Amelioration of the Cost of Conjugative Plasmid Carriage in *Eschericha coli* K12

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ABSTRACT

Although plasmids can provide beneficial functions to their host bacteria, they might confer a physiological or energetic cost. This study examines how natural selection may reduce the cost of carrying conjugative plasmids with drug-resistance markers in the absence of antibiotic selection. We studied two plasmids, R1 and RP4, both of which carry multiple drug resistance genes and were shown to impose an initial fitness cost on *Escherichia coli*. To determine if and how the cost could be reduced, we subjected plasmid-containing bacteria to 1100 generations of evolution in batch cultures. Analysis of the evolved populations revealed that plasmid loss never occurred, but that the cost was reduced through genetic changes in both the plasmids and the bacteria. Changes in the plasmids were inferred by the demonstration that evolved plasmids no longer imposed a cost on their hosts when transferred to a plasmid-free clone of the ancestral *E. coli*. Changes in the bacteria were shown by the lowered cost when the ancestral plasmids were introduced into evolved bacteria that had been cured of their (evolved) plasmids. Additionally, changes in the bacteria were inferred because conjugative transfer rates of evolved R1 plasmids were lower in the evolved host than in the ancestral host. Our results suggest that once a conjugative bacterial plasmid has invaded a bacterial population it will remain even if the original selection is discontinued.

OUR capacity to decrease antibiotic resistance among clinically important bacteria depends in part on the fitness costs associated with the resistances. If a cost is associated with a resistance phenotype, sensitive strains should be able to invade and outcompete resistant bacterial populations when the use of antibiotics is reduced. However, several studies examining the effects of reducing antibiotic use have shown that for chromosomally encoded resistances the cost is often ameliorated by compensatory mutations without loss of resistance (Shrag and Perrot 1996; Shrag *et al.* 1997; Björkman *et al.* 1998, 2000; Reynolds 2000; Nagaev *et al.* 2001).

The potential cost to a bacterial host of plasmid-borne antibiotic resistance may be associated with the resistance function itself or with plasmid regulation. For instance, plasmid-borne antibiotic resistances often act through alteration or efflux of the antibiotic and these mechanisms may disturb bacterial growth. In addition, plasmid replication and gene expression may interfere with bacterial growth in ways independent of any resistance functions carried by that plasmid. These costs can be expected to be specific for each plasmid/bacteria combination.

In the absence of the selecting antibiotics, a cost associated with plasmid carriage has been observed for nonconjugative plasmids bearing antibiotic-resistance genes (Zünd and Lebek 1980; Helling *et al.* 1981; Noack *et al.* 1981; Caulcott *et al.* 1983; Dykhuizen and Hartl 1983; Cooper *et al.* 1987; Lenski and Bouma 1987; Bouma and Lenski 1988; Modi and Adams 1991). Since these elements are also transmitted vertically during cell division one would anticipate that natural selection operating on the host, on the element, or both would either ameliorate the fitness costs or favor the ascent of bacteria that have lost these elements, *i.e.*, segregation (Levin and Lenski 1983). Amelioration of the cost of carriage for simpler, nonconjugative plasmids has been obtained by host evolution after evolution with selecting antibiotics (Bouma and Lenski 1988) as well as plasmid-host coevolution when evolution occurred without antibiotic selection (Modi and Adams 1991). Although the data are scarce, naturally occurring conjugative resistance plasmids are also generally considered costly when there is no selection for the resistance phenotype (Godwin and Slater 1979; Zünd and Lebek 1980). A major difference between the high-copy-number cloning vector type of plasmids used in the experiments mentioned above and many of the conjugative low-copy-number plasmids is, in addition to their potential for horizontal dissemination within and between bacterial species, the presence of stability systems in the latter. These systems are found in most conjugative plasmids and ensure faithful inheritance to the daughter cells at cell division.

Since plasmids contribute greatly to antibiotic-resistance development in bacteria, it is fundamentally important to understand how plasmid-borne resistances may respond to a decreased use of antibiotics. This report focuses on two well-characterized plasmids with different replication systems, R1 (~100 kb) and RP4 (60 kb). We examine how the costs of these plasmids on an *Escherichia coli* host can be ameliorated by natural selection in the absence of antibiotics.

