Reprinted from Nature, Vol. 335, No. 6188, pp. 351-352, 22 September 1988

Evolution of a bacteria/plasmid association

Judith E. Bouma & Richard E. Lenski*

Department of Ecology and Evolutionary Biology,
University of California, Irvine, California 92717, USA

Associations between bacteria and their accessory elements (viruses, plasmids and transposons) range from antagonistic to mutualistic[1,2]. A number of previous studies have demonstrated that plasmid carriage reduces bacterial fitness in the absence of selection for specific functions such as antibiotic resistance[3–13]. Many studies have demonstrated increased fitness of evolving microbial populations in laboratory environments[3,8,14–21], but we are aware of only one study in which fitness gains were partitioned between a plasmid and its host[8]. Here, we examine the evolution of an association between a plasmid and its bacterial host. Carriage of the non-conjugative plasmid pACYC184 initially reduced the fitness of *Escherichia coli* B in the absence of antibiotic. We then cultured plasmid-bearing bacteria for 500 generations in the presence of antibiotic. The fitness of each combination of host and plasmid, with and without the culture history, was determined by competing it against a baseline strain. The results indicate adaptation by the host genome, but no plasmid adaptation. We also competed the evolved host, transformed with the baseline plasmid, against its isogenic plasmid-free counterpart. The plasmid now increased the fitness of its host.

E. coli B[22,23] was transformed with the 4-kilobase (kb) non-conjugative plasmid pACYC184, which encodes resistance to chloramphenicol and tetracycline[24,25]. We cultured the plasmid-bearing bacterial population for 75 days in a glucose-limited minimal salts medium[22] supplemented with 10 $\mu g\ ml^{-1}$ chloramphenicol, which selected against plasmid-free segregants. Each day, 0.1 ml of culture was transferred into 9.9 ml of fresh medium; the cultures were maintained in 50-ml Erlenmeyer flasks at 37 °C and 120 revs per minute. The 100-fold daily increase in cell numbers corresponds to 6.64 doublings, so that 75 days of culture represented approximately 500 generations of binary fission.

To allow us to distinguish different strains in our assays of relative fitness, we used an arabinose marker. The parental *E. coli* B strain is unable to grow on arabinose. We selected mutants capable of using arabinose, which served as reference competitors. Ara^+ strains form white colonies on tetrazolium arabinose plates [tryptone agar supplemented with arabinose (L+isomer) and triphenyltetrazolium chloride (TTC)]. The derivations of the strains used in this study are described in Table 1.

To determine the effects of plasmid carriage on host fitness before the 75 days in culture, we competed the plasmid-bearing strain JB13 against its plasmid-free counterpart JB12 in the glucose-limited minimal medium, without chloramphenicol. As a control for the arabinose marker, we also competed the two plasmid-free strains, JB11 and JB12. Each replicate experiment ran for six days, with samples plated daily. Some colonies from each sample were also tested on agar supplemented with chloramphenicol to monitor for segregants appearing during the competition experiments, but they never became a large fraction of the population. Any slight increase in the frequency of segregants weakens statistical comparisons between plasmid-bearing and plasmid-free strains, so our conclusions are conservative. The arabinose marker had no significant effect on fitness, whereas there was a significant reduction in fitness of the bacterial host associated with carriage of the plasmid in the antibiotic-free environment (Table 2).

* To whom correspondence should be addressed.

Table 1 Bacterial strains

Strain	Relevant characteristics*
JB11	plasmid-free *Escherichia coli* B
JB12	spontaneous Ara^+ mutant of JB11†
JB13	transformant of JB11 using plasmid pACYC184
JB14	spontaneous Ara^+ mutant of JB13†
JB38	derivative of JB13 cultured for 500 generations
JB44	spontaneous plasmid-free segregant of JB38‡
JB54	transformant of JB11 using plasmid from JB13§
JB55	transformant of JB44 using plasmid from JB13
JB56	transformant of JB11 using plasmid from JB38
JB57	transformant of JB44 using plasmid from JB38§
JB85	spontaneous Ara^+ mutant of JB44†
RL861	transformant of JB85 (Ara^+) using plasmid from JB13

All strains were stored at −80 °C immediately after their isolation to preclude further genetic changes.

* Ara^+ indicates ability to use arabinose; all strains are Ara^- unless otherwise indicated.

† Isolated by plating on minimal arabinose agar.

‡ Isolated by testing clones on agar with and without supplemental antibiotics.

§ JB54 and JB57 are identical to JB13 and JB38, respectively; they were used to control for any possible effects of segregation and transformation on fitness.

We investigated whether the 500 generations of culture history conferred increased fitness; and, if so, whether genetic changes in the bacterial chromosome, plasmid, or both were responsible for this adaptation. All combinations of plasmid and host, with and without the culture history (JB54–JB57) were competed against JB14, the baseline plasmid-bearing strain with the alternative arabinose marker, in medium supplemented with chloramphenicol. We obtained three replicate estimates of the selection coefficient for each of the strains, as described (Table 2). Analysis of variance indicates a highly significant effect associated with the bacterial chromosome, but no significant effects associated with either the plasmid or the chromosome/plasmid interaction (Table 3). Thus, the bacteria/plasmid association underwent significant adaptation during the 500-generation experiment; this adaptation was the result of genetic change in the bacterial chromosome, but not in the plasmid.

