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Antibiotic resistance correlates with transmission in plasmid evolution

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Conjugative (horizontally transmissible) plasmids are autonomous replicators, whose "self-interests" do not necessarily overlap with those of their hosts. This situation causes plasmids and bacteria to sometimes experience differing selection pressures. *Escherichia coli* plasmid pB15 contains genes for resistance to several antibiotics, including tetracycline. When plasmid-bearing cells were experimentally evolved in the laboratory, changes in resistance level in the unselected tetracycline marker coincided with changes in plasmid rates of vertical versus horizontal transmission. Here, we used minimum inhibitory assays that measure resistance levels as quantitative traits to determine phenotypic correlations among plasmid characters and to estimate divergence among plasmid lineages. Results suggested that plasmid-level evolution led to formation of two phenotypically dissimilar groups: virulent (highly infectious) and avirulent (weakly infectious) plasmids. In contrast, measures of carbon-source utilization, and fitness assays relative to a common competitor revealed that bacterial hosts generally converged in phenotypic performance, despite divergence among their associated plasmids. Preliminary sequence analyses suggested that divergence in plasmid conjugation was due to altered configurations of a shufflon region (a site-specific recombination system), where genetic rearrangements affect conjugative ability. Furthermore, we proposed that correlated resistance and transmission in pB15 derivatives were caused by a tetracycline-resistance transposon inserted into a transfer operon, allowing transcription from its promoter to simultaneously affect both plasmid resistance and transmission.

KEY WORDS: **Bacteria, *Escherichia coli*, experimental evolution, shufflon, virulence.**

Plasmids are ubiquitous genetic elements in bacterial populations (Walsh 2003), with varying effects on bacterial fitness. Some plasmids harbor beneficial genes, such as carbon-source utilization, antimicrobial toxins and resistance to phage, antibiotics, and heavy metals (Dionisio et al. 2002; Riley and Wertz 2002). Other plasmids may exist as pure parasites if rapid conjugation (cell-to-cell spread) offsets slower growth of infected cells (i.e., reduced vertical transfer of plasmids; Stewart and Levin 1977; Bergstrom et al. 2000; Turner 2004). Segregants that spontaneously lose a costly plasmid can invade a plasmid-bearing population; perhaps in response, some plasmids have evolved mechanisms such as

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postsegregational killing (PSK) that prevent plasmid-free (PF) segregants from outcompeting plasmid-bearing cells during nonselective conditions (e.g., Gerdes et al. 1986; Thisted et al. 1994). Thus, plasmids are intimately associated with bacteria, but these symbiotic elements range from mutualists to parasites (Harrison and Brockhurst 2012).

Regardless of their fitness effects, plasmids may contribute to divergence among bacterial populations. Beneficial plasmids may foster competitive release, where infected bacteria radiate into new or less-occupied niches; e.g., *Bacillus anthracis* harbors plasmids with pathogenicity islands, allowing opportunistic bacterial infection and anthrax disease in mammals (Leppla 1995). Coevolution between plasmids and their hosts should further enforce such mutualisms. Even if selection for plasmid carriage is only occasional (e.g., fluctuating antibiotics), compensatory mutations may evolve to reduce plasmid costs, perhaps explaining why resistance plasmids are found in bacteria recently unexposed to drugs (Hughes and Data 1983; Svara and Rankin 2011). Last, nonessential or deleterious plasmids can coevolve to become bacterial mutualists (Bouma and Lenski 1988; Dionisio et al. 2005; De Gelder et al. 2008; San Millan et al. 2014). Thus, plasmids can affect evolutionary trajectories of bacteria, causing infected hosts to diverge from PF counterparts. But it is less certain whether plasmids and bacteria can diverge, independently of their symbiotic partners.

Experimental Plasmid/Host Associations

We previously allowed *Escherichia coli* and the deleterious plasmid pB15 to evolve for 500 generations in the presence of abundant glucose, in treatments with differing immigration of PF cells (low, medium, or high opportunity for conjugative transfer; Turner et al. 1998). Results did not support the idea that increasing susceptible-host density should favor evolution of increased parasite virulence (and vice versa; Levin and Pimentel 1981; May and Anderson 1983), evaluated by changes in plasmid conjugation rates and costs of carriage (Turner et al. 1998). However, data showed that conjugation ability coincided with presence/absence of subclinical tetracycline (Tet) resistance in evolved plasmids, strongly suggesting a tight correlation between these evolved traits.

Here, we quantitated the phenotypic correlation between antibiotic resistance and horizontal transmission in this collection of evolved plasmids, and determined whether resistance to antibiotics other than tetracycline was involved. We also sought preliminary evidence that the phenotypic divergence among these genetic elements occurred independently of phenotypic convergence or divergence in their host bacteria, especially in the presence of plasmid/bacteria coevolution. Last, we used whole genome sequencing of the ancestral plasmid to infer the mechanism underlying the correlation between antibiotic resistance and plasmid conjugation. Our results suggested that Tet resistance correlated with transmission ability, whereas other measured resistance traits did not. We also found that plasmid divergence did not generally coincide with divergence in host bacteria. Rather, the evolved host/plasmid associations underwent striking fitness convergence in the high-glucose environment, independent of underlying plasmid divergence and possible host/plasmid coevolution. The genomic analysis suggested that a shufflon region in plasmid pB15 could mechanistically explain how conjugative ability differed among evolved plasmid derivatives, and that insertion of a Tet-resistance transposon into a transfer operon could explain the correlation between resistance and conjugation traits in evolved plasmids.

Materials and Methods

CULTURE CONDITIONS

Unless otherwise indicated, bacteria grew for 24 h at 37°C in nonshaking 18 × 150 mm glass tubes containing 10 mL Davis minimal (DM) broth (Carlton and Brown 1981) with 1000 μg/mL glucose or maltose. Here, the *E. coli* achieved stationary phase density of $\sim 2 \times 10^9$ cells/mL. Daily serial transfer occurred by gentle vortexing followed by 100-fold dilution into fresh medium, resulting in ~6.64 (= $\log_2$ 100) generations of binary fission during the return to stationary cell density.

STRAINS

Table 1 lists the strains studied. The ancestral plasmid-bearing host was REL5382: *E. coli* B evolved for 2000 generations in DM with 25 μg/mL glucose (strain no. REL1206, Lenski et al. 1991), and then infected with plasmid pB15. This ancestor is denoted Ara^-_0/pB15 due to host inability to grow on L-arabinose; Ara^+ and Ara^- strains form white and red colonies on tetrazolium-arabinose (TA) indicator agar (Levin et al. 1977). Strains were evolved for 500 generations in DM with 1000 μg/mL glucose (Turner et al. 1998). Two treatments affected horizontal-transfer opportunity by controlling the percent of PF immigrant bacterial cells that entered the population. To do so, every 3 d (20 gen), populations in the High (H) opportunity for horizontal-transfer treatment were serially transferred into new medium along with 99% PF immigrant cells. Populations experiencing medium (M) opportunity for horizontal transfer were serially transferred along with 50% immigrant cells. The low (L, 0% immigrants) treatment never received immigrants (although conjugation with spontaneous segregants was possible). These treatments experienced kanamycin selection (25 μg/mL) every 3 d to ensure plasmid maintenance. A control (C) treatment was identical to the L treatment but without antibiotic, and a PF treatment was identical to

Table 1. Key bacterial strains used in this study, and their phenotypic traits.