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TABLE 1

***Escherichia coli* strains and plasmids**

Strain or plasmid	Designation[a]	Relevant characteristics[b]	Reference or source[c]
Strain			
J53-1	(b_0)	K12 F$^-$ pro$^-$, met$^-$, Nal^R	NCTC
CD100		As J53-1 but resistant to phage T5	This study
CD104	(b_0p_0)	As J53-1 but carrying plasmid R1	This study
CD105	(b_0p_0)	As J53-1 but carrying plasmid RP4	This study
CD111–113	(b_1p_1)	Derivatives of CD104 randomly isolated from populations 1–3, respectively, after 166 days of serial transfer	This study
CD114–116	(b_1p_1)	As CD111–113 but derivatives of CD105 isolated from populations 4–6, respectively	This study
CD117	(b_1*)	As CD111–113 but derivative of J53-1 isolated from population 7	This study
CD120–124	(b_1)	Plasmid-free derivatives of CD111–116	This study
CD125–127	(b_1p_0)	CD120–122 carrying plasmid R1	This study
CD128–129	(b_1p_0)	CD123–124 carrying plasmid RP4	This study
CD130–135	(b_0p_1)	J53-1 carrying the plasmids from respectively CD111–116	This study
CD139		As CD112 but isolated on basis of its resistance pattern, (Nal^R)	This study
CD142–143		As CD113 but isolated on basis of their resistance patterns, (Nal^R) and (Nal^R, Km^R)	This study
CD148–149	(b_1*p_0)	CD117 carrying plasmid R1 or RP4	This study
Plasmid			
R1		~100 kb, IncF11, Cm^R, Km^R, Ap^R, Sm^R/Su^R	NCTC
RP4		60 kb, IncP, Km^R, Ap^R, Tc^R	NCTC
pFF1ts97		RP4 replicon, Cm^R, Ap^R	Fang and Helinski (1991); Valla *et al.* (1991)
pKG339		pSC101 replicon, *copA*, Tc^R	Jensen *et al.* (1995)

[a] In strain designations *b* refers to the host and *p* to the plasmid (see Figure 1 legend for details).
[b] Nal, nalidixic acid; Cm, chloramphenicol; Km, kanamycin; Ap, ampicillin; Sm, streptomycin; Tc, tetracycline.
[c] NCTC, National Collection of Type Cultures, London.

MATERIALS AND METHODS

Strains and designations: Table 1 lists the *E. coli* K12 strains and plasmids used in this study. The plasmids R1 and RP4 were selected for this study on the basis of their differences in replication control and host range. Plasmid R1 has an inhibitor-target mechanism of replication control and has a narrow host range whereas RP4 has an iteron-based control of replication and a very broad host range. A notation where *b* refers to the bacterial host and *p* to the plasmid it carries was used for this study. The clones used to found long-term serial cultures were hence designated b_0p_0 or b_0 and clones isolated at the end of the experiment were designated b_1p_1 or b_1* (b_1* was used for clones that had evolved in the absence of plasmids to distinguish them from constructions where b_1p_1 were turned into b_1 by curing). Further strain constructions made from these isolates were designated as shown in Figure 1 and Table 1. Strain J53-1 was used as the host in all long-term selection experiments. Strains CD104 and CD105 are derivatives of J53-1 where plasmid R1 or plasmid RP4 was, respectively, introduced by conjugation. Strain CD100 is a spontaneous mutant of J53-1 resistant to bacteriophage T5 and was used as a common competitor in all the pairwise competition experiments (see below).

Growth media: The bacterial populations were propagated in Davis minimal (DM) medium (Carlton and Brown 1981) supplemented with 230 μg/ml proline, 45 μg/ml methionine, and with a glucose concentration of 100 μg/ml for liquid cultures. This glucose concentration gave a stationary-phase bacterial concentration of ~5×10^8 cells/ml. DM agar with a glucose concentration of 1000 μg/ml was used for plating. Final concentrations of antibiotics in Luria agar were 100 μg/ml nalidixic acid, 25 μg/ml kanamycin sulfate, 10 μg/ml tetracycline, 25 μg/ml chloramphenicol, 20 μg/ml streptomycin, and 100 μg/ml ampicillin. For phage resistance selection, a lysate of phage T5 corresponding to 10^9 phages was spread onto DM plates prior to spreading of the bacteria.

Long-term serial cultures: A schematic presentation of the experimental evolution and the construction of new plasmid-host combinations are shown in Figure 1. Three replicate populations 1, 2, and 3 of CD104 and three replicate populations 4, 5, and 6 of CD105 were initiated from stationary-phase cultures grown in LB with selective antibiotics for the respective plasmid. Following the notation developed for this study (see *Strains and designations*), the bacteria and plasmid used to start either of these triplicate populations were all designated b_0p_0. Population 7 was started with the plasmid-free J53-1 and the starting clone was given the designation b_0. The populations were serially propagated every 24 hr with 40 μl of culture into 4 ml of fresh DM without selecting antibiotics in 16-mm tubes. The cultures were incubated at 37° in a slanted rack, shaking at 250 rpm. Aerosol-

