Evolutionary Paths That Expand Plasmid Host-Range: Implications for Spread of Antibiotic Resistance

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Associate editor: Eduardo Rocha

Abstract

The World Health Organization has declared the emergence of antibiotic resistance to be a global threat to human health. Broad-host-range plasmids have a key role in causing this health crisis because they transfer multiple resistance genes to a wide range of bacteria. To limit the spread of antibiotic resistance, we need to gain insight into the mechanisms by which the host range of plasmids evolves. Although initially unstable plasmids have been shown to improve their persistence through evolution of the plasmid, the host, or both, the means by which this occurs are poorly understood. Here, we sought to identify the underlying genetic basis of expanded plasmid host-range and increased persistence of an antibiotic resistance plasmid using a combined experimental-modeling approach that included whole-genome resequencing, molecular genetics and a plasmid population dynamics model. In nine of the ten previously evolved clones, changes in host and plasmid each slightly improved plasmid persistence, but their combination resulted in a much larger improvement, which indicated positive epistasis. The only genetic change in the plasmid was the acquisition of a transposable element from a plasmid native to the *Pseudomonas* host used in these studies. The analysis of genetic deletions showed that the critical genes on this transposon encode a putative toxin–antitoxin (TA) and a cointegrate resolution system. As evolved plasmids were able to persist longer in multiple naïve hosts, acquisition of this transposon also expanded the plasmid's host range, which has important implications for the spread of antibiotic resistance.

Key words: **antibiotic resistance, horizontal gene transfer, broad host range plasmid, Pseudomonas, epistasis, toxin–antitoxin, resolvase, transposon, experimental evolution.**

Introduction

Horizontal gene transfer mediated by mobile genetic elements such as plasmids allows bacterial populations to rapidly adapt to novel environments (Frost et al. 2005). The most alarming example of this is the rapid horizontal spread of antibiotic resistance genes among bacterial pathogens. In the last few decades many pathogens responsible for nosocomial and other infections have become resistant to almost all available antibiotics, hindering the ability to effectively treat serious and sometimes lethal infections (Kåhrström 2013). In particular, species such as *Klebsiella pneumoniae* and *Escherichia coli* are of international concern as they have acquired plasmid-encoded resistance to third-generation cephalosporins and increasingly to carbapenems (WHO 2014; Holt et al. 2015). In many settings within the United States more than half of these Gram-negative bacteria tested have become resistant to common antimicrobials (WHO 2014). Similarly, multiple drug resistance (MDR) in Gram-positive pathogens such as *Staphylococcus aureus* and *Mycobacterium tuberculosis* has become a great global public health concern (WHO 2014). Plasmids are one of the main contributors to this crisis because they replicate separately from the chromosome (Del Solar et al. 1998) and can transfer multiple antibiotic resistance genes all at once by conjugation or mobilization (Smillie et al. 2010; Phan et al. 2015), leading to the creation of MDR pathogens.

Plasmids do not always persist in bacterial populations in the absence of selection for the traits they encode. The

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persistence of a plasmid is promoted by at least four underlying processes: Proper plasmid segregation into daughter cells during cell division (Ebersbach and Gerdes 2005), growth inhibition or killing of plasmid-free cells by means of plasmid-encoded toxin–antitoxin (TA) systems (Hayes 2003), reinfection of plasmid-free cells by conjugative transfer, and a low plasmid cost that prevents plasmid-bearing cells from being rapidly outcompeted by plasmid-free cells (Stewart and Levin 1977; Ponciano et al. 2007). In some hosts the systems required to ensure plasmid persistence may function suboptimally, resulting in plasmid loss in the absence of selection. These hosts are said to be outside the "long-term host range" of that plasmid (De Gelder 2007). Although we and others have demonstrated that the fitness cost of plasmids and their ability to persist in naïve bacterial hosts can evolve under conditions that select for plasmid-encoded traits (Bouma and Lenski 1988;Modi and Adams 1991; Modi et al. 1991; Turner et al. 1998; Dahlberg and Chao 2003; Dionisio et al. 2005; Heuer et al. 2007; De Gelder et al. 2008; Sota et al. 2010; San Milan, Heilbron, et al. 2014; San Milan, Peña-Miller, et al. 2014; Harrison et al. 2015), the underlying molecular mechanisms are still poorly understood. Studies that have attempted to describe the mechanisms of plasmid cost or host range changes point to multiple solutions, so far often involving the replication initiator protein (Maestro et al. 2003; Sota et al. 2010; San Milan, Peña-Miller, et al. 2014; Harrison et al. 2015). Given the alarmingly rapid spread of antibiotic resistance, the evolutionary mechanisms must be determined whether we are to understand how shifts in the host range of antibiotic resistance plasmids affect the dissemination of resistance genes among pathogens.

In a previous study, we evolved a mini-replicon derived from a broad-host-range IncP-1β plasmid under antibiotic selection in *Pseudomonas moraviensis* for 1,000 generations. The mini-replicon includes the origin of replication and its cognate initiator protein, which alone is sufficient for core replication (Durland and Helinski 1987), and other genes required for maintenance and control; it however lacks the two transfer operons and the *parA* gene located in-between them (Sota et al. 2010). By using a conjugation-deficient mini-replicon we could focus on mutations that affect vertical inheritance and persistence of plasmids in the absence of horizontal plasmid transfer. We showed that evolved plasmids were more persistent in their coevolved hosts but not in the ancestral host. At that time, it was not determined whether increased persistence of the plasmid was due to genetic changes in the host alone or epistatic interactions that developed through plasmid–host coevolution (Sota et al. 2010). Here, we report the findings of studies done using a joint experimental-modeling approach to determine the molecular mechanisms that facilitated stabilization of previously evolved plasmids. We demonstrate that acquisition of a transposon-encoded putative TA and cointegrate resolution system in combination with host mutations improved plasmid persistence in the coevolved host, indicative of plasmid–host epistasis. More importantly, this transposition also expanded the plasmid's long-term host range by allowing it to persist in multiple naïve hosts. This study demonstrates for the first time that acquisition of plasmid persistence functions through interplasmid transposition can expand the long-term host range of a plasmid in a single step.

Results

Evolution of Plasmid Persistence

The persistence of plasmids in evolved clones and in permutations constructed by pairing plasmids and hosts from various clones was determined in 10-day plasmid persistence assays as previously described (De Gelder et al. 2007) (see supplementary methods, Supplementary Material online). The results of these assays are time series, and we refer to them as "plasmid persistence profiles."

To quantify changes in plasmid persistence after 1,000 generations of plasmid–host coevolution with antibiotic selection, we determined the plasmid persistence profiles for ten clones from five independently evolved populations of *P. moraviensis* containing plasmid pMS0506. We hereafter refer to these five populations as lineages (L1–L5). A plasmid population dynamics model (De Gelder et al. 2004; Joyce et al. 2005; Ponciano et al. 2007) was used to formulate hypotheses based on the observed trends in the data. In particular, this mechanistic model provided a means to estimate the two main parameters that affect the persistence of a nonconjugative plasmid (supplementary table S1, Supplementary Material online), namely the frequency of plasmid loss λ and the fitness cost σ of plasmid carriage. The model is a discrete time population dynamics system of equations, whose time unit is given in number of generations per day (see Joyce et al. 2005; Ponciano et al. 2007). It basically states that from one generation to the next the number of plasmid-carrying cells is twice the number present in the previous generation minus the fraction (λ) lost due to missegregation. Plasmid-free cells multiply at a rate of $2^{1+\sigma}$, where σ represents the fitness advantage of not carrying the plasmid, thus the plasmid cost. Single values of λ and σ were estimated per plasmid–host pair, and the question whether any two sets of plasmid persistence profiles were similar or distinct was then addressed mathematically by assigning one pair of λ and σ values to the first set of plasmid persistence profiles and another one to the second set. The Bayesian Information Criterion (BIC) was used to compare the likelihood of both dynamics arising under a single set of parameter values to the likelihood that two sets of parameters were needed to explain the data. We denoted as "BICjoint" the BIC statistic value for the models assuming a single dynamic generated two different plasmid persistence profiles, and as "BICsep" the BIC statistic value for the models assuming separate dynamics. Selecting the most likely model among a set of two (or more) models according to the BIC amounts to choosing the model with the lowest BIC score, which is the model that minimizes the error in simulating the biological mechanism generating the data. The difference in scores (BICsep − BICjoint) was expressed as ΔBIC. More negative ΔBIC values were thus indicative of larger differences between plasmid persistence profiles (see Materials and Methods).