				Tet							
Strain	Properties	Symbol[1]	Ara	KBDD	E-test	Nm	Kan	Sm	$\log_{10}\gamma$[2]	Δc[3]	Copy number
REL5382	Ancestor	ANC	−	17.3	6.0	15.7	>256	>256	−12.629	–	0.912 ± 0.078
REL1206	Plasmid-free ancestor	Plasmid-free ANC	−	21.6	0.583	21.3	6	>256	−∞	–	–
PET297	Evolved control	C1	−	16.7	6.667	13.7	>256	>256	−11.245	–	0.913 ± 0.047
PET298	Evolved control	$C2^{-}$	−	17.3	9.333	14.3	>256	>256	−11.293	0.050	0.940 ± 0.036
PET299	Evolved control	$C2^{+/-}$	+/−	24.3	0.25	12.7	>256	>256	−13.287	−0.054	0.941 ± 0.041
PET300	Evolved control	C3	−	16.0	8.0	13.7	>256	>256	−11.401	0.128	0.942 ± 0.057
PET315	Evolved, low transfer	L1	−	22.4	0.5	10.1	>256	>256	−13.755	−0.050	0.910 ± 0.046
PET316	Evolved, low transfer	L2	−	19.0	1.0	12.2	>256	>256	−13.746	−0.113	0.901 ± 0.030
PET304	Evolved, low transfer	L3	−	14.2	6.0	9.5	>256	>256	−11.970	0.143	0.910 ± 0.025
PET305	Evolved, medium transfer	$M1^{r}$	+	15.3	4.0	12.8	>256	>256	−11.901	0.091	0.925 ± 0.037
PET314	Evolved, medium transfer	$M1^{s}$	+	24.0	0.75	12.6	>256	>256	−∞	ND	0.916 ± 0.049
PET306	Evolved, medium transfer	M2	+	16.9	4.0	13.2	>256	>256	−11.896	0.006	0.917 ± 0.054
PET307	Evolved, medium transfer	M3	+	16.0	4.0	13.0	>256	>256	−11.899	0.111	0.930 ± 0.029
PET309	Evolved, high transfer	H1	+	25.6	0.337	10.8	>256	>256	−∞	ND	0.795 ± 0.021
PET310	Evolved, high transfer	H2	+	15.6	4.0	12.7	>256	>256	−11.953	0.028	0.931 ± 0.044
PET313	Evolved, high transfer	H3	−	22.4	0.337	11.0	>256	>256	−13.685	−0.097	0.913 ± 0.045
REL1207	Ara^{+} mutant of REL1206	$Ara^{+}{}_{0}$	+						–	–	–
REL5384	REL1207 bearing pB15	$Ara^{+}{}_{0}$/pB15	+						–	–	0.893 ± 0.055
PET318	Nal^{r} mutant of REL1207	$Ara^{+}{}_{0}[Nal^{r}]$	+						–	–	–
PET319	Nal^{r} mutant of REL1206	$Ara^{-}{}_{0}[Nal^{r}]$	−						–	–	–
PET354	PET318 bearing pB15	$Ara^{-}{}_{0}[Nal^{r}]$/pB15	−						–	–	–

[1] + and − indicate ability and inability to grow on L-arabinose (Ara). r and s indicate resistance and sensitivity to nalidixic acid (Nal); all strains are Nal^{s} unless otherwise indicated.

[2] conjugation rate ($\log_{10}\gamma$) below the limit of detection is indicated by rate equal to negative infinity (−∞).

[3] ND is not determined.

the C treatment, but founded by $Ara^{-}{}_{0}$. All treatments had three replicate lineages.

RESISTANCE ASSAYS

We measured resistance using Kirby–Bauer disc diffusion (KBDD; BBL™ Sensi-Discs™, Becton Dickinson) and E-test strips (AB Biodisk). For both, a colony-purified strain was grown on TA agar, and then placed in 1 mL sterile 0.85% saline solution and vortexed to match 0.5 McFarland turbidity standard. A sterile swab was used to confluently spread the mixture on Muller–Hinton agar. For KBDD, we applied an antibiotic (30 μg/mL) paper disc to the agar surface and incubated for 18 h at 37°C. Antibiotic diffusion killed or inhibited growth, and the zone of inhibition (mm diameter) indicated strain susceptibility, and mild (intermediate) or full (clinical) resistance (NCCLS 2003). We had no a priori expectations about possible resistance genes on plasmid pB15 beyond those reported earlier (Lundquist and Levin 1986), and arbitrarily chose to assay resistance to 24 antibiotics based on available reagents in our laboratory. These antibiotics were amikacin, amoxicillin, ampicillin, cefazolin, cefepime, cefotaxime, ceftazidime, chloramphenicol, ciprofloxacin, doxycycline, gentamycin, kanamycin, meropenem, nalidixic acid, neomycin, nitrofurantoin, piperacillin, streptomycin, spectinomycin, tetracycline, ticarcillin, tobramycin, trimethoprim, and trimethoprim/sulfamethoxazole. E-test strips accurately determine minimum inhibitory concentration (MIC; bacterial growth relative to a calibrated gradient) of bactericidal and bacteriostatic antibiotics. An E-test strip was placed on the agar surface, and incubated 16 h at 37°C, followed by photographic analysis using manufacturer guidelines. We used E-tests to examine KBDD-confirmed markers, except commercially unavailable neomycin.

CONJUGATION RATE ASSAY

We measured conjugation rate using published methods (Simonsen et al. 1990; Turner et al. 1998; Turner 2004), by mixing donors (D) and Nal^{r} (nalidixic acid resistant) recipients (R) ~1:100 in static culture for 24 h; the Nal^{r} marker facilitated visualization of rare transconjugants (T) on selective plates with 25 μg/mL Kan and 15 μg/mL Nal. Final densities of D, R, and T were determined by colonies formed on TA agar. Growth rate per hour (ψ) of mating cultures was estimated by regressing the

natural log (ln) of total density ($N = T + R + D$) versus time during exponential phase. We estimated conjugation rate (γ) using the formula of Simonsen et al. (1990):

$$\gamma = \psi \times ln[1 + (T/R)(N/D)]/(N - N_0),$$

where N_0 is initial population size. This measure is in units of milliliter per cell hour, indicating the volume, during 1 h, in which a plasmid-bearing cell can successfully "search for" and infect a PF cell.

RELATIVE FITNESS AND COST OF PLASMID CARRIAGE

We measured relative fitness by preconditioning two differentially Ara-marked strains separately in the experimental medium for 24 h (ensuring comparable physiology), and then mixing 1:1 for 24-h competition under the above culture conditions. We estimated initial $N_i(0)$ and final $N_i(1)$ densities of each competitor by plating on TA agar. We calculated the time-average rate of increase, m_i, for the competitor as

$$m_i = ln[N_i(1)/N_i(0)]/(24h).$$

The fitness of strain i relative to strain j is expressed simply as the dimensionless ratio of their rates of increase (Lenski et al. 1991):

$$W_{ij} = m_i/m_j.$$

To determine the cost of plasmid carriage, c, we competed a plasmid-bearing strain versus an isogenic PF strain that differed by a neutral marker. The difference between 1.0 and the estimated fitness equals c, where $c > 0$ indicates that a plasmid reduces host fitness. (Turner [2004] showed that segregants and transconjugants were small minorities arising during competitions between plasmid-bearing and PF cells; thus, excluding them from calculations did not significantly affect fitness estimates.) We measured the *change* in the cost of plasmid carriage, Δc, by competing PET354 (Ara^-_0[Nal^r]/pB15; Table 1) versus an isogenic strain (PET319) carrying an evolved plasmid (Turner et al. 1998). We estimated fitness of the plasmid-bearing strain relative to PET354, and subtracted the estimate from 1.0 to calculate Δc; $\Delta c > 0$ indicated an evolved plasmid increased its cost compared to the ancestor plasmid, whereas $\Delta c < 0$ indicated an evolved plasmid decreased in cost (or was beneficial if $c + \Delta c < 0$).

ACQUISITION OF SEGREGANT GENOTYPES

To isolate a spontaneous segregant, we grew a plasmid-bearing strain in DM + 1000 μg/mL glucose for 24 h, and plated a diluted sample on TA agar to obtain individual colonies. We used replica plating (Sambrook and Russell 2001) from TA onto TA + 30 μg/mL Kan to identify Kan-sensitive segregants; we then confirmed a purported segregant by sterile tooth-picking onto both plate types.

We used two protocols to induce plasmid segregation because replica plating sometimes failed to yield segregants needed to create transconjugant strains. The Thymine Protocol of Trevors (1986) induces segregation by restricting thymine availability to cells. We grew a single colony for 24 h in DM + 1000 μg/mL glucose containing 4 μg/mL thymine. We then transferred a 0.1 mL sample into 10 mL of thymine-free medium for 24-h growth, and plated a diluted sample on Luria broth (LB) agar plus thymine. The Elevated Temperature Protocol induces segregation through heat shock. We grew a single colony for 24 h in DM + 1000 μg/mL glucose at 43°C, followed by 1:20 serial dilution for additional 24-h growth under identical conditions. Both protocols used replica plating and confirmatory tooth-picking, as described above.