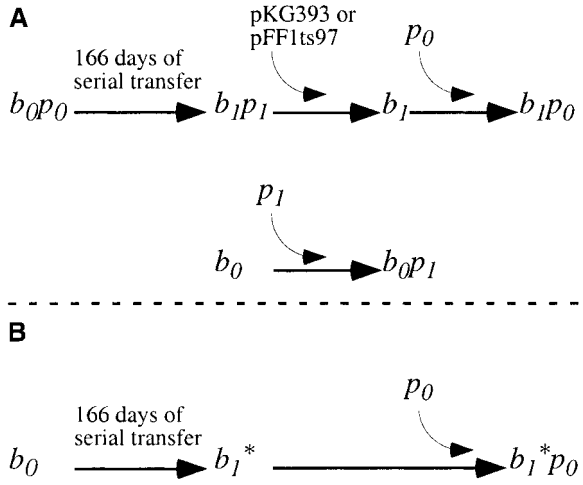

FIGURE 1.—Experimental evolution and strain construction. *b* refers to the bacterial host and *p* to the plasmid it carries. (A) Three independent populations of the *E. coli* host J53-1 carrying either plasmid R1 or plasmid RP4 (b_0p_0) were propagated by daily serial transfer. Clones were isolated from each population after 166 days (b_1p_1). One random clone from each population was subjected to curing, leading to a plasmid-free evolved host (b_1). Curing was accomplished through infection by an incompatible replicon: pKG339 for R1-carrying clones and pFF1ts97 for RP4-carrying clones (see main text for details). The respective ancestral plasmid (p_0) was further transferred into the cured clones (b_1p_0). Finally constructs were made where the evolved plasmids were transferred into the ancestral plasmid-free host (b_0p_1). (B) One population of the plasmid-free *E. coli* host J53-1 (b_0) was propagated by daily serial transfer. A random clone from this population was isolated after 166 days (b_1*). In addition, constructions were made where either of the ancestral plasmids R1 or RP4 was transferred into this evolved host (b_1*p_0).

resistant filter tips were used in all work associated with transfers of the evolving populations. The transfers continued for 166 days corresponding to ~1100 generations. About every 50 generations the presence of the plasmids was determined on the appropriate antibiotic plates for 100 colonies from each population. Plasmid isolation was used to verify the presence of plasmids if resistance markers were absent. One random clone from each end-population was isolated for further analysis [CD111–116 (b_1p_1) and CD117 (b_1*) (Table 1)]. Clones with new resistance phenotypes that had evolved during the experiment were also isolated (CD139 and CD142–143) and further analyzed when they represented >5% of the total population. All clones isolated at the end of the experiment are referred to as evolved in the following text.

Curing and construction of new plasmid-host combinations from evolved clones: The plasmids from the evolved clones were transferred into the ancestral host J53-1. Transfer was done either by conjugation in two steps, where *E. coli* LE392-1 served as an intermediate host, or by transformation using the $CaCl_2$ procedure (MANIATIS *et al.* 1989), and resulted in clones CD130–135 (b_0p_1). The plasmids R1 and RP4 both carry multiple stability systems, including postsegregational killing and partitioning, which makes natural segregant formation very rare. Clones cured of their plasmids (b_1) were instead obtained by using two slightly different protocols. Plasmid pKG339 (JENSEN *et al.* 1995), which contains the R1 *copA* gene under control of a LacI-regulated promoter, was transformed into CD111–113. Transformants were isolated and selected clones were plated on isopropyl thiogalactoside (IPTG)-containing plates. The addition of IPTG results in a large production of CopA RNA that will inhibit R1 replication and make the isolation of evolved R1 segregants possible. After confirmation that the evolved R1 plasmid had been lost the clones were grown in the absence of selecting antibiotics and natural segregants of pKG339 were isolated [CD120–122 (b_1)]. For clones CD114–116 the mini-RP4 replicon pFF1ts97 (FANG and HELINSKI 1991; VALLA *et al.* 1991) was used for curing. This temperature-sensitive replicon was transformed into the cells and transformants were grown at 30°. The pFF1ts97 replicon is incompatible with the wild-type RP4 plasmid and selection for pFF1ts97 made it possible to select for segregants of the evolved RP4 plasmids. After one additional plating and confirmation that the evolved RP4 plasmid was lost, the clones were allowed to grow without selective antibiotics at 37°, which would lead to segregation of pFF1ts97, and clones CD123–124 (b_1) were then isolated. Each step in the curing procedures was monitored by electrophoresis of plasmid isolations. Controls were made where the plasmids that had been removed by either of the curing procedures above were reintroduced into their host. The ancestral plasmids were finally introduced into the evolved, cured clones and CD117, resulting in CD125–129 (b_1p_0) and CD148–149 (b_1*p_0).

Estimates of fitness: Pairwise competition experiments were performed against a common competitor, CD100. The competing clones were separately preconditioned in DM for one 24-hr cycle. For each competition experiment cultures were inoculated with the preconditioned cultures of the competitors at a 1:1 ratio and were then propagated for one 24-hr cycle as described above. The initial and final ratios of the two competitors were assayed on DM plates with and without phage T5. The relative fitness (see method of fitness estimation below) of the T5-resistant common competitor was 0.992 ± 0.014 relative to the J53-1 ancestor. At least 10 independent competitions were conducted for each strain. An increased sensitivity can also be obtained by measuring fitness over several cycles but this method was avoided to minimize potential effects of plasmid transfer between the competitors.