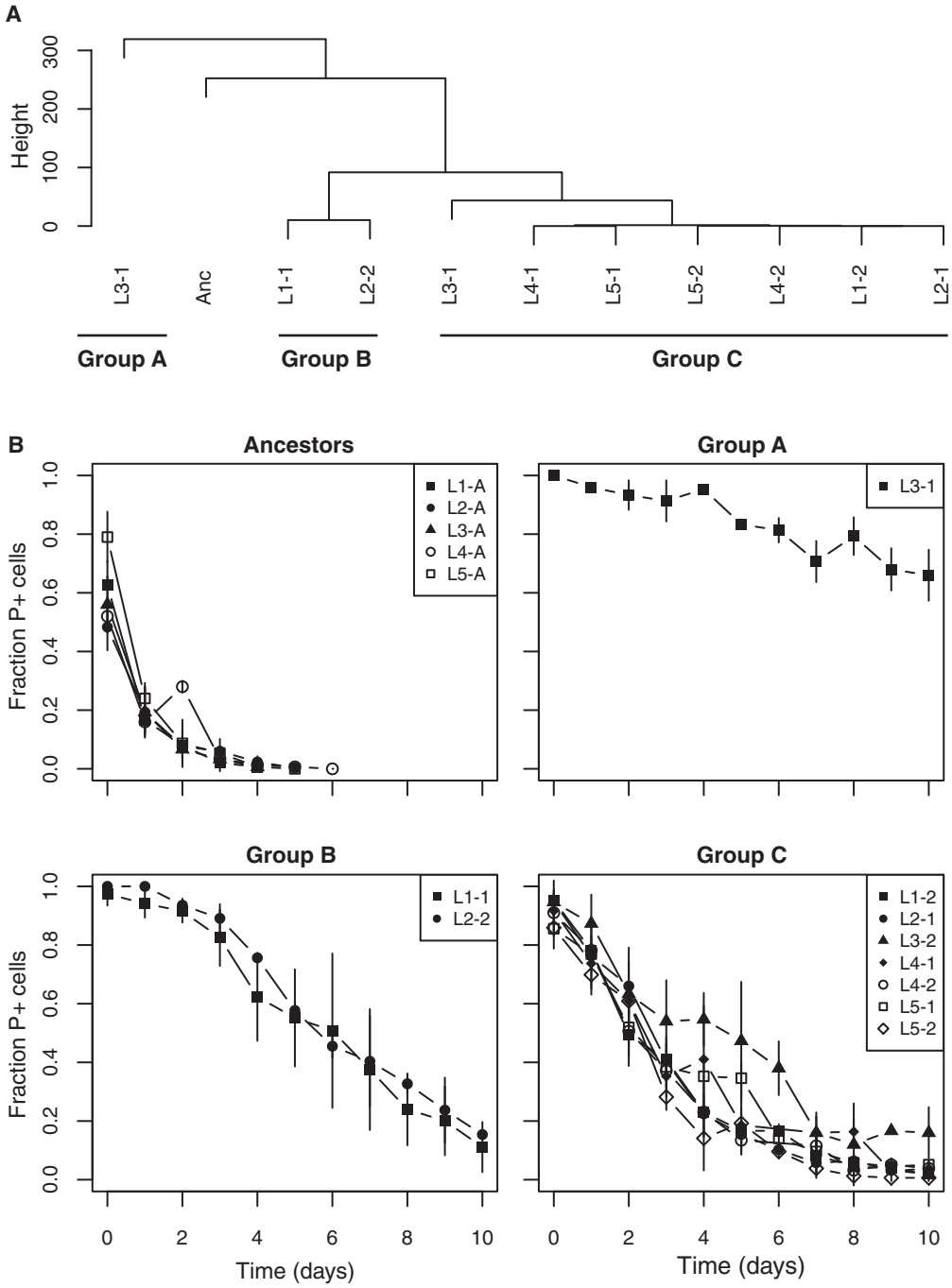

Fig. 1. The plasmid persistence profiles of the evolved clones suggest multiple genetic solutions to improved persistence. Based on single linkage cluster analysis of the pairwise distances in plasmid persistence dynamics as measured by the ΔBIC (*A*), the persistence profiles (*B*) of the ancestral host–plasmid pairs and ten evolved clones from the five replicate lineages (L1–L5) were grouped in four categories (Anc [ancestor], and groups A, B, and C). The number of replicates (*n*) was 6 for L2-A, L3-A and L1-1, and 3 for all other clones. Data points and error bars represent the averages and standard deviations. See Materials and Methods for an explanation of ΔBIC.

All ten evolved clones showed improved plasmid persistence compared with the ancestral plasmid–host pairs (fig. 1). In the ancestors, the cost of plasmid carriage was estimated to be very low and a high frequency of loss explained the low plasmid persistence. The plasmid persistence profiles were clustered into four statistically distinct groups based on all possible pairwise comparisons (fig. 1). The decrease in plasmid loss frequency in clones of groups A and B was approximately an order of magnitude larger than for group C, whereas the fitness costs of plasmid carriage were about the same. Notably, clones from the same lineage did not necessarily cluster together. These data suggest that although a decrease in plasmid loss frequency explained most of the increased persistence, there were multiple genetic solutions within and among populations.

Table 1. Summary of Mutations in the Plasmid pMS0506 and the R28-S Host Genome.

		pMS0506		pR28		Chromosome															
Group[a]	Clone[a]	Tn*6231* Insertion[b]	Tn*6231* OrfF	pR28	Tn*6231* OrfF	Putative *dnaB* Promoter[7]	pyocin R	CapD	FleQ	FliF	Putative Helicase	Vgr	Hypothetical Protein	AtoC Response Regulator	No Annotation	Biotin Synthase	TonB	No Annotation	No Annotation	CheY	Laccase	30S Ribosomal Protein S5	No Annotation
Anc	L1-A	—	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
A	L3-1	v1[b]	v1	+	v1	—	—	v1	v1	—	v1	—	v1	—	v1	—	v1	—	—	v1	—	v1	—
B	L1-1	v2	v2	—	v2	—	—	—	v2	—	—	—	—	—	—	v1	—	—	—	v2	v1	v2	—
B	L2-2	v3	v2	—	v2	—	—	—	—	v1	—	—	—	—	—	—	—	—	—	v3	—	v3	—
C	L1-2	v2	v2	+	v2	—	—	—	v2	—	—	—	—	—	—	v1	—	—	—	v2	v1	v2	—
C	L2-1	v3	—	+	—	—	—	—	—	v1	—	—	—	—	—	—	—	v1	—	v3	—	v3	—
C	L3-2	—	—	+	v1	v1	—	v1	v1	—	—	—	—	—	v1	—	—	v1	—	v1	—	v1	—
C	L4-1	v2	—	+	—	—	—	—	v2	—	—	—	—	—	—	—	—	—	v1	v4	—	v2	v1
C	L4-2	v4	—	+	—	—	—	—	v2	—	—	—	—	—	—	—	—	—	—	v4	—	v2	—
C	L5-1	v5	—	+	—	—	—	—	v3	—	—	v1	—	—	—	—	—	—	—	v5	—	v2	—
C	L5-2	v6	—	+	—	—	v1	—	v4	—	—	—	—	v1	—	—	—	—	—	—	—	v2	—

NOTE—Anc, ancestor; INDEL, insertion–deletion; +, present; —, absent. SNP, single nucleotide polymorphism; all SNPs within coding sequences were nonsynonymous.