BIOLOG ANALYSES

We sought to determine whether evolved strains had diverged during their evolution, which can be efficiently examined by measuring changes in carbon-source utilization (Cooper and Lenski 2000; Cooper 2002). Biolog plates quantify strain ability to metabolize each of 95 carbon sources via extent of tetrazolium-dye reduction, gauged by OD_{600}. We grew a strain for 24 h in 10 mL static LB culture from frozen stock; we added 25 μg/mL Kan to plasmid-bearing cultures to ensure plasmid retention. We then diluted the culture 1:100 into fresh LB, and repeated the 24-h growth and dilution. We specifically excluded glucose from this process to avoid its effect of repressing many metabolic functions. After 8-h growth to produce a late exponential-phase culture, we centrifuged the culture at 4000 rpm, resuspended the pellet in phosphate-buffered saline (PBS), and repeated the process to achieve a 15 mL PBS resuspension at optical density (OD_{600}) of 0.50. We added the diluted sample to Biolog GN2 plates (Biolog, Hayward, CA) at 150 μL per well.

We analyzed plates using a Tecan Infinite plate reader, obtaining readings at 0, 2, 4, 6, 8, 12, 18, 24, and 48 h; we held plates in a static 37°C incubator in between readings. We estimated relative performance on each carbon source by calculating area under the curve using the trapezoidal approximation (Cooper and Lenski 2000; Cooper 2002). We measured the Biolog profile of each strain three times in complete blocks. We summarized these data as means and coefficients of variation, and used ANOVA to estimate variance within assays (genotype and genotype × block effects) and between assays (blocks). We also estimated the sum of growth on all carbon sources (total growth) for each strain.

SEQUENCING

Sequencing of the ancestral pB15 plasmid used data from several sources. Data from whole genome sequencing of REL5382

containing pB15 were performed at the Yale University Center for Genomics & Proteomics using a Roche/454 Genome Sequencer FLX.

Partial assembly of the pB15 sequence was accomplished by subtracting out the *E. coli* genome sequence and assembling contigs using the GS de novo Assembler Application version 2.3 (Roche, Branford, CT). These data were later combined with additional REL5382/pB15 genomic sequence in the form of 80 base-paired end reads from an Illumina Solexa Genome Analyzer IIx performed at the Centre for the Analysis of Genome Evolution and Function (CAGEF) at the University of Toronto. The final assembly of the pB15 ancestral sequence was done by hand and verified by sequence data generated by primer walking and sequence from a clone library of pB15 using Sanger dideoxy sequencing on an Applied Biosystems (Foster City, CA) 3730xl DNA Genetic Analyzer at the Yale DNA Analysis Facility. The evolved nonconjugative plasmid in PET309 was also sequenced at CAGEF on their Illumina Solexa Genome Analyzer IIx.

The multiple different sequencing methods were employed because of difficulty in assembling the pB15 genome. This was primarily due to the Tn5/Tn5393 transposon cluster containing multiple tandem and inverted repeats, and the multiple configurations of the active shufflon region in the genomic DNA preparations. It has been our experience that multiple shufflon arrangements in template DNA will prevent successful PCR amplification of the shufflon region, which significantly complicates sequencing these regions.

PLASMID COPY NUMBER ESTIMATION

Plasmid copy number per chromosome was estimated via simplex Real-Time PCR by calculating the copy number of the plasmid kanamycin-resistance gene (*aph*) relative to the chromosomal housekeeping gene encoding isocitrate dehydrogenase (*icd*). The assay was calibrated using *E. coli* strain JW1116–1 (CGSC no. 9047), which had the *aph* gene inserted into its chromosome in very close proximity to *icd*, thus insuring equal copy numbers of both targets. Template DNA was from total DNA preparations made from overnight cultures in DM1000 broth without kanamycin, incubated with shaking at 37°C, using the method of Maloy (1990). The primers for *icd* were as follows: Forward 5′-GCC AGG AAC TGG ATC TCT ACA TCT GC-3′ and reverse 5′-GAA GAT AAC CAT ATC GGT CAG TTC AGG GTG-3′. The primers for *aph* were as follows: forward 5′-GCA GCT GTG CTC GAC GTT GTC-3′ and reverse 5′-GAG CAA GGT GAG ATG ACA GGA GAT CC-3′. Twenty microliters PCR reactions were set up using Fast SYBR Green Master Mix (Applied Biosystems), with each primer at a concentration of 0.3 μM, and ∼0.25 μg of template DNA. Samples were run in an Applied Biosystems 7500 Fast Real-Time PCR System using the following program: Stage 1: 60°C for 1 min; Stage 2: 95°C for 10 min; Stage 3 (40 cycles): Step 1: 95°C for 15 sec, Step 2: 60°C for 1 min. Two reactions were run for each target gene for each sample on the same plate. The experiment was then replicated on a second plate, so each target gene for each sample was assayed four times. Relative plasmid copy numbers were calculated using the comparative C_T method. The copy number is reported as the average of the ratios of the C_T values for the plasmid and chromosomal sequences, normalized to the average ratio of the target sequences in the calibrator sample (JW1116–1). The relative efficiency of the two PCR reactions was very similar, and the measured relative copy number of the *aph* to *icd* genes in the calibrator sample was 1.013 ± 0.021.

SHUFFLON CONFIGURATION AND *tetA* GENE SCREEN

A PCR screen was developed to determine which of the seven shufflon segments was fused to the *pilV* gene in each evolved plasmid. Seven PCR reactions were run for each plasmid (one for each shufflon segment), using the same template DNA as was used for the plasmid copy number determination. The same pilV forward primer (5′-CGG TCA GGT AAA AGG CAA TAG CGG-3′) was used for each reaction, in conjunction with a reverse primer specific to each shufflon segment (A, A', B, B', C, C', and D'). The sequences of the shufflon-specific primers were as follows: A: 5′-GGT TCA CAT AGA GGT TCA TTC TCA GGG C-3′, A': 5′-GCA ATT CGA CTT CTG TGC CAT TGC C-3′, B: 5′-GTA TTG ACG GGC GTG AAA TGG CAG-3′, B': 5′-CAG CAC AGG CGA CAG GTT GGC-3′, C: 5′-ATA TTG CTC ATG GAC TTA CCT GAA TGG C-3′, C': 5′-ACT TTA TCG GCG GTA AGA GTG GTG G-3′, and D': 5′-TTC TGG TAG TAC GGT GAT AAC TGG TCG-3′. Fifty microliters PCR reactions were prepared with a final concentration of 0.3 μM each of forward and reverse primer, 3.5 mM $MgCl_2$, 0.2 mM of each dNTP, ∼0.3 μg template DNA, and 1.25 U GoTaq DNA Polymerase (Promega, Fitchburg, WI). The reactions were run using the following cycling protocol: 95°C for 5 min, then 30 cycles of 30 sec at 95°C, 30 sec at 58°C, and 1 min at 72°C with a final incubation of 5 min at 72°C.

Additionally a PCR screen was used to determine the presence/absence of the *tetA* tetracycline-resistance gene. The primers used for the screen were as follows: forward 5′-GAC TAC GCG ATC ATG GCG ACC-3′, and reverse 5′-GCT CAT GAG CCC GAA GTG GC-3′. The PCR reactions and cycling protocol were the same as listed above with the exception of the annealing temperature for the *tetA* screen was 56°C. The results of both screens were visualized via agarose gel electrophoresis stained with EtBr.