Calculation of Malthusian parameter: Relative fitness (W) was determined as described by LENSKI (1988) and is the ratio of the number of doublings for the tested clone and the common competitor

$$W_{ij} = \frac{\log_2(N_i(1)/N_i(0))}{\log_2(N_j(1)/N_j(0))},$$

where $N_i(0)$ and $N_j(0)$ are the initial densities of the tested clone and the common competitor, respectively, and $N_i(1)$ and $N_j(1)$ are their corresponding final densities.

Determination of relative copy number changes: Minimal inhibitory concentrations of ampicillin were determined for the ancestral and evolved strains as an indirect measure of plasmid copy number (UHLIN and NORDSTRÖM 1977). Drops (10 μl) corresponding to 10^2 cells from overnight cultures were spotted onto plates containing different concentrations of ampicillin. The lowest concentration of ampicillin where inhibition of growth was detected was determined after overnight incubation at 37°. In a second approach to determine copy number change, DNA band intensities on agarose gels from plasmid isolations were measured and expressed as a ratio to an internal standard in the plasmid isolation. Equal volumes from overnight cultures of CD104 (b_0p_0) and CD111–113 (b_1p_1) were each mixed with an equal volume from one single overnight culture of a tester plasmid containing strain (the relevant characteristic of the tester plasmid is that it differs in size from the plasmids under study). Plasmid DNA was isolated from the mixtures and run on an agarose gel. The tester plasmid was included to make the assay independent of

the DNA extraction efficiency and functioned as a standard for quantification. A similar procedure was done for CD105 (b_0p_0) and CD114–116 (b_1p_1). The gel was then stained for 2 hr in ethidium bromide. The intensity of the ancestral or evolved R1 or RP4 plasmid band was determined and normalized against the intensity of the coextracted tester plasmid band using Scion Image Version 3b (Scion Corporation, Frederick, MD). The relative copy number of each evolved plasmid is expressed as the ratio of the evolved plasmid and the tester plasmid band intensities from one coextraction, divided by the ratio of the ancestral plasmid and its corresponding tester plasmid band intensity. Any differences in yield between the ancestral and evolved plasmids in the overnight cultures were first corrected for.

Phenotypic and molecular analysis of evolved plasmids: The resistance phenotype of 100 isolates from each end population was determined by replica plating on antibiotic-containing media. Restriction enzyme analysis was performed on plasmid DNA that was isolated by alkaline lysis (Birnbom and Doly 1979) and digested with either *Eco*RI (R1) or *Eco*RV and *Pst*I (RP4). Digestions were performed according to the manufacturer's recommendations (New England Biolabs, Beverly, MA) and separated on 0.9% agarose TAE gels. The rate of conjugal transfer (Simonsen *et al.* 1990) was determined for evolved and ancestral plasmids in evolved and ancestral hosts in experiments equal to one 24-hr cycle of serial transfer. The number of transconjugant CD100 cells was determined on DM plates supplemented with kanamycin and phage T5.

RESULTS

Plasmid stability and general fitness changes: Plasmid-free cells were never detected in the populations founded by R1- or RP4-bearing clones over the ~1100 generations of selection in an antibiotic-free environment. The plasmids initially decreased the fitness of *E. coli* J53-1, with 6% (*t*-test, $P < 0.002$) for R1 (CD104, b_0p_0) and 21% ($P < 0.001$) for RP4 (CD105, b_0p_0; Table 2). The random clones isolated at the end of the experiment (b_1p_1) had increased their fitness, with up to 70% as compared to their plasmid-bearing ancestors (b_0p_0; Table 2).

Compensatory evolution: The respective contributions of host- and plasmid-encoded mutations to the general fitness increase observed in the evolved clones (b_1p_1) were measured on combinations of ancestral and evolved hosts and plasmids.

The costs of the evolved plasmids were tested in both the evolved and ancestral host backgrounds. With one exception (see below, clone CD122) there were no significant differences in fitness between the evolved clones (b_1p_1) and the evolved clones cured of their plasmids (b_1). The cost of the evolved plasmids on the ancestral host (b_0p_1) was further tested and showed that five of the six evolved plasmids had a lower cost than the corresponding ancestral plasmid (b_0p_0). In contrast, one of the evolved plasmids, the R1 derivative in clone CD112, showed an increased cost on the ancestral host (CD131, 17%, $P < 0.001$).

The effect of host evolution was assessed by measuring the cost of the ancestral plasmids on the evolved hosts. Where the ancestral plasmids were transferred to the evolved hosts that were cured of their evolved plasmids (b_1p_0) we found no significant costs of the plasmids. For the host that had evolved in the absence of plasmids reduced costs of the ancestral plasmids ($b_1{*}p_0$) were demonstrated, as compared to the costs on the ancestral host (b_0p_0). For the ancestral plasmid R1 on this host (CD148, $b_1{*}p_0$), we found no significant cost and the ancestral plasmid RP4 (CD148, $b_1{*}p_0$) had reduced its cost to 9% ($P < 0.002$; Table 2).