[a]Refer to figure 1 for group and clone descriptions.

[b]Insertion of Tn*6231* into pMS0506; different variants represent different insertion sites. See supplementary table S4, Supplementary Material online, for details.

[c]Variant identity (location of mutation within gene).

Coevolution between Plasmid and Host Genome Was Required for Increased Persistence

To determine whether mutations in the plasmid, the host genome, or both accounted for increased plasmid persistence, we focused our analyses on four clones: One each from groups A and B and two from group C. For each clone, we determined plasmid persistence in four plasmid–host pairs: Namely the evolved plasmid (P_E) in either the ancestral (H_A) or evolved (H_E) host and the ancestral plasmid (P_A) in either the ancestral (H_A) or evolved (H_E) host (fig. 2). In the ancestral host each evolved plasmid was only marginally more stable than the ancestral plasmid, suggesting that plasmid evolution alone did not explain the improved persistence in the evolved clones (fig. 2*A*–*D*). Two lines of evidence are consistent with this observation: The model fitting diagnostics as measured by the change in the Bayesian Information Criterion (ΔBIC) and the model predictions. First, the ΔBIC statistic showed that the plasmid persistence profiles of the ancestral host containing an evolved plasmid were more similar to those of the same host with the ancestral plasmid than they were to those of the evolved host with the same evolved plasmid (supplementary table S2, Supplementary Material online; a more negative ΔBIC means a larger difference between two profiles). The second line of evidence came from using the model to predict the amount of time required for 99% plasmid loss, which is reported here as 1% plasmid retention ($T_{1\%}$; supplementary table S3, Supplementary Material online). The Maximum-Likelihood Estimates (MLEs) of $T_{1\%}$ for ancestral hosts with the four evolved plasmids were quite similar to those for the same hosts with ancestral plasmid, but different from the $T_{1\%}$ MLEs for evolved hosts with evolved plasmids. Together these data indicate that evolution of the plasmid alone could not fully account for its markedly improved persistence in the coevolved hosts.

Host evolution alone did also not account for all of the improved plasmid persistence in three of four cases, as the ancestral plasmid was only slightly more stable in the evolved host than in the ancestral host (fig. 2*A*–*C*). This was again supported by comparison of the ΔBIC statistics and the $T_{1\%}$ values for these three clones (supplementary tables S2 and S3, Supplementary Material online). These findings exemplify positive epistasis because mutations in both the plasmid and host were required for the highest persistence. In contrast, mutations in the chromosome alone seemed sufficient to explain the increased persistence of the plasmid from clone L3-2 because the ancestral plasmid was as stable as the evolved plasmid in the evolved host (fig. 2*D* and supplementary tables S2 and S3, Supplementary Material online). These results support the hypothesis that there were at least two evolutionary pathways that resulted in increased plasmid persistence: The coevolution of host and plasmid, and host evolution only.

To understand whether the plasmid and host-encoded mutations truly exemplify positive epistasis in three of the four clones, we compared the magnitude of change in plasmid loss frequency across these three sets of evolved and ancestral plasmid–host permutations (supplementary table

Table 2. Plasmid and Transposon Copy Number in Ancestral and Evolved R28-S Clones.

Group[b]	Clone	Copy number[a]		
		pR28	pMS0506	Tn*6231*
Anc	L1-A	1.9	5.3	1.7
A	L3-1	3.0	9.4	13.9
B	L1-1	Absent	4.8	5.4
B	L2-2	Absent	3.4	3.9
C	L1-2	1.8	3.0	5.3
C	L2-1	3.7	4.7	5.3
C	L3-2	2.3	9.0	2.3
C	L4-1	1.6	4.1	6.7
C	L4-2	2.0	3.9	6.8
C	L5-1	2.3	6.1	9.8
C	L5-2	2.3	3.9	7.3

Note.—Anc, ancestor.

[a]Based on average sequence coverage relative to the chromosome.

[b]Groups as defined in figure 1.

S3, Supplementary Material online). The frequency of plasmid loss decreased 42-fold relative to its ancestor for the most stable evolved clone (L1-1), whereas for the other two clones this decrease in frequency was between 3- and 10-fold. In contrast, the plasmid loss frequencies for the intermediate plasmid–host pairs (H_E*P_A and H_AP_E) did not differ much from those of the ancestral plasmid–host pairs. The loss frequency of the evolved hosts with ancestral plasmid (H_E*P_A) was only 2- to 5-fold lower than that of the ancestral plasmid–host pair, and the loss frequency for the ancestral host with evolved plasmid (H_AP_E) was virtually unchanged from or even slightly higher than that of the ancestral pair. As the products of these relative decreases in loss frequency for H_E*P_A and H_AP_E were lower than the estimated decreases for the three evolved clones H_EP_E, there must be positive epistasis between host and plasmid mutations, indicative of plasmid–host coevolution.

Plasmid Mutations Expand the Host Range

To determine whether the evolved plasmids had also expanded their host range we compared the persistence of the evolved plasmid from clone L1-1 in three naïve hosts, namely the beta-proteobacterium *Cupriavidus necator* JMP228 and two gamma-proteobacteria, *E. coli* BW25113 and *Psuedomonas putida* KT2440 (fig. 3). In all three hosts, the evolved plasmid showed very high persistence whereas the ancestral plasmid was unstable. Thus chromosomal mutations were apparently not required for increased plasmid persistence in these three naïve hosts, which is in stark contrast to the positive epistasis that emerged during the coevolution of the plasmid in *P. moraviensis*. These findings clearly demonstrate that plasmid adaptation to one host can lead to an expanded plasmid host range that might be key to promoting the spread of antibiotic resistance across phylogenetically distinct bacterial populations.

Genetic Changes in the Plasmid Explain Improved Persistence and Host Range Expansion

To identify the genetic basis for the increase in plasmid persistence and expanded host range, the genomes of ten selected evolved clones (fig. 1) and one ancestor were completely sequenced and compared (table 1). These genomes included the *P. moraviensis* chromosome, the non-self-transmissible IncP-9 plasmid pR28 that is indigenous to *P. moraviensis* and our model plasmid pMS0506. The most striking genetic change observed was the transposition of a 7.1-kb transposon from the native plasmid pR28 (Hunter et al. 2014) into pMS0506 in all five lineages. This insertion was confirmed by gel electrophoresis, which showed enlarged plasmids (supplementary fig. S1, Supplementary Material online), and by an increase in transposon copy number (table 2). The transposon was named Tn*6231*. There were no other mutations in any of the plasmids. In lineages L4 and L5, independent transposition events must have occurred as the location of the insertion differed between clones (supplementary table S4, Supplementary Material online). Only in one of the ten clones (L3-2) did plasmid pMS0506 not acquire this transposon, which is consistent with our earlier conclusion that in this clone plasmid persistence improved due to chromosome evolution only (fig. 2). Thus acquisition of the transposon from pR28 played a major role in improving the persistence of pMS0506 and expanding its host range.