We then determined whether adaptation by the bacterium had altered the effect of plasmid carriage on fitness. We competed JB55, the evolved bacterium transformed with the baseline plasmid, against JB85, the isogenic plasmid-free strain, in the absence of chloramphenicol. To ensure that the arabinose marker had no effect on fitness in the evolved bacterium, we included a control in which JB44 was competed against JB85. As shown in Table 4, the arabinose marker again had no significant effect on fitness, whereas there was a highly significant enhancement of host fitness associated with plasmid carriage, even in the antibiotic-free environment, after the host had evolved in the presence of the plasmid for 500 generations. Given this surprising result, we performed another control: we competed RL861 (a transformant of JB85) against the plasmid-free JB44. The highly significant enhancement of fitness was also observed in this evolved host possessing the alternative

Table 2 Effect of plasmid carriage on host fitness before culture history

Competitors A	Competitors B	n	Mean	Standard error	t
JB13	JB12	26	−0.055 per day	0.018	3.084 ($P < 0.01$)
JB11	JB12	30	−0.003 per day	0.006	0.500 (NS)

A control for the effect of the arabinose marker was included (JB11 versus JB12). The n estimates of the selection coefficient were calculated by regressing the natural logarithm of the ratio of the two competitors (A/B) against time. A positive value indicates that A is more fit than B; a negative value that B is more fit than A. NS, not significant; t = t-statistic.

arabinose marker (Table 4).

Our results can be summarized as follows. (1) The newly introduced plasmid was antagonistic in the absence of antibiotic. (2) The host/plasmid association underwent adaptation during 500 generations in the presence of antibiotic. This adaptation was due to genetic change by the host. (3) After 500 generations, the plasmid enhances the fitness of its host 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. (4) The genetic change responsible for the new mutualistic effect arose in the host chromosome, because the evolved bacterium experiences the benefit when transformed with the baseline plasmid.

In the one previous study where fitness gains were partitioned between a plasmid and host, Helling and co-workers[8] observed that increased fitness was due to genetic change in the host, and not in the plasmid, but they did not determine whether the cost of plasmid carriage had changed as the result of bacterial adaptation. Several other studies have demonstrated that accessory elements can enhance bacterial fitness even in the absence of selection for specific functions such as superinfection immunity or antibiotic resistance[26-31]. However, we believe that this is the first study in which an evolutionary transition from antagonism to mutualism has been observed directly for a bacterial accessory element.

What is the nature of the genetic change in the host that causes it to benefit from plasmid infection, where previously it was harmed? The evolved bacterial host could have become tolerant of some cost imposed by the plasmid, thereby allowing a previously existing plasmid-encoded benefit to be manifest. It is interesting that a portion of pACYC184 is derived from P15A, a non-conjugative, naturally occurring plasmid that does not code for any known phenotypic properties[25]. It is not clear how cryptic plasmids are maintained in bacterial populations. Mathematical analyses[32,33] have shown that the conditions for such plasmids to be maintained through mobilization by conjugative plasmids are extremely restrictive. Such plasmids may carry functions that benefit their hosts, but which have not been detected because they confer no obvious phenotype. We will test this hypothesis by deleting portions of the pACYC184 genome and determining whether any particular region is essential for its fitness-enhancing effect in the evolved host.

There is an important practical implication of our results. It is often assumed that the introduction of DNA for biotechnological functions will reduce the fitness of recombinant organisms, owing to costs of carriage and expression of foreign DNA. According to this 'excess baggage' hypothesis, the deliberate release of recombinant organisms into the environment is inherently safe because these costs will prevent their unintended spread[4,34-36]. Our results caution against uncritical acceptance of this hypothesis.

We thank Pete Atsatt, Rick Condit, Loan Duong, Ralph Evans, Sue Hattingh, Keith Justice, Bruce Levin, Judy MacDougall, Toai Nguyen, Trinh Nguyen, Mayur Patel, Quang Phan, Michael Rose, Brad Shaffer and Lone Simonsen for their valuable comments, discussion, or assistance. Support for this work was provided by grants from the National Institutes of Health (GM-37105) and the Gulf Breeze Laboratory of the US Environmental Protection Agency (CR-813401) to R.E.L.

Table 3 Contributions of bacterial chromosome and plasmid to genetic adaptation

Source	Degrees of freedom	Sums of squares	Mean square	F
Chromosome	1	3.6725	3.6725	119.625 ($P<0.001$)
Plasmid	1	0.0069	0.0069	0.225 (NS)
Interaction	1	0.0117	0.0117	0.381 (NS)
Error	8	0.2457	0.0307	
Total	11	3.9368		

Fixed effects model analysis was used to determine the contribution to adaptation during the 500 generations of culture history. NS, not significant; $F = F$ statistic.