Control experiments where the evolved plasmid was reintroduced into the cured host (*i.e.*, reconstruction of b_1p_1 from b_1) showed that the curing procedure did not affect fitness (data not shown) with one exception. This cured clone CD122 showed a higher fitness when its plasmid had been reintroduced compared to the fitness of the plasmid-bearing clone before curing.

A second problem we encountered when curing the evolved clones was observed in the evolved RP4-bearing clone CD116 (b_1p_1). During curing when this clone was transformed with the plasmid pFF1ts97 that carries the same basic replicon as the ancestral RP4, the expected loss of the resident evolved RP4 plasmid was not seen. Both plasmids were still present, as determined by plasmid isolation, after several successive platings on media selective for pFF1ts97.

Phenotypic evolution: Clones that had lost one or more antibiotic resistances emerged in all R1 populations but represented a minority of the cells (Table 3). The plasmids from representatives of these minority phenotypes were further analyzed by restriction endonuclease digestion and showed loss of restriction fragments of the size corresponding to the respective antibiotic-resistance genes (Figure 2A; Clerget *et al.* 1981). Consequently all resistance gene fragments were absent in the digestions of the clones CD139 and CD142 that had lost all the plasmid resistance phenotypes. Clone CD143 had retained only those resistance gene fragments containing the kanamycin resistance and its flanking insertion sequence (IS1) elements.

Several changes in the evolved plasmids from the randomly drawn clones were also revealed. The RP4 isolates CD114 and CD115 (b_1p_1) differed from the ancestral plasmid in their restriction endonuclease digestion patterns (Figure 2B) and showed several novel restriction sites in the transfer and replication regions (data not shown). These changes were probably associated with transposition of chromosomally located IS elements into the plasmids. For the plasmids in both CD114 and CD115 possible insertions into the *trbE* region that is involved in pilus production were found (Pansegrau *et al.* 1994). An additional insertion in the *oriV* region of the plasmid in CD115 was also found. One structural change was detected in the random R1 clones as CD113 showed a reduction in size of the restriction fragment that in the ancestral R1 plasmid harbors one IS1 copy and a kanamycin resistance gene (Figure

TABLE 2

Fitness of ancestral and evolved strains and their combinations, relative to the plasmid-free ancestor

Population	Ancestral host/ancestral plasmid (b_0p_0) or ancestral host (b_0)		Evolved host/evolved plasmid (b_1p_1)		Evolved host (b_1 or b_1^*)		Evolved host/ancestral plasmid (b_1p_0 or $b_1^*p_0$)		Ancestral host/evolved plasmid (b_0p_1)	
	Strain	Fitness (±SE, no. of exp.)[a]	Strain	Fitness (±SE, no. of exp.)	Strain	Fitness (±SE, no. of exp.)	Strain	Fitness (±SE, no. of exp.)	Strain	Fitness (±SE, no. of exp.)
1	CD104	0.942 (±0.014, n = 26)	CD111	1.286 (±0.021, n = 15)	CD120	1.300 (±0.018, n = 17)	CD125	1.286 (±0.020, n = 13)	CD130	1.019 (±0.021, n = 15)
2	CD104	0.942[b]	CD112	1.303 (±0.016, n = 15)	CD121	1.306 (±0.020, n = 15)	CD126	1.281 (±0.020, n = 13)	CD131	0.835 (±0.042, n = 15)
3	CD104	0.942[b]	CD113	1.309 (±0.012, n = 15)	CD122	1.231[c] (±0.039, n = 19)	CD127	1.302[c] (±0.035, n = 13)	CD132	0.992 (±0.013, n = 15)
4	CD105	0.792 (±0.023, n = 20)	CD114	1.260 (±0.016, n = 18)	CD123	1.294 (±0.022, n = 12)	CD128	1.233 (±0.024, n = 12)	CD133	0.984 (±0.015, n = 15)
5	CD105	0.792[d]	CD115	1.167 (±0.020, n = 17)	CD124	1.190 (±0.015, n = 10)	CD129	1.200 (±0.017, n = 10)	CD134	0.954 (±0.017, n = 15)
6	CD105	0.792[d]	CD116	1.349 (±0.019, n = 18)	ND[e]		ND		CD135	0.982 (±0.028, n = 14)
7	J53-1	1.000			CD117	1.236 (±0.011, n = 15)	CD148	1.234 (±0.017, n = 15)		
							CD149	1.127 (±0.038, n = 14)		

Fitnesses were measured as the fitness relative to the common competitor CD100. ± standard error. All fitness values were corrected for the effect of the T5-resistance marker in CD100.

[a] Standard error, number of experiments.