Based on sequence similarity Tn*6231* belongs to the Tn*3* family of genetic elements that transpose by a "copy and paste" mechanism and it bears 99% nucleotide identity to Tn*4662* on the *P. putida* HS1 plasmid pDK1 (Yano et al. 2010). Tn*6231* contains eight open-reading frames (fig. 4A) that are likely transcribed from a central divergent promoter region and have high amino acid (aa) identity with the following proteins from Tn*4662*: A transposase (TnpA; 99.8%), a resolvase involved in cointegrate resolution and with putative repressor activity (TnpR; 100%), and six hypothetical proteins OrfA–F (99–100% aa identity for OrfA–E and 85.3% for OrfF). Additional analyses done using Basic Local Alignment Search Tool suggested that OrfA and OrfB form a two-component TA system that has 75% and 70.3% aa identity to a functional Tad/Ata TA system from *Paracoccus aminophilus* (Dziewit et al. 2007). The central divergent promoter region upstream of this TA system is the only identifiable promoter-like region. We expect that autoregulatory activity from the TA system affects expression of other genes on this transposon. Immediately following OrfB of the TA system is a putative transposase repressor (TnpC; 100% aa identity), two proteins without an identifiable function, and OrfF, which showed 100% aa identity to RpfG, a cyclic diGMP phosphodiesterase response regulator. In five of the ten clones we found that a 7-bp indel introduced a frameshift in *orfF*, but this likely did not affect plasmid persistence, as the persistence profile of a clone with mutated *orfF* was similar to that of clones with unchanged *orfF* (fig. 1 and table 1).

We focused our attention on the putative TA system encoded by *orfAB* and the resolvase TnpR because TA

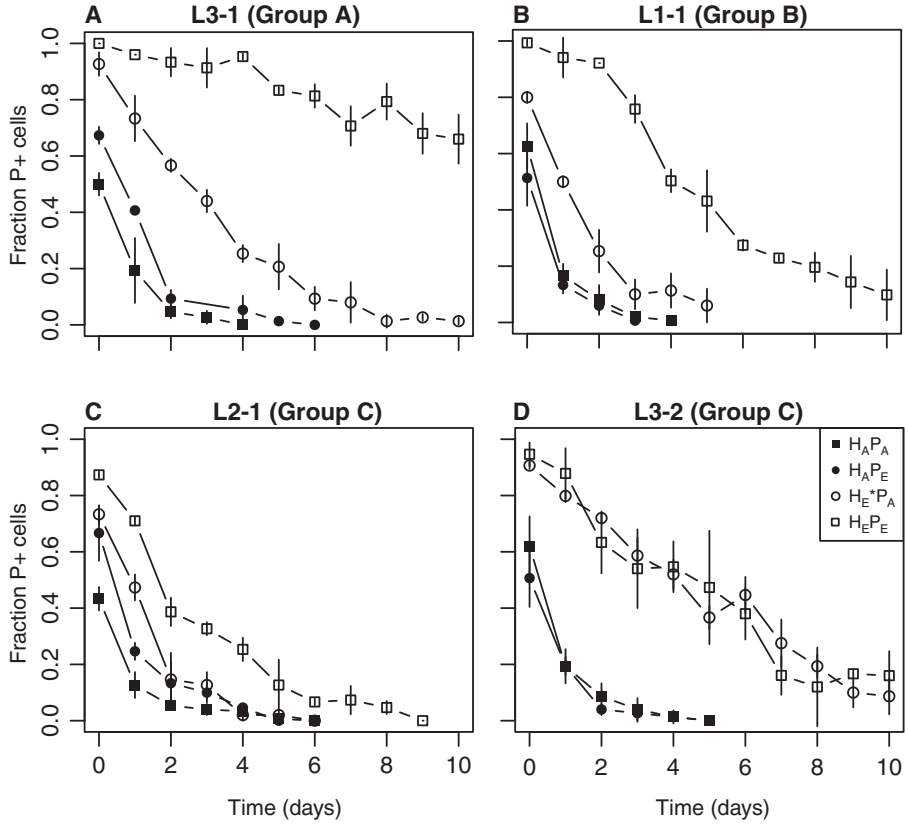

Fig. 2. Plasmid persistence evolved through mutations in plasmid and host, or host only. The persistence profiles of H_AP_E and $H_E{}^*P_A$ compared with H_AP_A and H_EP_E suggest that mutations affecting persistence occurred in both the plasmid and the chromosome (*A*–*C*) or only in the chromosome (*D*). The particular evolved clone used and its persistence profile group are indicated above each figure. H_AP_A, ancestral host and plasmid; H_AP_E, ancestral host with evolved plasmid; $H_E{}^*P_A$, cured evolved host with ancestral plasmid; H_EP_E, evolved host and plasmid. The number of replicates was 6 for L1-1 H_AP_E and 3 for all other clones. Data points and error bars represent the averages and standard deviations.

systems are known to improve plasmid persistence by inhibiting or killing plasmid-free cells (Dziewit et al. 2007) and resolvases probably help resolve plasmid multimers before cell division (Grindley 2002), thus reducing plasmid loss frequency. To test this hypothesis, five deletion mutants were constructed in the evolved plasmid pL1-1 and their effect on plasmid persistence in their coevolved host L1-1 was determined. These deletions in Tn*6231* were the promoter region that also includes the target for the TnpR resolvase, *tnpR*, *orfA–F*, *orfB–F*, and *tnpC–orfF* (fig. 4A). Plasmid persistence was reduced by all the deletions tested except the *tnpC–orfF* deletion. Deletions of the promoter and *orfA–F* had the most significant effects, followed by *orfB–F* (supplementary fig. S2, Supplementary Material online), whereas deletion of the TnpR resolvase only had a minor effect. These results suggest that acquisition of the putative TA system encoded by *orfAB* had a central role in improving the persistence of evolved pMS0506.

We also used the population dynamics model to determine the effects of these deletion mutants on the plasmid loss frequency and cost (fig. 4 and supplementary table S5, Supplementary Material online). Deletion of the promoter on Tn*6231* increased the loss frequency 20-fold while not affecting the cost of plasmid carriage. Similarly, deletion of *orfA–F* resulted in a 12-fold increase in loss frequency, but also incurred a 2.5-fold increase in cost. This supports our tentative conclusion that the TA system accounted for most of the improved persistence of pMS0506 observed in nine of ten evolved clones. It also suggests that deletion of *orfAB* and hence the autoregulatory activity of the TA system at the central promoter region may have increased plasmid cost through increased transcription of *tnpR* and *tnpA* (fig. 4, supplementary fig. S2 and table S5, Supplementary Material online). Consistent with this, deletions of *tnpR* and *orfB–F* each had a greater effect on cost than on loss. We therefore posit that the products of *tnpR* and *orfB* regulated the cost of the acquired transposon, whereas *orfAB* greatly decreased plasmid loss through TA-like activity.

To determine whether the host range expansion was also mostly a result of acquiring the TA system, we compared the persistence of several transposon deletion mutants of pL1-1 in the naïve *E. coli* host BW25113 (fig. 5 and supplementary table S5, Supplementary Material online). Deletion of the central promoter region resulted in greatly decreased plasmid persistence, whereas deletion of *orfA–F* or *tnpR* had a less drastic effect. In contrast, deletion of *tnpC–orfF* had no measurable effect. These results suggest that both the TA system and TnpR were required for complete persistence of our antibiotic resistance plasmid in this naïve *E. coli* host, and thus for expansion of its long-term host range.