Results and Discussion

ANCESTRAL TRAITS

To gauge whether pB15 conferred resistance to 24 antibiotics, we conducted replicated ($n = 2$) preliminary KBDD assays

(see Methods) comparing Ara$^{-}_{0}$/pB15 and Ara$^{-}_{0}$ (control). Data (Table 1) showed that pB15 conferred full resistance to kanamycin (Kan) and neomycin (Nm), and intermediate resistance to tetracycline (Tet); we did not observe previously reported pB15 resistance to ampicillin (Lundquist and Levin 1986). The assays also confirmed full resistance of Ara$^{-}_{0}$ to streptomycin (Sm), a marker on the *E. coli* B chromosome (Souza et al. 1997); assays with pB15 in a Sm-sensitive host confirmed the marker was also on the plasmid. Transfer rates and relative fitness assays (see Methods) confirmed Ara$^{-}_{0}$/pB15 transfer rates reported earlier (Table 1; Turner et al. 1998; Turner 2004).

EVOLUTION OF QUANTITATIVE PLASMID TRAITS

Resistance variation

To examine whether evolved strains underwent changes in antibiotic resistance, we used replicated ($n = 3$) KBDD assays to quantify resistance of a representative plasmid-bearing genotype isolated from each treatment (H, M, L) and control (C) population at generation 500. As a control, alongside these assays we also re-assayed ($n = 4$) resistance in strains Ara$^{-}_{0}$/pB15 and Ara$^{-}_{0}$. Population M1 was ~1:1 polymorphic for Tet-resistant and sensitive genotypes (Turner et al. 1998), and population C2 showed a minority subpopulation of Tet-sensitive plasmids within "leaky" Ara-marked hosts (i.e., Ara$^{+/-}$; pink colonies on TA agar). Thus, we examined 14 total plasmid/host associations from the 12 evolved populations (Table 1).

Treatment populations occasionally experienced Kan selection to ensure plasmid maintenance, whereas C lineages did not (Turner et al. 1998). KBDD results showed that all evolved plasmid-bearing strains retained maximal Kan and Sm resistance (no zone of inhibition), with no among-replicate variability (data not shown). Thus, Kan selection during experimental evolution did not affect Kan resistance.

In contrast, we observed variable resistance to Tet and Nm in evolved plasmids. In KBDD assays for Tet, the zone of inhibition (mm diameter) defines full resistance ≤14 mm, intermediate resistance 15–18 mm, and susceptibility ≥19 mm (NCCLS 2003). Whereas the ancestor Ara$^{-}_{0}$/pB15 had intermediate resistance, six of the 14 evolved associations had become Tet susceptible, and one (L3) was fully resistant (Table 1). KBDD assays define Nm full resistance ≤12 mm, intermediate resistance 13–16 mm, and susceptibility ≥17 mm (NCCLS 2003). The ancestor had intermediate resistance to Nm, and nine out of 14 evolved associations remained intermediate; however, five were categorized fully resistant or borderline fully resistant (Table 1). We found no significant correlation between Nm and Tet resistance in evolved strains ($r = -0.313$, $n = 14$, $P = 0.276$), suggesting the traits evolved independently.

E-test strips (see Methods) provide highly accurate measures of an antibiotic's MIC. Consistent with KBDD data, replicated ($n = 3$) E-test assays (Table 1) showed that the ancestor and all evolved strains were maximally resistant to Kan and Sm (MIC > 256 μg/mL), with no among-replicate variation (data not shown). E-tests for Tet defined susceptibility: $MIC_S \leq 4$ μg/mL, intermediate resistance $MIC_I = 5$ μg/mL, and full resistance $MIC_R \geq 8$ μg/mL. E-test data for Tet (Table 1) echoed results from KBDD. E-tests for Nm were not commercially available.

We showed that plasmids evolved measurable differences in subclinical Tet resistance. In contrast, the other two known plasmid-specific resistance traits either did not undergo any measurable change (Kan), or were measurably different but within the values constituting resistance defined as full or intermediate (Nm). Although Kan and Nm resistances are conferred by the same gene (Apirion and Schlessinger 1968), this does not result in the same level of resistance for both antibiotics. Typically the version of the *aph* gene found on Tn5 confers a >fivefold higher resistance level to Kan than to Nm (Melnikov et al. 2014). The MIC for Kan for plasmid pB15 and its evolved derivatives (Table 1) are above the threshold of detection for assays that measure clinically relevant MICs, whereas the MIC values for Nm are still within the detectable range. This difference in sensitivity explains how we could detect differences in Nm-resistance levels among the evolved plasmids, but could not observe any such differences in Kan-resistance levels, even though both traits are coded by the same *aph* gene. It is possible that variance in Kan resistance would track variance in Nm resistance for the collection of evolved plasmids, if this assay method actually measured the Kan resistance in the relevant range. More importantly, from an evolutionary standpoint, it is plausible that high levels of Kan and Nm resistance were maintained because they were not expected to be strongly selected against. It is possible (and often assumed) that bacteria evolved in the absence of antibiotic selection may decrease, or altogether lose, expression of resistance; e.g., Godwin and Slater (1979) showed chemostat-evolved conjugative plasmids lose resistance at unselected loci when cultured in absence of antibiotic. Instead, we suggest that resistance to Kan and Nm in our experimental populations was maintained because these resistance traits are "cheap"; they occur through an enzyme that modifies or deactivates the drug, and the cell does not have to consume ATP to be resistant. (Also, we note that evolution of reduced resistance [or its complete disappearance] can occur at unselected loci through effects of genetic drift, but that this process was unlikely in our study due to its relatively short duration [500 bacterial generations].)

In contrast, Tet resistance is achieved through an efflux pump mechanism, making it relatively more costly for the cell because ATP must be consumed to pump out the drug when it continuously reenters the cell from the extracellular environment. However, we

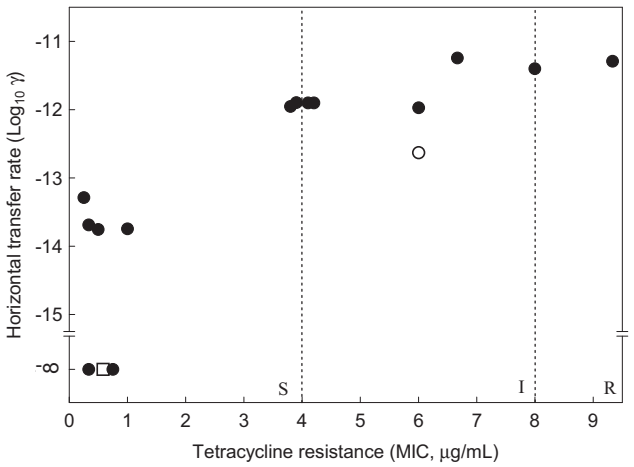

Figure 1. Genetic correlation between horizontal transmission rate and tetracycline resistance in pB15 and its evolved derivatives. Filled circles are 14 evolved plasmids, open circle is the ancestral plasmid, and open square is the plasmid-free bacterial host; conjugation ability below the limit of detection is indicated by rate equal to negative infinity ($-\infty$). Vertical dashed lines indicate levels of minimum inhibitory concentration (MIC) for susceptibility (S), intermediate resistance (I), and resistance (R) to tetracycline.

noted that Tet resistance in the ancestral and evolved plasmids was at a very low level, which was generally subclinical. Because resistance to Tet was not a component of the selective environment, we believe that the evolved changes in Tet resistance had to do with its particular association with plasmid transmission traits, which we further examined below.

Phenotypic correlations between plasmid traits

To examine changes in $\log_{10}$ conjugation rate (γ; see Methods) for the evolved plasmid/host associations, we conducted replicated ($n = 3$) conjugation rate assays. Results (Table 1) confirmed earlier observations (Turner et al. 1998) of variability among treatment-evolved plasmids, including transfer rates faster and slower than the ancestor, and measurably undetectable conjugation. Control-evolved plasmids were also variable, but all retained conjugation ability (Table 1).

Figure 1 shows a positive relationship between mean transfer ability and mean Tet resistance (E-test data); data from evolved plasmids that retained conjugation showed a highly significant positive correlation between these quantitative traits ($r = 0.916$, $n = 12$, $P < 0.0001$). Similarly, we observed a negative correlation between $\log_{10} \gamma$ and size of KBDD-assayed zone of inhibition (i.e., Tet sensitivity) in these genotypes ($r = -0.798$, $n = 12$, $P = 0.002$). Because the results closely agreed, we justifiably examined the relationship between $\log_{10} \gamma$ and Nm-resistance assayed via KBDD alone; these traits were uncorrelated ($r = 0.523$, $n = 12$, $P = 0.081$; Fig. S1). We concluded that horizontal transfer and Tet resistance were tightly associated through genetics, whereas other known resistance loci (Kan, Sm, Nm) seemed unassociated with conjugation ability.

