

Adaptive plasmid evolution results in host range expansion of a broad-host-range plasmid

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ABSTRACT

Little is known about the range of hosts in which broad-host-range (BHR) plasmids can persist in the absence of selection for plasmid-encoded traits, and whether this ‘long-term host range’ can evolve over time. Previously, the BHR multi-drug resistance plasmid pB10 was shown to be highly unstable in *Stenotrophomonas maltophilia* P21 and *Pseudomonas putida* H2. To investigate whether this plasmid can adapt to such unfavorable hosts, we performed evolution experiments wherein pB10 was maintained in strain P21, strain H2, and alternatingly in P21 and H2. Plasmids that evolved in P21 and in both hosts showed increased stability and decreased cost in ancestral host P21. However, the latter group showed higher variability in stability patterns, suggesting that regular switching between distinct hosts hampered adaptive plasmid evolution. The plasmids evolved in P21 were also equally or more stable in other hosts compared to pB10, which suggested true host range expansion. The complete genome sequences of four evolved plasmids with improved stability showed only one or two genetic changes. The stability of plasmids evolved in H2 improved only in their co-evolved hosts, not in the ancestral host. Thus a BHR plasmid can adapt to an unfavorable host and thereby expand its long-term host range.

INTRODUCTION

Recently published analyses of prokaryotic gene and whole genome sequences have revealed that horizontal gene transfer (HGT) between closely and very distantly related Bacteria and Archaea plays a far more important role in the evolution of these organisms than had been previously recognized (DOOLITTLE and BAPTESTE 2007; GOGARTEN and TOWNSEND 2005; JAIN *et al.* 2002; KOONIN 2003; LAWRENCE and HENDRICKSON 2003; SMETS and BARKAY 2005). Moreover, many studies have provided evidence that different gene transfer mechanisms contribute to the extensive gene flux among bacteria in microbial communities (DRÖGE *et al.* 1999; SØRENSEN *et al.* 2005; VAN ELSAS and BAILEY 2000). Among these mechanisms, conjugative gene transfer mediated by so-called broad-host-range (BHR) plasmids is thought to play a very important role in gene spread among distantly related hosts (THOMAS 2000). Because of their ability to transfer and replicate in quite distinct phylogenetic lineages, these extrachromosomal mobile replicons can shuffle drug resistance and many other genes among a wide range of hosts (MAZODIER and DAVIES 1991). In spite of their importance in bacterial adaptation, such as in the rapid spread of multi-drug resistance (MCGOWAN 2006; PATERSON 2006), we currently do not know if and how their host range expands or contracts over evolutionary time.

While it is obvious how BHR plasmids can improve the fitness of their host by providing it with 'ready-made' genes that encode beneficial traits such as drug resistance, it is much less clear how well they persist in the absence of selection for plasmid-encoded genes. Although most BHR plasmids confer a low burden (fitness cost) to many of their hosts (THOMAS 2004), highly costly plasmid carriage has been documented in a few strains (DAHLBERG and CHAO 2003; DE GELDER *et al.* 2007; HEUER *et al.* 2007). When cells without such high-cost plasmids emerge in a bacterial population through imperfect plasmid segregation, they can quickly sweep through in the absence of selection, unless they get reinfected by the plasmid at a high enough rate (BERGSTROM *et al.* 2000; STEWART and LEVIN 1977). Therefore, analogous to parasites, the most persistent and successful plasmids are those with the best inheritance system, the lowest fitness cost, and the highest infection rate (SØRENSEN *et al.* 2005). We have previously shown that the stability of a BHR plasmid is highly

variable within the range of hosts in which it transfers and replicates (DE GELDER *et al.* 2007). Within a time period of 100 generations, the model plasmid used in our previous and this present study was lost in 95% or more of the population in three hosts, while there was 0% detectable plasmid loss in 16 other hosts. Therefore, we define the plasmid's 'long-term host range' as the range of hosts in which a plasmid is stably maintained for at least 100 generations without selection. We also designate hosts in which the plasmid is unstable or stable within this period as 'unfavorable' or 'favorable' hosts, respectively. It is presently not known whether BHR plasmids could evolve to adapt to some of these unfavorable hosts by improving their stability.