[b] Fitness measurement is the same as for population 1.

[c] See RESULTS for details.

[d] This fitness measurement is the same as for population 4.

[e] ND, not determined.

TABLE 3

Distribution of plasmid-resistance phenotypes within the three evolved R1 populations expressed in percentage of the total population

	Population		
Resistance phenotype[a]	1	2	3
Cm^R, Km^R, Ap^R, Sm^R	93	85	66
Km^R, Ap^R, Sm^R	1		
Cm^R, Ap^R, Sm^R	3	1	
Cm^R, Km^R, Sm^R	3		
Cm^R, Sm^R	1		
Km^R	4	11	
—[b]	1	9	22

[a] Cm, chloramphenicol; Km, kanamycin; Ap, ampicillin; Sm, streptomycin.
[b] All antibiotic resistances expressed by the plasmid were absent.

TABLE 4

Transfer rates of ancestral and evolved R1 plasmids

Donor strain[a]	Transfer rate[b]
CD104 (b_0p_0)	2.21 *e*-11 ± 3.08 *e*-12 (n = 7)
CD148 (b_1*p_0)	3.07 *e*-11 ± 3.51 *e*-12 (n = 5)
CD130 (b_0p_1)	3.01 *e*-11 ± 7.89 *e*-12 (n = 5)
CD111 (b_1p_1)	2.25 *e*-12 ± 6.20 *e*-13 (n = 5)
CD131 (b_0p_1)	2.63 *e*-11 ± 5.78 *e*-12 (n = 6)
CD112 (b_1p_1)	1.14 *e*-12 ± 3.04 *e*-13 (n = 6)
CD132 (b_0p_1)	2.76 *e*-11 ± 5.11 *e*-12 (n = 6)
CD113 (b_1p_1)	9.14 *e*-12 ± 1.71 *e*-12 (n = 6)

[a] Paired strains contain the same plasmid.
[b] Transfer rate (milliliter per cell per hour) ± standard error, number of experiments in parentheses.

2A). The clone CD113 (b_1p_1) was kanamycin resistant and the size reduction was probably due to excision of the IS1 element.

The evolved RP4 plasmids in clones CD114 and CD115 were transfer deficient in both the evolved and the ancestral host. The evolved RP4 plasmid in clone CD116 had retained its transferability but the transfer rates of this plasmid and of the ancestral RP4 could not be determined for the liquid culture conditions used in these experiments, as they did not exceed the transfer that occurred after plating on the selective media. The evolved R1 clones (b_1p_1) showed lower transfer rates compared to the ancestral R1 clone (b_0p_0). The reduction in transfer rate of the evolved plasmids was not seen in the ancestral host (b_0p_1; Table 4). Transfer of the ancestral plasmid R1 was also measured from the host that had evolved in the absence of plasmids, CD148 (b_1*p_0). This transfer rate was of the same magnitude as transfer from the ancestral host (b_0p_0; Table 4).

The values obtained by the two different methods used for copy number measurements did not fully correlate. Only when both methods showed a similar deviation in copy number was an average of these values used to assign changes in copy number. The RP4 derivative in clone CD116 (b_1p_1) showed an increase in copy number of ~70% and the R1 clone CD111 (b_1p_1) showed a 20% reduction in copy number (Table 5).

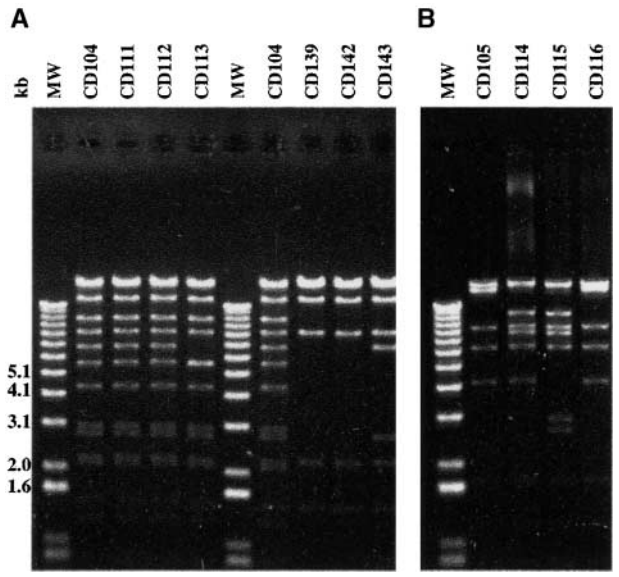

FIGURE 2.—Restriction fragments of ancestral and evolved (A) R1 derivative plasmids digested with *Eco*RI and (B) RP4 derivative plasmids digested with *Eco*RV and *Pst*I. The molecular weight marker (M_r) is a 1-kb ladder.