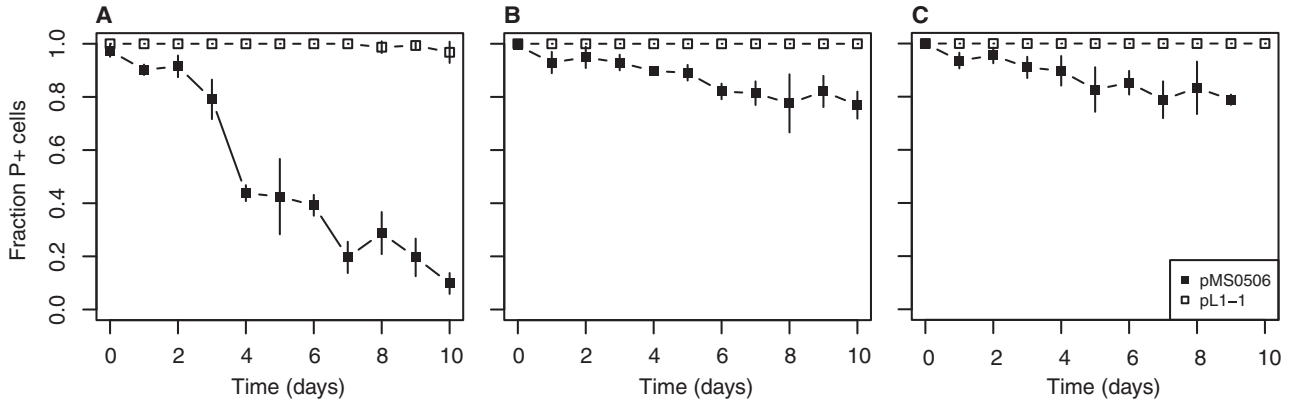

FIG. 3. Acquisition of the transposon by evolved plasmid pL1-1 increased persistence in other gamma- and beta-proteobacteria. (*A*) *Escherichia coli* (ΔBIC = −1,586.745), (*B*) *P. putida* (ΔBIC = −312.58) (both gamma-proteobacteria), and (*C*) *C. necator* (ΔBIC = −555.49; beta-proteobacteria). Data points and error bars represent the averages and standard deviations of three replicates.

To obtain corroborating evidence that the TA system and TnpR played a role in expanding the plasmid's host range, we cloned *orfAB* with or without *tnpR* into a low copy number vector (Gerdes et al. 1985), and the persistence of these constructs in *E. coli* BW25113 was compared with that of the vector only (fig. 6). The construct with *orfAB* was slightly more persistent than the vector alone. This rather small net increase in plasmid persistence appears to be the combined result of a 100-fold lower loss frequency that was counteracted by a 20-fold increase in cost (supplementary table S5, Supplementary Material online). Addition of *tnpR* did not further enhance persistence of the vector. Taken together these findings demonstrate that *orfAB* of Tn*6231* encodes a TA system that not only enhances plasmid persistence but also functions to expand a plasmid's host range.

Genetic Changes in the Host Genome That May Affect Plasmid Persistence

To identify the chromosomal mutations that may have improved plasmid persistence through genetic interaction with the transposon acquisition, the chromosome sequences of the ten evolved clones were analyzed. Filtering the data produced a shortlist of 29 unique chromosomal mutations in the 19 genes or regions listed in table 1. As clone L3-2 was the only clone wherein pMS0506 had not undergone any genetic change, mutated genes common to all other nine clones but not present in L3-2 would be candidates for epistatic interactions with evolved plasmid pMS0506. However, no such genes were found. Mutations involved in DNA processing functions were surprisingly uncommon and only found in two clones of lineage 3. These included a single nucleotide polymorphism in a putative promoter upstream of the DnaB helicase-encoding gene *dnaB* in L3-2 and in a putative helicase in L3-1. The majority of the chromosomal mutations was found in genes likely involved in metabolism, biosynthesis, energy consumption, bacteriocin production, chemotaxis, and motility. Of all the chromosomal genes that underwent mutations, only two were common to all replicate lineages and encoded chemotaxis regulatory protein CheY (9/10 clones) and 30S ribosomal protein S5 (10/10 clones). Identifying the specific chromosomal mutation(s) that explain the observed epistatic interactions between plasmid and host will thus require systematic reconstruction of individual mutations and combinations of mutations.

The two evolved clones that showed higher persistence of pMS0506 than other clones from their lineages had lost the native plasmid pR28 that encodes the transposon with the putative TA system (fig. 1 and table 2). To determine the timing of pR28 loss relative to that of the transposition event, plasmid DNA was extracted from the L1 ancestor and randomly chosen clones isolated from that lineage every 100 generations (supplementary fig. S1, Supplementary Material online). Transposition was first detected after 600 generations and loss of pR28 only after 1,000. We posit that transposition of Tn*6231* into pMS0506 weakened selection for the maintenance of pR28 because the Tad/Ata-like TA system would have been expressed from two different plasmids, only one of which (pMS0506) was under antibiotic selection. Loss of the native plasmid from these two clones must have improved the positive effect of the TA system on persistence of our drug resistance plasmid.

As the copy number of a plasmid can affect its loss frequency, we compared that of pMS0506 between the evolved and ancestral strains based on its average sequence coverage relative to the chromosome (table 2). In seven of the ten evolved clones the copy number was slightly lower, but in clones L3-1 and L3-2 it was much higher. As chromosomal changes explained all and much of the increased persistence in L3-2 and L3-1, respectively, this plasmid stabilization may be partially due to the higher copy number caused by chromosomal mutations such as those involving helicase-like genes. These findings reaffirm that there are multiple evolutionary pathways to improving persistence of the same plasmid in a novel host.

Discussion

We showed for the first time that an antibiotic resistance plasmid could expand its host range by acquiring a TA system and resolvase from a coresiding indigenous plasmid