Turner et al. (1998) observed a trade-off between transmission modes in treatment-evolved plasmids because increased conjugation rate coincided with reduced vertical transfer (revealed through measuring Δc, see Methods); these Δc data are reprinted in Table 1. We measured Δc for control-evolved plasmids with replication ($n = 4$), and observed a qualitative similarity to earlier results; the three plasmids with higher conjugation rates were more costly than the ancestor, whereas the plasmid with lower $\log_{10} \gamma$ was less costly (Table 1). Again, using data only from evolved conjugative plasmids, the phenotypic correlation between $\log_{10} \gamma$ and Δc was highly significant ($r = 0.845$, $n = 12$, $P = 0.001$). Last, we found that Δc was highly correlated with Tet resistance in these evolved plasmids (E-test data: $r = 0.774$, $n = 12$, $P = 0.003$; KBDD data: $r = -0.809$, $n = 12$, $P = 0.001$).

Overall, these data showed that experimental evolution caused plasmids to genetically diverge into two distinct groups: relatively virulent plasmids (high conjugation, low vertical transfer, Tet resistance) and relatively avirulent plasmids (low/immeasurable conjugation, high vertical transfer, Tet sensitivity).

TESTING DIVERGENCE AMONG EVOLVED HOST/PLASMID ASSOCIATIONS AND AMONG SEGREGANTS

Glucose is the preferred carbohydrate for *E. coli* and multiple regulatory mechanisms ensure that the sugar is utilized prior to all other carbohydrates (Postma 1993). Although maltose is diglucose, it is much less preferred and its utilization seems to exert little regulatory control catabolism of other carbohydrates (Saier 1987). Travisano (1997) showed that these two sugar environments are sufficiently different that *E. coli* adaptation to glucose does not necessarily carry over to improved performance in maltose; thus, fitness assays using the two sugars provide a convenient method for gauging divergence among bacterial strains.

Performance in glucose and maltose environments

To examine whether plasmid-level and cell-level divergence coincided, we first measured ($n = 5$) fitness of evolved plasmid-bearing clones relative to a common ancestral competitor ($Ara^-{}_0$/pB15 or $Ara^+{}_0$/pB15) in two habitats: the evolutionary environment DM + 1000 μg/mL glucose, and the alternate habitat DM + 1000 μg/mL maltose (preliminary assays confirmed the common competitors were equally fit in the two environments; data not shown). Because treatment and control populations showed equivalent correlations between quantitative plasmid traits (Table 1), we restricted the following analysis to the 10 treatment-evolved strains.

Results showed that the 10 strains improved equally in fitness on glucose (Table S1, first column) and on maltose (Table S2, second column), despite treatment differences in imposed immigration. A *t*-test for paired comparisons indicated that the difference in extent of adaptation to these two sugars was not significant (Table S1, third column). Therefore, the evolved plasmid/host associations, as a group, adapted as well to high-glucose medium as they had to the unselected high-maltose medium.

Lack of evidence for phenotypic divergence among evolved associations

After the method of Travisano (1997), we used analyses of variance to examine statistical significance of the among-clone genetic variation, estimated as the difference in the group and error mean squares, divided by the number of replicate assays performed for each group (see Sokal and Rohlf 1981). These tests showed no statistically significant genetic variation for fitness among the 10 clones in glucose medium, and in maltose medium (for complete description, see Supporting Information). This correlated response to selection argued that the 10 host/plasmid associations did not diverge, despite extensive divergence of their resident plasmids.

Acquisition of PF segregants

To separately examine evolutionary changes in plasmids versus those in host chromosomes, we attempted to repeat the glucose/maltose fitness analyses using PF segregants, but found an interesting result. We readily obtained spontaneous segregants of the ancestor, $Ara^{-}{}_{0}$/pB15, via replica plating between nonselective (TA) and selective agar (TA with 25 μg/mL Kan); e.g., typically $\sim 10^{-2}$ spontaneous-segregant frequencies. In contrast, evolved associations showed orders of magnitude reduced spontaneous segregation; e.g., strain PET298 (clone $C2^{-}$) had $\sim 10^{-3}$ spontaneous-segregant frequencies, and we were unable to find segregants of other evolved strains ($<10^{-3}$) despite extensive searching efforts.

We therefore used other methods (thymine starvation, elevated temperature; see Methods) to attempt improved acquisition of segregants. Thymine starvation proved highly effective for obtaining segregants of $Ara^{-}{}_{0}$/pB15; e.g., one experiment showed 314 segregants in 321 colonies screened (98% segregants). However, the method only slightly improved segregant acquisition from evolved host/plasmid associations, compared to replica plating. Five of the 10 evolved clones used in above fitness analyses yielded the following low percent segregants per colonies screened: M1r, 0.12%; M2, 0.27%; H1, 0.56%; H2, 0.27%; H3, 0.22%. Elevated temperature completely failed for obtaining segregants of pB15 and evolved plasmids.

Our difficulty in obtaining segregants suggested that one of the targets for plasmid evolution might have been increased stability in host cells. This change could occur through evolution of higher copy number. To examine this possibility, we measured ($n = 4$) plasmid copy number (see Methods) for each of the ancestral and evolved strains with replication. Results showed that the mean copy number per chromosome was highly similar among the strains, ranging between 0.795 and 0.942 copies per cell (Table 1). That is, all of the ancestral and evolved strains showed roughly one plasmid copy per cell, and we found no evidence that a change in this trait likely explained the sometimes substantial changes in Tet resistance among plasmids (Table 1), such as due to evolution of multiple plasmid copies per cell where multiple resistance genes might explain greater resistance to Tet.

Sequence analysis of the ancestral plasmid revealed the existence of both a ParAB plasmid partitioning system and genes homologous to the RelE PSK plasmid stability system (Fig. S2). Under this type of PSK system, the segregant daughter cell inherits a plasmid-encoded toxin in its cytoplasm, but fails to inherit the plasmid that carries a gene needed to manufacture the antitoxin; thus, plasmid-stability mechanisms generally boost the likelihood that plasmids function as very stable mini-chromosomes. Improvements in any one or more of these systems would decrease the segregation rate, and we have yet to determine the mechanism of the observed increased stability.

Lack of evidence for divergence among segregants

Due to the generalized difficulty in obtaining PF segregants, the following analysis was restricted to only a subset of the 10 evolved strains. But we note that the test group equally represented segregants from virulent and avirulent plasmid types. We measured ($n = 3$) fitness of five evolved segregants relative to the marked ancestor ($Ara^{-}{}_{0}$/pB15 or $Ara^{+}{}_{0}$/pB15) in high-glucose and high-maltose environments. Data showed that segregants did not vary in fitness in glucose, nor in maltose (for complete description, see Supporting Information). We conservatively concluded that the host bacteria in our study did not diverge, despite distinct genetic changes that occurred on their resident plasmid and the chromosome.

Metabolic profiling of host/plasmid genotypes

We measured growth of each genotype on 95 different carbon sources using Biolog plates to examine whether changes in the physiology of the host/plasmid association coincided with plasmid divergence. Results described in the Supplemental Online Material showed little divergence among evolved strains in their total diet-breadth, although we observed that virulent strains were quantitatively superior to avirulent strains on 11 substrates. Such differences would not meet more rigorous statistical criteria correcting for multiple tests.

Summary of divergence assays

The available data showed that the evolved plasmid/host associations achieved very similar fitness gains in the evolved environment, and in maltose (Table S1), and the obtainable segregants as well. Although we cannot rule out the possibility that the bacterial hosts diverged during our experiment, the available data suggest that they converged evolutionarily. Thus, they may be climbing the same fitness peak in the DM1000 environment. One possibility is that the particular plasmid that dominated each population after 500 generations hitchhiked to fixation alongside its host that was favored by selection, or within a beneficial immigrant that entered the treatment population. Thus, plasmids may sometimes enter adaptive host backgrounds via conjugation, whereas in other cases they are reached via vertical inheritance. This possibility suggests that complicated dynamics could have occurred within our experimental populations, which would be challenging but interesting to mathematically model (e.g., De Gelder et al. 2004). Below we suggest a mechanism whereby host convergence and plasmid divergence could simultaneously evolve.