When plasmid-host adaptation occurs, it could either represent a host shift, whereby plasmid adaptation to one particular host negatively affects its stability in other hosts, or a true host range expansion, when there is no trade-off between improved stability in a new host and stability in previously favorable hosts. The phenomenon of shifts in bacterial hosts has been observed for phage (CRILL *et al.* 2000; DUFFY *et al.* 2007; FERRIS *et al.* 2007), but so far as we know, not for plasmids. Several very valuable experimental evolution studies have demonstrated that plasmids can adapt to a bacterial host, or that the host adapts to the plasmid, but none examined evolutionary changes in the plasmid's long-term host range (BOUMA and LENSKI 1988; DAHLBERG and CHAO 2003; DIONISIO *et al.* 2005; HEUER *et al.* 2007; LENSKI *et al.* 1994; MODI and ADAMS 1991; MODI *et al.* 1991; TURNER *et al.* 1998). Given that many BHR plasmids are involved in the rapid spread of multiple antibiotic resistance determinants, there is a need to investigate if and how these plasmids can shift or further expand their long-term host range, and thus persist longer in unfavorable hosts, including potential human, animal, or plant pathogens.

While it is conceivable that a plasmid will adapt to one unfavorable host, plasmids might encounter multiple distinct unfavorable hosts within short time spans through conjugative transfer in a bacterial community. As the molecular causes of instability can be different in different hosts, plasmid mutations that increase stability in one host might be neutral or even detrimental in other hosts. Due to this form of antagonistic pleiotropy one might expect that plasmid adaptation would be different when the plasmid resides in distinct hosts over evolutionary time, as compared to when

it is maintained in a single genetic background. Nothing is known about the effect of such host switches on plasmid-host adaptation.

In order to elucidate the ability of BHR plasmids to adapt to unfavorable hosts by improving their stability and/or fitness cost in that host, we sought to answer four questions. First, can BHR plasmids adapt to unfavorable hosts? Second, is adaptive plasmid evolution different when the plasmid is regularly switched between two distinct hosts? Third, have host-adapted plasmids merely shifted or truly expanded their host range? Fourth, what is the molecular basis of plasmid-host adaptation? To answer these questions, we performed evolution experiments with the BHR plasmid pB10 (SCHLÜTER *et al.* 2003) in two hosts in which pB10 is highly unstable (DE GELDER *et al.* 2007) under three protocols: long-term propagation in *Stenotrophomonas maltophilia* P21 only, in *Pseudomonas putida* H2 only, and alternatingly between both hosts. We then tested the stability and cost of evolved plasmids in their ancestral host. The results show that a BHR plasmid can adapt to an unfavorable host and thereby undergo host range expansion, while regularly switching between different hosts can slightly hamper plasmid adaptation. Moreover, plasmid host range expansion was accomplished by as little as a single mutation in a 64.5-kb plasmid.

MATERIALS AND METHODS

Culture conditions: All experiments were carried out using Difco® Tryptic Soy Broth (TSB) or Tryptic Soy Agar (TSA) and at 30°C. All liquid cultures were incubated on a rotary shaker (200 rpm). Antibiotics were used at the following concentrations: 100 mg/L tetracycline (Tc), 100 mg/L amoxicillin (Amx), 50 mg/L streptomycin (Sm), 250 mg/L rifampicin (Rif), and 250 mg/L naladixic Acid (Nal). Media are abbreviated as follows: TSA-RifTc stands for TSA medium with rifampicin and tetracycline at the concentrations listed above. Strains and cultures were archived at -80° after mixing 1 mL of liquid culture with 0.3 mL of glycerol. Dilutions and cell suspensions were made in sterile saline (8.5 g/L NaCl).