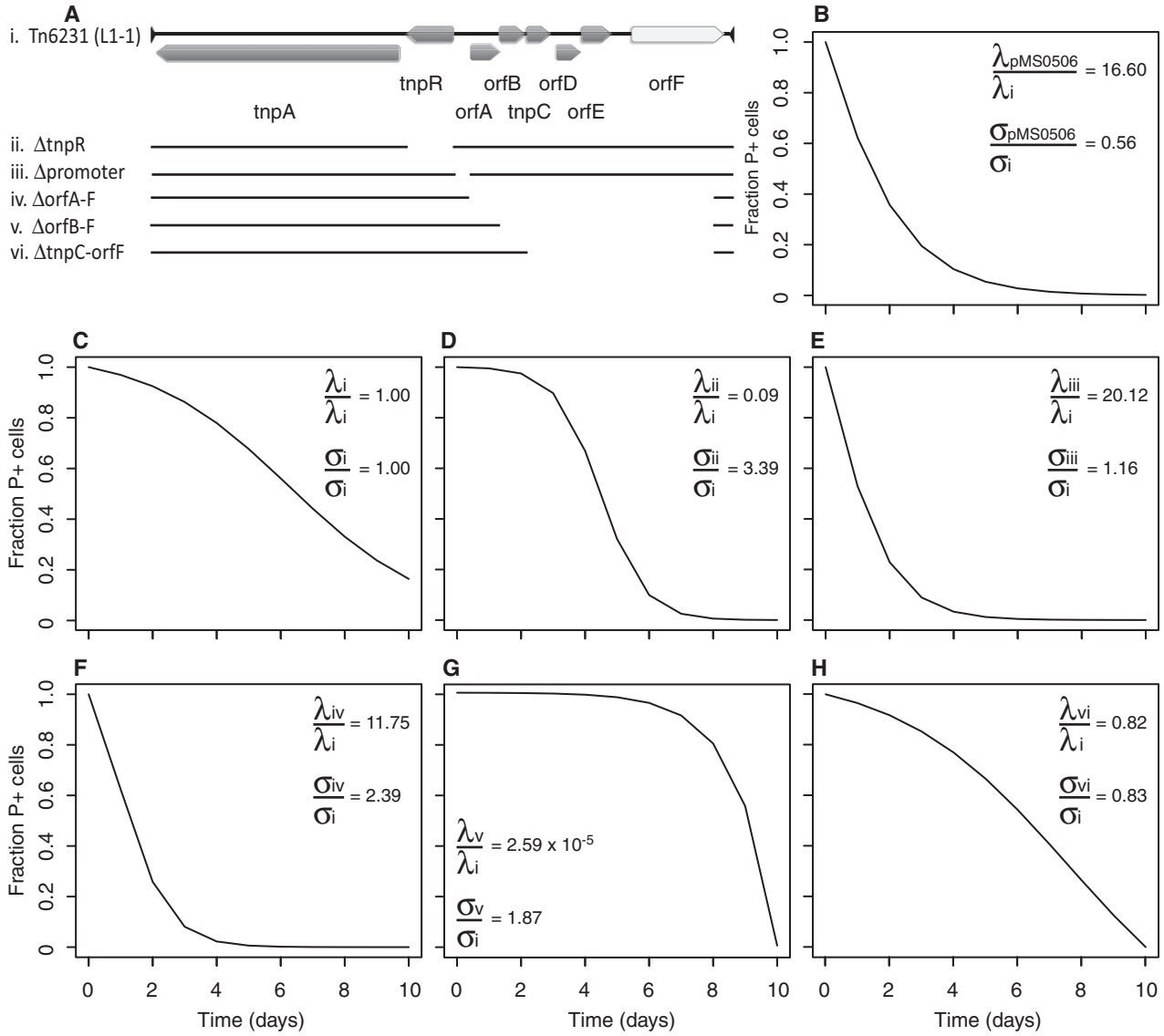

FIG. 4. Improved persistence in evolved host L1-1 is mediated by *orfAB* and *tnpR* on evolved plasmid pL1-1. Panel (*A*) shows the wild-type transposon (i) and the various deletion derivatives (ii–vi), with deleted segments shown as gaps in the lines under the transposon map. The persistence profiles in (*B*)–(*H*) were predicted from the estimated segregational loss frequencies (λ) and cost (σ) for each plasmid: Ancestral plasmid in ancestral host (pMS0506, panel *B*), evolved pL1-1 with intact transposon in its coevolved host (i, panel *C*), and five deletion derivatives in that same evolved host (ii–vi, panels *D*–*H*); the initial fraction of plasmid-bearing (P+) cells was set at 1. For each plasmid, the ratios λ/λ_i and σ/σ_i in panels (*B*)–(*H*) indicate whether the segregational loss frequency or cost was higher (>1) or lower (<1) than for evolved pL1-1 (i). Thus, values above 1 in panels (*D*)–(*H*) suggest that the deleted region positively contributed to decreasing plasmid loss or cost of pL1-1. Compared with pL1-1 four of the five deletion mutants had a higher loss frequency, cost, or both, and their plasmid persistence profiles were very different, as indicated by very negative ΔBIC values: −448.7 (*tnpR*), −1,586.6 (promoter), −1,613.1 (*orfA-F*), and −1,574.4 (*orfB-F*). Only deleting *tnpC–orfF* had a slightly positive effect on plasmid loss and cost (ΔBIC = −16.4). The plasmid persistence profile of the promoter deletion mutant was indistinguishable from that of the ancestral plasmid (ΔBIC= 1.0).

by transposition. TA systems are known to be effective mechanisms to avoid plasmid loss from bacterial populations (Hayes 2003; Goeders and Van Melderen 2014). If the TA encoding genes are lost or no longer transcribed the labile antitoxin is rapidly degraded, freeing the stable toxin to arrest growth or cause cell death. TA systems thus promote persistence of plasmids and other mobile genetic elements by inhibiting or killing cells that no longer maintain those elements (Gerdes et al. 1986; Yarmolinsky 1995). Based on sequence similarity the TA system on Tn*6231* on the native plasmid of *P. moraviensis* R28 is related to the widely distributed Tad/Ata- (Dziewit et al. 2007) and the well-studied RelEB (Christensen et al. 2001) systems. The Tad/Ata-like system was shown to stabilize plasmids in some but not all hosts tested, likely due to inhibition of cell division (Dziewit et al. 2007). In our host, the Tad/Ata-like system on Tn*6231* also appears to be bacteriostatic rather than bactericidal as plasmid-free segregants were still observed after plasmid loss. The

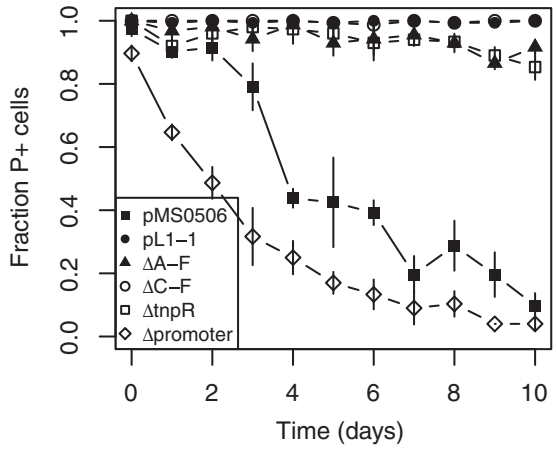

Fig. 5. Both *orfAB* and *tnpR* are required for persistence of pL1-1 in *Escherichia coli*. Deletion of either region resulted in a small but measurable decrease in persistence (in comparison to pL1-1 ΔBIC was −85.15 for the *orfA-F* deletion, −107.00 for the *tnpR* deletion, and $T_{1\%} > 100$ for both). Inactivation of both functions through deletion of the promoter completely destabilized the plasmid (ΔBIC for comparison to pL1-1: −705.20; $T_{1\%} = 14.1$). Deletion of *tnpC–orfF* had no measurable effect (ΔBIC = 11.40). Data points and error bars represent the averages and standard deviations of three replicates.

efficiency of TA systems can be host-dependent (Smith and Rawlings 1998; Dziewit et al. 2007), but given the lower efficiency of the Tad/Ata-like system on Tn*6231* in its native host than in two naïve hosts, this host may also have previously evolved some immunity to the addictive effect, for example, by counteracting the effect of the toxin. The loss of the native plasmid in two clones that showed higher persistence of our resistance plasmid suggests that interplasmid transposition of TA systems can be detrimental to maintaining a plasmid that is not under selection.

In addition to the genes encoding a putative TA system, the resolvase TnpR of Tn*6231* also improved plasmid persistence in the coevolved *Pseudomonas* and naïve *E. coli* hosts. Resolvases are known to resolve cointegrates including plasmid multimers, which are often formed after replication (Field and Summers 2011). Interestingly TA systems are often found integrated into regulatory regions such that their activity becomes integrated into operon function, resulting in symbiotic-like coexistence with the genome (chromosome or plasmid) (Matcher and Rawlings 2009; López-Villarejo et al. 2015). This is consistent with the modeling outcome of Mongold (1992), suggesting that postsegregational killing did not evolve as a "spiteful" act. Integration of the TA system into Tn*6231* of *P. moraviensis* R28 such that all the transposon genes shared a single promoter region may be an example of this. Coordinated expression of the TA system and the resolvase may facilitate temporary inhibition of cell division while resolving the multimers, thereby promoting plasmid persistence. This hypothesis is supported by our finding that both the resolvase and TA encoding genes were required for higher persistence and expanded host range.