GENOME OF pB15 REVEALS A SHUFFLON

To infer the mechanism responsible for a correlation between Tet resistance and plasmid transfer, we used a pyro-sequencing approach to obtain the entire genome of ancestral plasmid pB15 (Genbank accession #KM409652; Fig. S2). A BLAST analysis revealed high degrees of sequence similarity to several IncI1 plasmids including the well-characterized R64 and ColIb-P9 plasmids. The *inc* and *repY* genes of pB15 were identical to these two plasmids indicating that it is also in the IncI1 incompatibility group (Sampei et al. 2010). Genome annotation (Fig. S2) showed that pB15, like R64 contained a group of nested insertion sequence (IS) elements and transposons downstream of the *repZ* gene. Although the location and the nested nature of the transposons are the same, the transposons themselves were different with pB15 having a Tn5/Tn5393 cluster (encoding kanamycin/neomycin, bleomycin, and two different streptomycin-resistance elements), in place of the Tn10/Tn6082 group found in R64. Additionally, pB15 contained an IS26-composite transposon conferring tetracycline resistance inserted into the *traE* and *traF* genes. Plasmid R64 is known to contain a shufflon region: A site-specific recombination system consisting of recombination sites and a recombinase gene (Komano 1999). Notably, genetic rearrangements in the plasmid R64 shufflon alter its conjugative ability; recombination changes the structure of the PilV adhesin located at the end of the thin type IV conjugative pilus. The PilV adhesin determines recipient specificity in liquid matings by binding to the lipopolysaccharide of recipient cells (Ishiwa and Komano 2004). Shufflon rearrangement can alter or even eliminate the ability of the thin pilus to bind to recipient cells, greatly reducing the conjugation frequency in liquid media. This reduction should be reversible through further shufflon recombination that restores efficient horizontal transfer.

Genome similarity to plasmid R64 is evidenced by pB15's identifiable shufflon region (Fig. 2). Shufflons by definition can rearrange; thus, the shufflon depicted in Figure 2 is only one of many possible configurations in plasmid pB15. Like the shufflon in R64, the pB15 shufflon has seven alternate C-terminal segments that can be switched onto the PilV adhesin. Not only is the structure very similar to the shufflon of R64, but the segments themselves share 95–98% sequence identity with the R64 shufflon. And in experiments measuring the conjugation efficiency of different shufflon segments for R64, it was determined that only the A' segment was able to perform a liquid mating to *E. coli* B-recipient cells (Komano et al. 1994). This is consistent with our observation that the A' segment was the only active shufflon segment detected when sequencing the ancestral pB15 plasmid.

MOLECULAR EVIDENCE TO EXPLAIN CORRELATED RESISTANCE AND TRANSMISSION

Differences in shufflon configuration among evolved plasmids

Presence of a shufflon in the pB15 ancestor offers a mechanism for how the evolved plasmids show differential conjugation rates. Presumably, during their evolution these plasmids diverged in shufflon configuration, such that by the end of the experimental evolution the treatment populations were dominated by variants with differing conjugation abilities. To examine this idea, we performed shufflon configuration screens (see Methods) for the ancestral and evolved plasmid-bearing strains. Results (Fig. 3A) showed that the shufflon in ancestral plasmid pB15 is active, yielding amplicons for all seven known shufflon configurations. Similarly, all eight of the evolved plasmids categorized as "virulent" (high conjugation, low vertical transfer, Tet resistance) showed shufflon activity equivalent to the ancestor (data not shown). In contrast, all six evolved plasmids that demonstrated a reduced conjugative ability and were categorized as "avirulent" (low conjugation, high vertical transfer, Tet sensitivity) presented a very different result. We observed that none of these plasmids gave a positive result for any shufflon configuration, indicating that these plasmids lacked a functional thin pilus. Additionally, these plasmids were found to have also lost the relatively closely linked *tetA* gene that confers resistance to tetracycline (Fig. 3B). Four of these plasmids (PET299, PET313, PET315, and PET316) retained the ability to conjugate, so the deletions in these plasmids must be limited to thin pilus formation and the *traEF* region and do not extend into the genes responsible for thick pilus formation or DNA transfer. The two plasmids that had completely lost conjugation ability would not be as constrained as to the extent of the sequence they deleted. Although complete sequence characterization of the evolved plasmids in our collection lies beyond

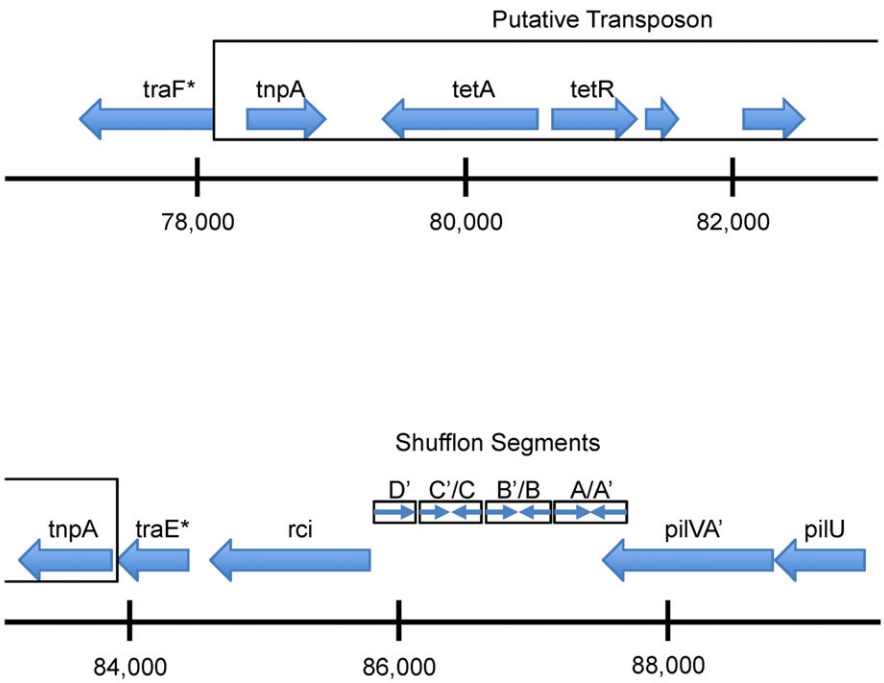

Figure 2. **Gene organization of the shufflon region of plasmid pB15. The *pilV* gene encodes the adhesin subunit of the type IV pilus. The shufflon consists of four segments, three of which are reversible and encode a total of seven alternate C-terminal domains for the PilV adhesin. The *rci* gene encodes the shufflon's site-specific recombinase. A transposon encoding the tetracycline-resistance cassette is integrated into the *traEFG* operon and displaced portions of both *traE* and *traF* as indicated by the asterisks.**

the scope of the current study, and will be pursued separately (Wertz and Turner, unpublished data), we did sequence one of the evolved descendants of pB15 that had lost its conjugative ability (PET309) to determine its genotype. This revealed that the loss of conjugation ability was due a large deletion of approximately 85% of its sequence (from base 11,381 to 98,973 in the ancestral sequence). This deletion removed virtually all of the transfer genes including the shufflon and the transposon containing the tetracycline-resistance genes, but the kanamycin resistant Tn5 transposon was retained. This dramatic reduction in size might explain the reduced cost of carriage of the evolved plasmid (Table 1).

Proposed mechanism for correlated resistance and transmission

The sequence data also showed that plasmid pB15 contained seemingly intact transposons (Fig. S2), including a transposon harboring *tetA* and *tetR* genes for Tet resistance (Fig. 2). Furthermore, this transposon was integrated into the *traEFG* operon replacing and deleting portions of the *traE* and *traF* genes. The function of the *traEFG* genes is not yet known, but it has been determined that they are not required for successful conjugative transfer (Komano et al. 2000). The location of the transposon puts the *tetA* gene in the proper orientation to be transcribed by the upstream *traE* promoter. This suggested that induction of the *traEFG* operon during conjugation would result in increased expression of Tet resistance, which would explain why conjugative transfer and Tet resistance were correlated traits in our study system.