DISCUSSION

Many antibiotic-resistance genes are carried by conjugative plasmids but studies to date on compensatory evolution of antibiotic resistance have focused on genes encoded either on chromosomes or on nonconjugative plasmids (Bouma and Lenski 1988; Modi and Adams 1991; Shrag and Perrot 1996; Björkman *et al.* 1998, 2000; Reynolds 2000; Nagaev *et al.* 2001). We examined how the cost of carrying conjugative antibiotic-resistance plasmids is affected by evolution in an antibiotic-free environment. Parallel populations of an *E. coli* host carrying the natural conjugative plasmids R1 or RP4 were propagated for ~1100 generations by serial transfer. The cost associated with plasmid carriage before and after experimental evolution was determined in competition experiments using a plasmid-free common competitor. Both plasmids R1 and RP4 imposed fitness costs on *E. coli* J53-1 at the start of the experiment (Table 2). At the end of the experiment all tested clones showed an increased fitness compared to the respective ancestral plasmid-host combination (Table 2). Genetic adaptation to the general growth conditions was expected to account for part of this fitness increase but

TABLE 5

Plasmid copy number

Strain	MIC[a]	Relative DNA content[b]
CD104 (b_0p_0)	300	1
CD111 (b_1p_1)	250	0.71
CD112 (b_1p_1)	400	0.75
CD113 (b_1p_1)	300	0.66
CD105 (b_0p_0)	4000	1
CD114 (b_1p_1)	4000	0.88
CD115 (b_1p_1)	4000	0.96
CD116 (b_1p_1)	7000	1.63

[a] MIC, minimal inhibitory concentration in micrograms per milliliter of ampicillin, based on three independent measurements.

[b] Relative change in plasmid DNA content compared to respective ancestral plasmid as determined by agarose gel electrophoresis and density measurements of relative band intensities. Average of two independent experiments is shown.

changes in response to the cost of plasmid carriage were also anticipated.

Reduction of the cost of plasmid carriage can be accomplished through two main processes: segregation and compensatory mutation. For plasmid-borne antibiotic resistance segregation will lead to the loss of resistance by the formation of plasmid-free cells whereas compensatory mutations may or may not abolish resistance. Both segregation and compensatory mutation are likely to occur in a large population, resulting in a race between the two. Under conditions of antibiotic-resistance selection, compensatory mutation is primarily available, but under conditions where antibiotic selection is released it is unclear which of these processes will dominate.

Like most natural conjugative plasmids, the plasmids used in this study have stability systems such as postsegregational killing and partitioning that make their segregation rates very low (Nordström *et al.* 1980; Schmidhauser and Helinski 1985). The fact that plasmid-free cells were never detected in the evolved populations was most likely affected by the action of these stability systems. Earlier studies have also shown that plasmid RP4 can be stably maintained in continuous culture (Melling *et al.* 1977; Jones *et al.* 1980). There is a possibility that segregants were reinfected if they appeared, but the very low transfer rates we found under these conditions (see below) makes this explanation unlikely.

Since segregation was not detected in these experiments we directed our efforts to examine the effect of compensatory evolution. The first indication of compensatory mutations was seen when the fitness of plasmid-free clones (b_1) constructed from evolved plasmid-bearing clones (b_1p_1) was measured. These measurements showed no difference in fitness between the pairs of plasmid-carrying (b_1p_1) and plasmid-free (b_1) clones with one exception (see below, clone CD122; Table 2). Compensatory mutations in either the chromosome or the plasmid could explain these results. The costs of five of the six tested evolved plasmids on the ancestral host (b_0p_1) were lower than the costs of the corresponding ancestral plasmid (b_0p_0; Table 2), suggesting that these evolved plasmids had acquired compensatory mutations. The fact that four of six evolved plasmids had no cost at all suggests that compensatory mutations on the plasmid may prevent selection for segregants and hence plasmid loss from the population.

If compensatory mutations on the evolved plasmids were alone responsible for the decreased cost one would expect that the ancestral plasmids would still impose a similar cost on the evolved hosts as on the ancestral host. This was, however, not seen; instead we found that the ancestral plasmids had no significant cost on the evolved hosts that were cured of their evolved plasmids (b_1p_0; Table 2). This result indicates that additional compensatory mutations had occurred in the chromosome. The surprising aspect of these results lies in the apparent redundancy of the compensatory mutations shown by the compensatory mutations occurring in both the plasmid and the host. When a mutation compensatory for the cost of plasmid carriage occurs on either the host or the plasmid replicon, selection for a second compensatory mutation on the second replicon should be abolished.

The host that evolved in the absence of plasmids (b_1*) expectedly demonstrated an increase in fitness in response to the growth conditions. However, a second surprising result showed that this well-adapted host naive to plasmid carriage was more tolerant to the cost of a plasmid. For the ancestral plasmid R1 on this host we found no significant cost and the ancestral plasmid RP4 had greatly reduced its cost (Table 2).