Like other mobile genetic elements, transposons have been described as "fundamentally self-interested DNA entities," as their most basic function is self-propagation that exacts a cost to the host, whereas at the same time they also often encode beneficial traits (Rankin et al. 2011). This characteristic was exemplified here by transposition of Tn*6231* from the native mercury resistance plasmid pR28 to our antibiotic resistance plasmid pMS0506 that has a higher copy number and a broader host range than pR28. No copies of Tn*6231* were found in the ancestral and evolved chromosomes. Thus by benefiting the persistence of a plasmid of higher copy number that was under antibiotic selection, the transposon ensured its own continued existence in all five evolving populations and thereby expanded its own host range.

Here, we showed that both transposon acquisition and chromosomal mutations were required for the full persistence phenotype in the coevolved host *P. moraviensis* R28-S but not in the naïve *E. coli*, *P. putida*, and *C. necator* hosts. Evidence for positive epistasis between plasmid- and host-encoded mutations has been obtained in other studies as well (Modi and Adams 1991; Shintani et al. 2010; Silva et al. 2011; San Millan, Heilbron, et al. 2014; San Millan et al. 2015) but the genetic mechanisms have not been determined. For example, San Millan, Heilbron, et al. (2014) showed that epistatic interactions between two coresident plasmids promote plasmid persistence. Other studies have shown that plasmids can have regulatory effects on the host chromosome and thereby alter host fitness (Shintani et al. 2010; Harrison et al. 2015; San Millan et al. 2015). Such plasmid–plasmid and plasmid–host interactions imply that there are multiple mechanisms by which plasmids and hosts may coevolve to form persistent partnerships.

Based on the distribution of the 29 mutations across the sequenced clones, the chromosomal mutations that most likely had a positive effect on plasmid persistence were the ones found in all five lineages. One example was mutations in CheY, which is part of a two-component regulatory system involved in chemotaxis (Falke et al. 1997) and a homolog of GacA (96.7% amino acid similarity). Mutations in such regulatory proteins may ameliorate plasmid cost through their effect on gene expression. Harrison et al. (2015) showed that deleting the genes encoding the GacA/GacS two-component regulatory system of *P. fluorescens* ameliorated the cost of a mercury resistance plasmid. Up to 17% of the genes in this host were upregulated by the plasmid and subsequently downregulated again upon inactivation of the GacA/GacS regulatory system. In addition to CheY, ribosomal protein S5 was the only other protein that underwent mutations in all replicate lineages (table 1). These S5 mutations may not have improved plasmid retention but could be compensating for the Str^R mutation in the ancestor, as previously described (Gregory et al. 2014). CheY is thus the most likely candidate to have affected plasmid persistence through epistasis with the transposition event on the plasmid, possibly decreasing the cost of transposon carriage.

The two mutations in lineage 3 that affected putative helicase functions (Singleton et al. 2007) may also have been critical to improving plasmid persistence in some instances. This is supported by recent work by San Milan, Peña-Miller, et al. (2014, 2015) who, following experimental evolution,

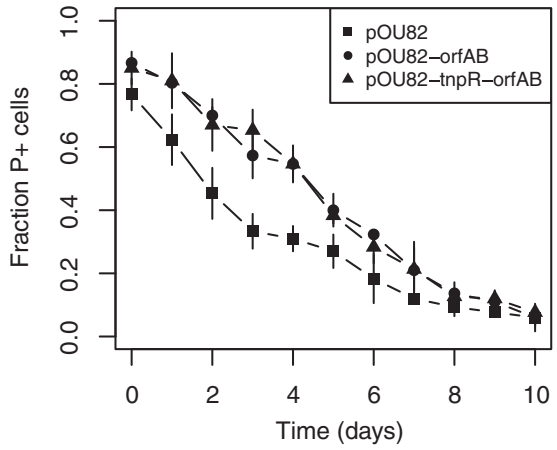

Fig. 6. Cloning of *orfAB* and *orfAB* plus *tnpR* into the unstable vector pOU82 increased its persistence. The constructed plasmids were slightly more stable than the vector alone (ΔBIC = −138.8 and −136.6; $T_{1\%}$: 22.3 and 23.2 compared with 18.1, respectively), due to a 100-fold decrease in loss frequency, partially counteracted by a higher plasmid cost. Data points and error bars represent the averages and standard deviation of three replicates.

found mutations in a putative helicase in *P. aeruginosa* carrying a small mobilizable plasmid. Introduction of the plasmid resulted in upregulation of approximately 13% of the host-encoded genes, including SOS response genes. Mutations in the helicase gene restored global gene expression to levels comparable to a plasmid-free strain and consequently decreased the plasmid cost. In our study, the helicase-associated mutations were found in the lineage with the only two clones that showed an elevated plasmid copy number and where chromosomal changes explained all of the increased plasmid persistence in one of them. Determining the individual or combined effects of the mutations through reconstruction in the ancestor as well as the underlying molecular mechanisms is beyond the scope of this work and will be the subject of future studies.

Inferring which underlying processes explained the observed plasmid host range and persistence changes was made possible by estimating the plasmid loss frequency and cost with our mechanistic mathematical model. Fitting this model allowed us to connect different plasmid persistence profiles with different genetic mechanisms underlying the observed changes. Because the criterion we chose for comparing plasmid persistence profiles, the BIC, is well known for favoring models that best approximate the underlying mechanism generating the data, we posit that using this criterion was key to identifying the genetic solutions to improved stability. Our model, like all mathematical models, is just an approximation to the processes behind the generation of the observed data. It does not, for instance, take into account multiple biological complexities, such as plasmid copy number variation, the TA system or other sources of stochastic variation. For bacteriostatic TA systems such as Tad/Ata, temporary growth inhibition of plasmid-free cells upon plasmid loss could lead to underestimating plasmid cost. In the future, we will expand the model to include this postsegregational mechanism. However, and despite being a simplification of reality, our model proved essential to obtain a process-based description and separation of the patterns observed in our stability curves (see fig. 1). Because our combined modeling and estimation approach teases apart changes in plasmid cost and hence host fitness, from other dynamic processes such as segregational loss, it allows precise predictions regarding plasmid persistence. Importantly, through these predictions the model led us to novel hypotheses regarding the observed changes in stability patterns, thus going well beyond the often sought-after compromise between biological realism and model size/number of parameters.

Together with our previous experimental evolution studies, this work shows that the same plasmid can either shift or expand its host range after specialization to different hosts. In our previous study with *Shewanella oneidensis*, mutations in plasmid pMS0506 that modified one of the two replication initiators improved plasmid persistence in that host but prevented replication in the previously permissive host *P. aeruginosa*, thus shifting its host range (Sota et al. 2010). In contrast, the present study shows that acquisition of a transposon improved the persistence of pMS0506 not only in its coevolved host but also in other naïve gamma- and beta-Proteobacteria, thus expanding its long-term host range. We conclude that the host range of a plasmid can be very dynamic, either shifting through "loss of function" mutations or expanding through gaining novel persistence functions such as resolvases and TA systems. The insights we are gaining into the evolutionary paths that expand, contract or shift a plasmid's host range will help control the alarmingly rapid spread of bacterial resistance to antibiotics of last resort.

Materials and Methods

Bacterial Strains, Plasmids, and Media

All strains and plasmids used in this study are listed in supplementary table S4, Supplementary Material online. Cultures of *E. coli* and *P. moraviensis* R28-S were grown in Luria–Bertani broth (LB) whereas cultures of *C. necator* were grown in one-tenth Tryptic–Soy broth, all at 30 °C. When needed media were supplemented with kanamycin (Km) at 50 μg ml^{-1} for *E. coli* and *P. moraviensis* and 25 μg ml^{-1} for *C. necator*. When needed ampicillin (Amp) at 100 μg ml^{-1} and X-gal at 30 μg ml^{-1} were used.