Based on the current evidence, we offer the following explanations for the molecular mechanisms underlying the combined trait differences (resistance, transmission) among the three groups of evolved plasmids in our study: nonconjugative avirulent, conjugative avirulent, and virulent.

First, the two nonconjugative avirulent plasmids experienced large deletions, including transfer genes and Tet-resistance genes. We presume that these genetic changes likely decreased their cost of carriage. However, we noted that the plasmids maintained their partition system and their PSK capabilities, which contributes to their stability in absence of positive selection for plasmid maintenance. Despite such stability mechanisms, nonconjugative plasmids may be lost from a bacterial population when spontaneous PF segregants experience a relative growth advantage. This vulnerability to loss may explain why nonconjugative avirulent plasmids were not found in control populations passaged in absence of periodic Kan selection (Table 1). That is, the two nonconjugative avirulent plasmids retained Kan resistance, which constituted a beneficial gene for the host bacteria in the treatment populations experiencing regular Kan selection. We concluded that this evolved avirulent plasmid strategy was only possible in the presence of Kan selection.

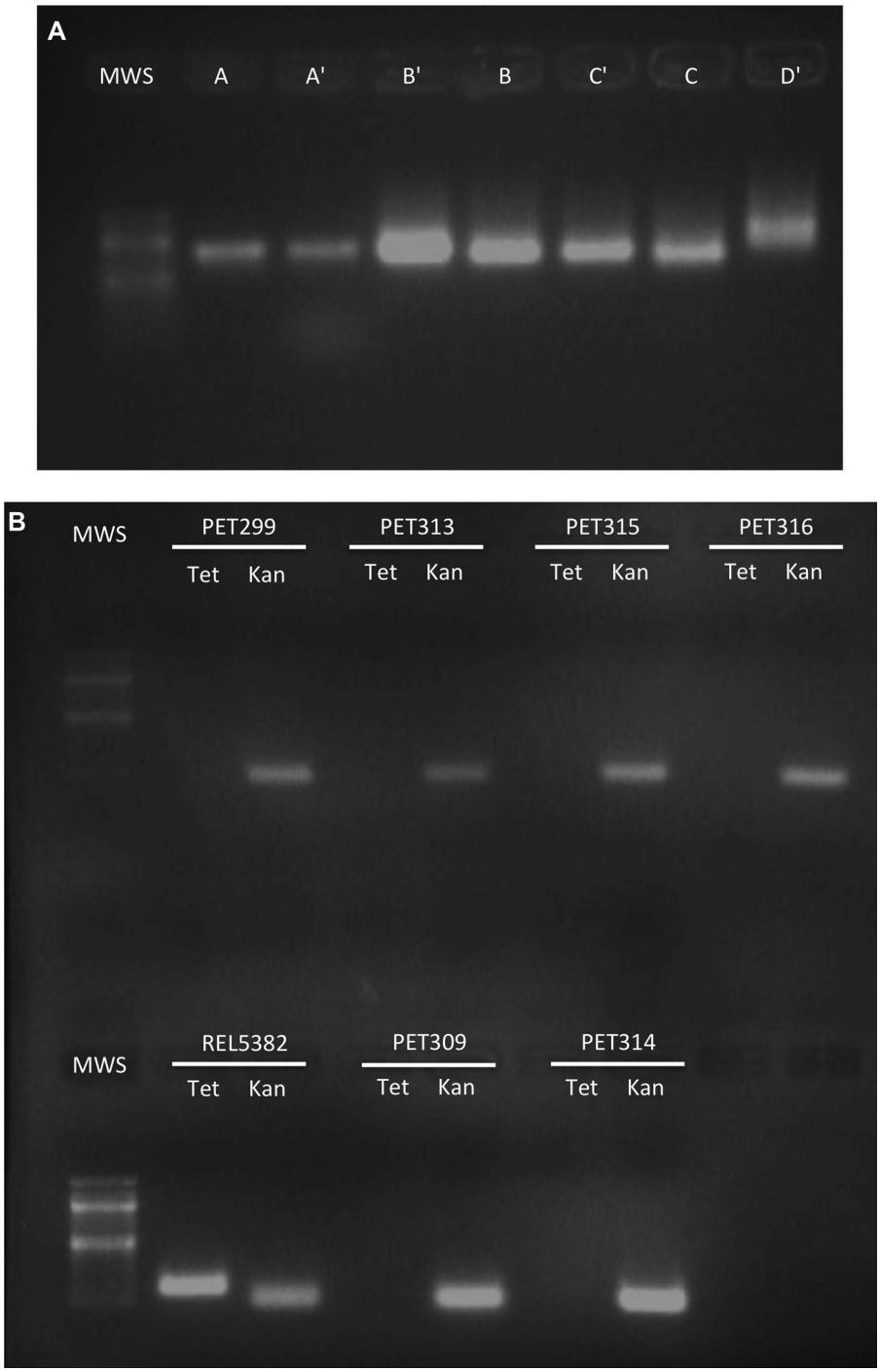

Figure 3. **Shufflon configuration and *tetA* PCR screen. (A) Results of the shufflon configuration screen for strain REL5382 containing the ancestral pB15 plasmid. Lanes are identified according to the shufflon segment fused to the *pilV* gene. (B) Results of the PCR screen for the *tetA* gene (lanes marked Tet), for the ancestral plasmid (REL5382) and all plasmids with a reduced conjugation rate. Lanes marked Kan are PCR reactions using the same *aph* primers used for the plasmid copy number RT-PCR assay and are included as a positive control. The molecular weight standard (MWS) for both gels is a 100 bp DNA ladder (NEB N3231).**

Second, it is likely that the conjugative avirulent plasmids improved their fitness by reducing their cost of carriage while maintaining significant conjugative ability. This could have been accomplished by a single deletion event that removed the unselected Tet-resistance gene as well as one or more of the genes in the *pil* operon, encoding the thin pilus. Apparently the increase in vertical transmission was more than sufficient to offset the reduction in horizontal transmission, relative to the ancestral pB15 plasmid. Moreover, we hypothesize that the lowered cost of carriage should reduce the competitive advantage of spontaneous

PF segregants, and that the moderately rapid conjugation rate of these plasmids may be sufficient to re-infect segregants, further decreasing the possibility for segregant invasion. We concluded that the conjugative avirulent plasmid strategy should be favored by selection, even in the absence of periodic Kan selection, explaining why this strategy evolved in both control and treatment populations (Table 1).

Third, we observed that the evolved virulent plasmids had active shufflons. This result suggested that a fraction of the plasmids in these populations should suffer reduced or ineffective conjugation at any time due to the particular shufflon configuration they harbored. In essence, this should constitute a "burden" on the resident plasmid population, in the sense that the plasmids with the incompatible shufflon configuration would be at a selective disadvantage relative to a plasmid with no thin pilus due to the cost of pilus production. Therefore, to compensate for this perpetual burden, we infer that the virulent plasmids evolved an upregulated conjugation system. Because Tet resistance and transmission are coupled genetically, this upregulation may explain why the virulent plasmids show higher levels of Tet resistance than the ancestor (Table 1). This evolved increase in horizontal transfer at the expense of reduced vertical transfer, also resulted in a higher fitness than the ancestral plasmid.

Last, the group of evolved virulent plasmids suggested that selection for increased transfer could simultaneously lead to evolution of increased Tet resistance. It is thus plausible that selection for antibiotic resistance may lead to selection for increased plasmid transfer. This outcome could be an overlooked consequence of the selection imposed by widespread antibiotic use—the popularity of antibiotic treatment may have produced strong selection for plasmids to transfer more rapidly, which may have exacerbated this ongoing biomedical problem.

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DATA ARCHIVING

The doi for our data is 10.5061/dryad.13f18.

LITERATURE CITED

Apirion, D., and D. Schlessinger. 1968. Coresistance to neomycin and kanamycin by mutations in an *Escherichia coli* locus that affects ribosomes. J. Bacteriol. 96:768–776.

