid carriage, and the coevolution of host-plasmid associations more generally, can stabilize these associations. This stability implies greater difficulty in controlling the proliferation of bacteria that carry plasmids encoding antibiotic resistance. Under this scenario, plasmid-encoded resistance spreads initially through a bacterial population as the result of antibiotic usage. If antibiotic usage was discontinued as soon as resistant strains of the target bacterium were encountered, then such resistance would tend to be eliminated because of the detrimental fitness effect of plasmid carriage for the naive bacteria, which had no previous opportunity to evolve in association with that plasmid. With the eventual loss of the plasmid-encoded resistance, the antibiotic might again become effective against the target bacterium. However, if antibiotic usage continues even after the emergence of plasmid-encoded resistance, then a prolonged opportunity is afforded for the host-plasmid association to evolve, which will tend to favor any mutations (in either the host chromosome or the plasmid genome) that reduce or even eliminate the deleterious side effects of plasmid carriage for the host (8, 26, 48, 49, 60; see also references 31 and 45 for evidence of similar phenomena in the case of bacterial resistance to bacteriophage and insect resistance to pesticide, respectively). Consequently, it becomes that much more difficult to rid a bacterial population of a plasmid encoding antibiotic resistance simply by discontinuing use of the antibiotic (60).

On the brighter side, the finding that plasmid carriage may be stabilized by an evolutionary history of association between plasmids and their hosts also suggests a general strategy for stabilizing those plasmids that are used as vehicles for gene expression in biotechnological applications. Such applications are often compromised by the instability of plasmids, which usually reflects the growth rate advantage that spontaneous segregants have relative to their plasmid-bearing counterparts. This problem is frequently combatted by periodically restarting the application with a fresh inoculum of plasmid-bearing cells or by imposing continuous selection for a plasmid-encoded function (e.g., antibiotic resistance), either of which may involve substantial costs. The results reported here indicate that it is possible to select for host-plasmid combinations in which the host is less burdened by, and sometimes even benefits from, plasmid carriage, so that segregants will overgrow their plasmid-bearing progenitors more slowly, if at all.

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