Plasmid pMS0506 has been described elsewhere (Sota et al. 2010). Briefly, it was constructed as a broad-host-range IncP-1β mini-replicon from the natural IncP-1β plasmid pBP136 (Kamachi et al. 2006). The conjugative transfer and mating pair formation regions and the segment in-between were removed, and a Km resistance gene from vector pUC4K and the origin of transfer from RP4 were inserted, allowing it to be mobilized by IncP-1 plasmids.

To prepare electrocompetent cells for transformation, 2 ml overnight cultures of *E. coli*, *P. moraviensis*, *P. putida*, and *C. necator* were washed at least three times with cold deionized water for *E. coli* and a cold solution of 300 mM sucrose for the other three species. Cells were resuspended in 200 μl cold

water or 300 mM sucrose to provide a dense stock culture. Approximately 100 ng of pMS0506 and 100 µl cell suspensions were mixed in a 1-mm gap cuvette and electroporated with a Gene Pulser Xcell (Bio-Rad, Hercules, CA). Pulse settings were 1.8 kV for *E. coli* and 2 kV for *P. moraviensis*, *P. putida*, and *C. necator*. After electroporation cells were suspended in 3 ml SOC (Hanahan 1983) for approximately 1 h, aliquots were spread onto the appropriate Km-containing medium.

Molecular and Genetic Methods

Conventional plasmid isolation and DNA manipulation techniques were used as described in Sambrook and Russell (2001). Restriction enzymes and high-fidelity Phusion DNA polymerase were obtained from New England Biolabs (Ipswich, MA). Primers used in this study are listed in supplementary table S6, Supplementary Material online. For subcloning into pOU82, pJET1.2 was used as an intermediate polymerase chain reaction (PCR) cloning vector (ThermoScientific, Waltham, MA). GeneJET PCR purification, plasmid miniprep, and gel extraction kits (ThermoScientific, Waltham, MA) were routinely used. Ligation products were transformed by heat shock into *E. coli* DH5α ultracompetent cells (Hanahan 1983) or by electroporation into *E. coli* EC100Dpir$^+$ (Epicentre, Madison, WI).

Deletions in Tn*6231* of evolved plasmid pL1-1 were constructed using a λ-Red-assisted recombination protocol in *E. coli* BW25113 as described by Datsenko and Wanner (2000). The λ-Red recombinase and chloramphenicol (Cm) resistance genes were from pKD46 and pKD3, respectively (supplementary table S4, Supplementary Material online). The Cm resistance cassette was removed by FLP-mediated recombination using pFLP3 (supplementary table S4, Supplementary Material online) (Choi et al. 2005). Deletion of the selected regions and the Cm resistance cassette were verified by PCR and sequence analysis. The primers used are listed in table 8.

Experimental Evolution

We previously evolved five replicate populations (referred to as lineages) of *P. moraviensis* R28-S (pMS0506) (Sota et al. 2010). This host was previously named *Pseudomonas koreensis* R28 but was recently renamed (Hunter et al. 2014). This strain was chosen as the plasmid host due to its inability to stably maintain IncP-1 plasmids (De Gelder et al. 2007; Hunter et al. 2014). Briefly, after transformation of pMS0506 in this strain and verification of plasmids in transformants, five were inoculated into five test tubes containing 5 ml of LB (Km). These cultures were considered to be generation 0 of each lineage. Subsequently 4.9 µl of culture was transferred daily to 5 ml fresh LB (Km) for 100 days (10 generations per day; 1,000 generations total). Every 100 generations 1 ml of culture for each lineage was stored in glycerol at −70 °C, and three randomly selected colonies were isolated and also archived at −70 °C. After 1,000 generations two clones from each of the five lineages were isolated randomly and their plasmid persistence and genome sequence determined. These clones are referred to by their lineage number followed by the clone number; for example, L1-2 represents clone 2 of lineage 1.

Additional Information on Using the BIC to Compare Plasmid Persistence Profiles

In this study, the BIC was used to determine whether the plasmid persistence profiles of different sets of clones were similar or distinct. The BIC criterion belongs to a class of information criteria that seeks to minimize the error in the causal structure in the data, rather than the error in prediction (Bozdogan 1987; Raftery 1995; Rice 1995; Taper 2004; Aho et al. 2014). Comparisons with this criterion penalize likelihood improvements due to the increase in the number of parameters in the model and at the same time avoid excessive Type I error from multiple pairwise testing. The BIC score is given by $-2 \times$ (maximized likelihood) + (number of model parameters) $\times$ log(sample size). Extensive simulation studies have been done that attempt to evaluate the quality of the inferences obtained by choosing models using the BIC. One of the most known studies carried out by Raftery (1995) arrived at a classification of BIC differences as "very strong evidence," "positive evidence," and "weak evidence" in favor of the distinction between any two models. According to Raftery (1995), an improvement in BIC score (usually denoted by ΔBIC) of more than 10 points amounts to very strong evidence of a difference between two models, between 6 and 10 to strong evidence, 2–6 to positive evidence, and 0–2 to weak evidence. However, these cutoffs not only change with the models and sample size, but in the case of Raftery (1995), they correspond to the case where the null hypothesis is assumed to be the true data-generating mechanism (for details, see Taper et al. 2008; Burnham et al. 2011; Aho et al. 2014; Taper and Ponciano 2016). In more complex biological scenarios, where mathematical models are a priori acknowledged (as we do here) as simple approximations to reality, current statistical research (Taper and Ponciano 2016) shows that these cutoffs tend to be in fact much larger, can be hard to determine, and that it is much more effective to compare differences in BIC values on a continuous scale as a way to get a post hoc interpretation of the strength of the evidence. Using this "evidential" approach allowed us to focus on assessing the magnitude of the difference between two persistence profiles, rather than on testing the "significance" of such difference. This change of focus is relevant because it frees our analysis from accepting to make a decision mistake 5% of the time thus acknowledging potential problems due to lack of power and experimental variability, while at the same time giving a solid assessment of which model better represents the underlying biological process (see Taper and Ponciano [2016] for a discussion on using Information Criteria). For more details about the model, how to fit it using Maximum Likelihood and these BIC model comparisons, we refer to Joyce et al. (2005), De Gelder et al. (2004, 2007, 2008), and Ponciano et al. (2007).

DNA Sequencing

The genome of *P. moraviensis* R28-S was recently sequenced by us (Hunter et al. 2014). To identify mutations in the evolved plasmids and host genomes, we sequenced two clones from each of the five independently evolved lineages of R28-S using both Roche 454 and Illumina MiSeq sequencing platforms in the University of Idaho IBEST Genomics Resources Core. More details on the DNA sequence analysis strategy and the algorithms used and can be found in supplementary methods, Supplementary Material online. All sequencing data pertaining to this project have been made available at the National Center for Biotechnology Information (SRA accession number SRP066179).

Supplementary Material

Supplementary methods, figures S1 and S2, tables S1–S6, and references are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

Acknowledgments

This work was supported by the National Institute of Allergy and Infectious Diseases at the National Institute of Health (grant number R01 AI084918), National Institute of General Medical Sciences at the National Institute of Health (COBRE grants P20RR16448 to and P20GM103397); the National Science Foundation BEACON Centre for the Study of Evolution in Action (grant number DBI-0939454); and Undergraduate Research Grants to J.M.H., R.S.S., and S.B. from the Department of Biological Sciences, University of Idaho. The authors are grateful to the staff of the IBEST Genomics Core facility for the genome resequencing work and to the National BioResource Project (NBRP) of National Institute of Genetics (NIG; Japan) for providing *Escherichia coli* BW25113.

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