Table 4 Effect of plasmid carriage on host fitness after culture history

Competitors A	Competitors B	n	Mean	Standard error	t
JB55	JB85	20	+0.171 per day	0.017	10.142 ($P<0.001$)
JB44	JB85	20	+0.009 per day	0.005	1.784 (NS)
RL861	JB44	20	+0.106 per day	0.009	11.800 ($P<0.001$)

A control for the effect of the arabinose marker was included (JB44 versus JB85). NS, not significant.

Received 4 March; accepted 18 July 1988.

1. Levin, B. R. & Lenski, R. E. in *Coevolution* (eds Futuyma, D. J. & Slatkin, M.) 99-127 (Sinauer Associates, Sunderland, Mass., 1983).
2. Davey, R. B. & Reanney, D. C. in *Evolutionary Biology*, Vol. 13 (eds Hecht, M. K., Steere, W. C. & Wallace, B.) 113-147 (Plenum, New York, 1980).
3. Dykhuizen, D. E. & Hartl, D. L. *Microbiol. Rev.* **47**, 150-168 (1983).
4. Lenski, R. E. & Nguyen, T. T. in *Planned Release of Genetically Engineered Organisms* (eds Hodgson, J. & Sugden, A. M.) 18-20 (Elsevier, Cambridge, 1988).
5. Lenski, R. E. & Bouma, J. E. *J. Bact.* **169**, 5314-5316 (1987).
6. Cooper, N. S., Brown, M. E. & Caulcott, C. A. *J. gen. Microbiol.* **133**, 1871-1880 (1987).
7. Lee, S. W. & Edlin, G. *Gene* **39**, 173-180 (1985).
8. Helling, R. B., Kinney, T. & Adams, J. *J. gen. Microbiol.* **123**, 129-141 (1981).
9. Noack, D. *et al. Molec. gen. Genet.* **184**, 121-124 (1981).
10. Levin, B. R. in *Antibiotic Resistance: Transposition and Other Mechanisms* (eds Mitsuhashi, S., Rosival, L. & Kremery, V.) 197-202 (Springer, Berlin, 1980).
11. Zund, P. & Lebeck, G. *Plasmid* **3**, 65-69 (1980).
12. Jones, I. M., Primrose, S. B., Robinson, A. & Ellwood, C. C. *Molec. gen. Genet.* **180**, 579-584 (1980).
13. Godwin, D. & Slater, J. H. *J. gen. Microbiol.* **111**, 201-210 (1979).
14. Lenski, R. E. *Evolution* **42**, 433-440 (1988).
15. Paquin, C. E. & Adams, J. *Nature* **306**, 368-370 (1983).
16. Chao, L., Vargas, C., Spear, B. B. & Cox, E. C. *Nature* **303**, 633-635 (1983).
17. Paquin, C. E. & Adams, J. *Nature* **302**, 495-500 (1983).
18. Dykhuizen, D. & Hartl, D. *Evolution* **35**, 581-594 (1981).
19. Graham, J. B. & Istock, C. A. *Science* **204**, 637-639 (1979).
20. Luckinbill, L. S. *Science* **202**, 1201-1203 (1978).
21. Atwood, K. C., Schneider, L. K. & Ryan, F. J. *Cold Spring Harb. Symp. quant. Biol.* **16**, 345-355 (1951).
22. Lenski, R. E. *Evolution* **42**, 425-432 (1988).
23. Lenski, R. E. & Levin, B. R. *Am. Nat.* **125**, 585-602 (1985).
24. Maniatis, T., Fritsch, E. F. & Sambrook, J. *Molecular Cloning* (Cold Spring Harbor Laboratory, New York, 1982).
25. Chang, A. C. Y. & Cohen, S. N. *J. Bact.* **134**, 1141-1156 (1978).
26. Edlin, G., Tait, R. C. & Rodriguez, R. L. *Biotechnol.* **2**, 251-254 (1984).
27. Hartl, D. L., Dykhuizen, D. E., Miller, R. D., Green, L. & DeFramond, J. *Cell* **35**, 503-510 (1983).
28. Biel, S. W. & Hartl, D. L. *Genetics* **103**, 581-592 (1983).
29. Edlin, G., Lin, L. & Bitner, R. *J. Virol.* **21**, 560-564 (1977).
30. Lin, L., Bitner, R. & Edlin, G. *J. Virol.* **21**, 554-559 (1977).
31. Edlin, G., Lin, L. & Kudrna, R. *Nature* **255**, 735-737 (1975).
32. Levin, B. R. in *Antibiotic Resistance Genes: Ecology, Transfer, and Expression* (eds Levy, S. B. & Novick, R. P.) 57-70 (Cold Spring Harbor Laboratory, New York, 1986).
33. Levin, B. R. & Stewart, F. M. *Genetics* **94**, 425-443 (1980).
34. Regal, P. J. *Recomb. DNA Tech. Bull.* **10**, 67-85 (1987).
35. Colwell, R. K., Norse, E. A., Pimentel, D., Sharples, F. E. & Simberloff, D. *Science* **229**, 111-112 (1985).
36. Brill, W. J. *Science* **227**, 381-384 (1985).

Printed in Great Britain by Turnergraphic Limited, Basingstoke, Hampshire