As an explanation for the results on compensatory evolution we suggest that during the experiment both the host and the plasmids acquire mutations that are selected but for different purposes. The plasmids evolve compensatory mutations that are selected because they enhance fitness of the host whereas the host evolves toward the general growth conditions with a side effect of decreasing the cost of plasmid carriage. If the plasmid evolves compensatory mutations more rapidly than the host evolves mutations with compensatory side effects this would result in the apparent redundancy of compensatory mutations. It would also explain why a host that has evolved in the absence of plasmids suffers a lower cost of an ancestral plasmid than an ancestral host does. This explanation is also consistent with the results that an evolved plasmid does better than its ancestor in the ancestral host.

The cured clone CD122 showed a higher fitness when its plasmid had been reintroduced compared to the fitness of the plasmid-bearing clone before curing in the control experiment. This suggests that chromosomal

genetic changes occurred during the curing procedure and the fitness values measured for the cured state of this clone are uncertain. A reasonable explanation for this result is that a mutualistic association resulting in strong plasmid dependency had evolved in this clone. We believe that the strong selection applied when the resident plasmid was forced out by an incompatible replicon during the curing procedure selected for a mutation compensatory for the loss of the plasmid. Bouma and Lenski (1988) and Modi and Adams (1991) previously showed that evolution leading to plasmid dependency occurred during experimental evolution of small nonconjugative plasmids. They were able to demonstrate, by the isolation of plasmid-free cells by natural segregation that had a significantly lower fitness compared to the plasmid-bearing cells, that plasmid dependency had evolved.

What was the basis for the decreased costs of the evolved plasmids from five out of six independent clones? First one may consider conjugation. Plasmid RP4 has a constitutively expressed transfer function whereas R1 is repressed for transfer. One part of the cost may be associated with pilus production. We found that two of the evolved RP4 isolates were transfer deficient and that this deficiency correlated with changes in their restriction endonuclease digestion patterns in genes involved in pilus production. The evolved R1 clones (b_1p_1) showed lower transfer rates than the ancestral R1 clone (b_0p_0). This reduction in transfer rate was likely due to host evolution resulting in a host-directed suppression of transfer since the reduced transfer of these plasmids was observed only in the evolved host (b_1p_1) and not in the ancestral host (b_0p_1; Table 4). The transfer rate of the ancestral R1 from the host that had evolved in the absence of plasmids ($b_1{*}p_0$) was of the same magnitude as transfer from the ancestral host (b_0p_0). This result supports the idea that the mechanism responsible for the reduced transfer rates in the former case evolved in direct response to plasmid carriage (Table 4). We did not attempt to localize the chromosomal mutations responsible for this effect but several host-encoded factors that influence plasmid transfer have been identified in previous studies. For example, the ArcA protein, involved in cellular redox sensing, affects R1 transfer (Strohmaier *et al.* 1998).

Copy number reduction is another possible route for cost reduction (Modi and Adams 1991). The copy number has been shown to be ~4–5/cell for plasmid R1 and 4–6 for plasmid RP4 (Figurski *et al.* 1979; Nordström *et al.* 1980). For one evolved R1 clone (CD111) we could observe a 20% reduction in copy number (Table 5). In contrast, an increased copy number corresponding to 7–10 copies per cell was observed for the evolved RP4 clone CD116 (Table 5). The mutation that made this plasmid increase its copy number could account for our failure to cure this clone by making it compatible with the original replicon.

Antibiotic-resistance expression is a third type of plasmid function that can be altered to reduce cost. Most of the clones from the evolved populations had retained all their antibiotic resistances and had obviously reduced their costs by other means. However, R1-carrying populations did contain minor fractions of clones that had lost resistances. The antibiotic sensitivities of these evolved clones correlated with deletions of the resistance genes and included loss of all the plasmid-borne resistances. Similar results have previously been presented for the conjugative plasmid TP120, where loss of resistances was observed after selection in chemostats (Godwin and Slater 1979).

Conclusions: These results demonstrate that the fitness cost of conjugative resistance plasmid carriage can be reduced and is often completely ameliorated after selection in an antibiotic-free environment. Reversion to sensitivity by plasmid segregation was not observed in these experiments. Instead, compensatory evolution was the major pathway. As previously suggested a decreased usage of antibiotics may hence not be sufficient for dealing with the antibiotic-resistance problem as sensitive bacteria are prevented from invading resistant bacterial populations after compensatory evolution has occurred. However, the emergence of plasmids with deletion of all antibiotic-resistance markers from the basic replicon in some of our experiments shows that there is still a potential for selection of antibiotic-sensitive populations. A consequence of the plasmid-encoded compensatory mutations is that the resistance genes associated with these plasmids may have an enhanced potential to invade new populations by horizontal transfer as compared to the more limited chromosomal compensatory mutations that still are confined to one population. It remains to be elucidated if plasmid compensatory mutations are specific for the strain they were selected in or if they also reduce the cost of the plasmid carriage when it transfers to other strains or species.

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