Bergstrom, C. T., M. Lipsitch, and B. R. Levin. 2000. Natural selection, infectious transfer and the existence conditions for bacterial plasmids. Genetics 155:1505–1519.

Bouma, J. E., and R. E. Lenski. 1988. Evolution of a bacteria/plasmid association. Nature 335:351–352.

Carlton, B. C., and B. J. Brown. 1981. Gene mutation. Pp. 222–242 *in* P. Gerhardt, ed. Manual of methods for general bacteriology. American Society for Microbiology, Washington, DC.

Cooper, V. S. 2002. Long-term experimental evolution in *Escherichia coli*. X. Quantifying the fundamental and realized niche. BMC Evol. Biol. 2:12.

Cooper, V. S., and R. E. Lenski. 2000. The population genetics of ecological specialization in evolving *Escherichia coli* populations. Nature 407:736–739.

De Gelder, L., J. M. Ponciano, Z. Abdo, P. Joyce, L. J. Forney, and E. M. Top. 2004. Combining mathematical models and statistical methods to understand and predict the dynamics of antibiotic-sensitive mutants in a population of resistant bacteria during experimental evolution. Genetics 168:1131–1144.

De Gelder, L., J. J. Williams, J. M. Ponciano, M. Sota, and E. M. Top. 2008. Adaptive plasmid evolution results in host-range expansion of a broad-host-range plasmid. Genetics 178:2179–2190.

Dionisio, F., I. Matic, M. Radman, O. R. Rodrigues, and F. Taddei. 2002. Plasmids spread very fast in heterogeneous bacterial communities. Genetics 162:1525–1532.

Dionisio, F., I. C. Conceicao, A. C. R. Marques, L. Fernandes, and I. Gordo. 2005. The evolution of a conjugative plasmid and its ability to increase bacterial fitness. Biol. Lett. 1:250–252.

Gerdes, K., P. B. Rasmussen, and S. Molin, 1986. Unique type of plasmid maintenance: postsegregational killing of plasmid-free cells. Proc. Natl. Acad. Sci. USA 83:3116–3120.

Godwin, D., and J. H. Slater. 1979. The influence of the growth environment on the stability of a drug resistance plasmid in *Escherichia coli* K12. J. Gen. Microbiol. 111:201–210.

Harrison, E., and M. A. Brockhurst. 2012. Plasmid-mediated horizontal gene transfer is a coevolutionary process. Trends Microbiol. 20:262–267.

Hughes, V. M., and N. Datta. 1983. Conjugative plasmids in bacteria of the 'preantibiotic' era. Nature 302:725–726.

Ishiwa, A., and T. Komano. 2004. PilV adhesins of plasmid R64 thin pili specifically bind to the lipopolysaccharides of recipient cells. J. Mol. Biol. 343:615–625.

Komano, T. 1999. Shufflons: multiple inversion systems and integrons. Ann. Rev. Genet. 33:171–191.

Komano, T., S. R. Kim, T. Yoshida, and T. Nisioka. 1994. DNA rearrangement of the shufflon determines recipient specificity in liquid mating of IncI1 plasmid R64. J. Mol. Biol. 243:6–9.

Komano, T., T. Yoshida, K. Narahara, and N. Furuya. 2000. The transfer region of IncI1 plasmid R64: similarities between R64 tra and legionella icm/dot genes. Mol. Microbiol. 35:1348–1359.

Lenski, R. E., M. R. Rose, S. C. Simpson, and S. C. Tadler. 1991. Long-term experimental evolution in *Escherichia coli*. I. Adaptation and divergence during 2,000 generations. Am. Nat. 138:1315–1341.

Leppla, S. H. 1995. Anthrax toxins. Pp. 543–572 *in* J. Moss, B. Iglewski, M. Vaughn, and A. T. Tu, eds. Bacterial toxins and virulence factors in disease. Marcel Dekker, New York, NY.

Levin, B. R., F. M. Stewart, and L. Chao. 1977. Resource-limited growth, competition, and predation: a model and experimental studies with bacteria and bacteriophage. Am. Nat. 111:3–24.

Levin, S., and D. Pimentel. 1981. Selection of intermediate rates of increase in parasite-host systems. Am. Nat. 117:308–315.

Lundquist, P. E., and B. R. Levin. 1986. Transitory derepression and the maintenance of conjugative plasmids. Genetics 113:483–497.

Maloy, S. R. 1990. Experimental techniques in bacterial genetics. Jones and Bartlett Publishers, Inc., Boston, MA.

May, R. M., and R. M. Anderson. 1983. Epidemiology and genetics in the coevolution of parasites and hosts. Proc. R. Soc. Lond. B 219:281–313.

Melnikov, A., P. Rogov, L. Wang, A. Gnirke, and T. S. Mikkelsen. 2014. Comprehensive mutational scanning of a kinase in vivo reveals substrate–dependent fitness landscapes. Nucleic Acids Res. 42:e112.

NCCLS. 2003. Disk diffusion supplemental tables, NCCLS document M100-S13 (M2). National Committee for Clinical Laboratory Standards, Wayne, PA.

Postma, P. W. 1993. Phosphoenolpyruvate: carbohydrate phosphotransferase systems of bacteria. Microbiol. Rev. 57:543–594.

Riley, M. A., and J. E. Wertz. 2002. Bacteriocins: evolution, ecology, and application. Ann. Rev. Microbiol. 56:117–137.

Saier, M. H. 1987. Enzymes in metabolic pathways. Harper and Row, New York.

Sambrook, J., and D. W. Russell. 2001. Molecular cloning: a laboratory manual. 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sampei, G., N. Furuya, K. Tachibana, Y. Saitou, T. Suzuki, K. Mizobuchi, and T. Komano. 2010. Complete genome sequence of the incompatibility group I1 plasmid R64. Plasmid 64:92–103.

San Millan, A., K. Heilbron, and R. C. MacLean. 2014. Positive epistasis between co-infecting plasmids promotes plasmid survival in bacterial populations. ISME J. 8:601–612.

Simonsen, L., D. M. Gordon, F. M. Stewart, and B. R. Levin. 1990. Estimating the rate of plasmid transfer: an end-point method. J. Gen. Microbiol. 136:2319–2325.

Sokal, R. R., and F. J. Rohlf. 1981. Biometry. 2nd ed. Freeman, San Francisco.

Souza, V., P. E. Turner, and R. E. Lenski. 1997. Long–term experimental evolution in *Escherichia coli*. V. Effects of recombination with immigrant genotypes on the rate of bacterial evolution. J. Evol. Biol. 10: 743–769.

Stewart, F. M., and B. R. Levin. 1977. The population biology of bacterial plasmids: a priori conditions for the existence of conjugationally transmitted factors. Genetics 87:209–228.

Svara, F., and D. J. Rankin. 2011. The evolution of plasmid-carried antibiotic resistance. BMC Evol. Biol. 11:130.

Thisted, T., A. K. Nielsen, and K. Gerdes. 1994. Mechanism of post-segregational killing: translation of Hok, SrnB and Pnd mRNAs of plasmids R1, F and R483 is activated by 3'-end processing. EMBO J. 13:1950–1959.

Travisano, M. 1997. Long-term experimental evolution in *Escherichia coli*. VI. Environmental constraints on adaptation and divergence. Genetics 146:471–479.

Trevors, J. T. 1986. Plasmid curing in bacteria. FEMS Microbiol. Lett. 32:149–157.

Turner, P. E. 2004. Phenotypic plasticity in bacterial plasmids. Genetics 167:9–20.

Turner, P. E., V. S. Cooper, and R. E. Lenski. 1998. Tradeoff between horizontal and vertical modes of transmission in bacterial plasmids. Evolution 52:315–329.

Walsh, C. 2003. Antibiotics: actions, origins, resistance. ASM Press, Washington, DC.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. No correlation between horizontal transmission rate and neomycin resistance in pB15 and its evolved derivatives.

Figure S2. Genome map of the ancestral conjugative plasmid pB15.

Table S1. Relative fitness, after 500 generations of selection, of plasmid-bearing clones in their selective environment, high-glucose medium, and in a novel environment, high-maltose medium.

Table S2. Relative fitness of evolved plasmid-free segregants in two environments.