Bacterial strains and plasmid: The 64.5-kb plasmid pB10, isolated from a wastewater treatment plant, is a self-transmissible, BHR IncP-1 β plasmid that mediates resistance against the antibiotics tetracycline, streptomycin, amoxicillin, and

sulfonamide, and against mercury ions (DRÖGE *et al.* 2000; SCHLÜTER *et al.* 2003). *Pseudomonas putida* H2 and *Stenotrophomonas maltophilia* P21 were recently isolated from creek sediment and activated sludge, respectively, and were found to poorly maintain pB10 in the absence of antibiotics (DE GELDER *et al.* 2007; DE GELDER *et al.* 2005; HEUER *et al.* 2007). Other strains used were *P. putida* UWC1 (McCLURE *et al.* 1989), *P. koreensis* R28 (DE GELDER *et al.* 2005), and *E. coli* K12 MG1655 (ATCC 47076).

Conjugative plasmid transfer: To transfer plasmids between strains, 2 mL of overnight grown cultures of the plasmid donor and recipient were centrifuged, the supernatants removed, and the pellets resuspended in 200 µL TSB. Twenty µL of each mating partner cell suspension was dropped on a TSA plate on top of each other for the actual conjugation, and separately as negative controls. After overnight incubation the entire cell mass of each control and conjugation mixture was harvested and suspended in 300 µL saline, from which dilutions were made to streak or plate on TSA selective for transconjugants.

Evolution experiments: Because initial fitness increases due to adaptation of the strains to the TSB medium may mask mutations that improve plasmid stability or cost, strains H2 and P21 were first pre-adapted to TSB for 100 generations. This was done because the strains were recent environmental isolates that had not been grown in TSB before, and because we have previously observed a rapid increase in carrying capacity of strain H2 within this period when grown in new medium (data not shown). One colony from a freshly streaked freezer stock was inoculated into 5 mL TSB, which was incubated for ca. 16 h, and subsequently 4.88 µL culture was transferred into 5 mL TSB (ca. 10 generations per day). The same culture volume was transferred daily for ten days (representing 100 generations of growth). A purified colony of each strain was inoculated in 5 mL TSB. After incubation, these two pre-adapted cultures were frozen and an aliquot was transferred to 5 mL TSB-Rif and TSB-Nal to obtain spontaneous Rif and Nal resistant mutants. After 24-48 hrs the cultures were turbid and an aliquot was streaked onto TSA-Rif or TSA-Nal. After incubation, one colony was restreaked which resulted in the strains P21_{anc}Rif, P21_{anc}Nal, H2_{anc}Rif and H2_{anc}Nal. These four strains constituted the pre-adapted, marked ancestral hosts without plasmid, and were archived

at -80° . P21_{anc}Rif and H2_{anc}Rif were used as recipients in conjugations with an overnight grown culture of DH5 α (pB10) to obtain P21_{anc}Rif(pB10) and H2_{anc}Rif(pB10), the ancestral strains used to start the evolution experiments. In addition, the Rif resistant hosts were used to determine stability and cost of ancestral and evolved plasmids (see further); for simplicity they are briefly named P21_{anc} and H2_{anc} in the text, table and Figs. 2, 3, and 4. All four plasmid-free ancestral strains were used as recipients for evolved plasmids after every cycle of the evolution experiment.

The experimental set-up of the evolution experiment is depicted in Fig. 1. In brief, there were three evolutionary protocols, each with five replicate lineages: Plasmid pB10 was maintained in host H2 (protocol H2), in host P21 (protocol P21), or alternatingly in both hosts (double host protocol, DH). Approximately every 70 generations, plasmids were switched to either a genetic variant of the same ancestral host (protocols H2 and P21) or to the alternate ancestral host (DH protocol) (Fig. 1). We chose to switch the plasmids between strains of the same host in the H2 and P21 protocols to avoid host adaptation and promote plasmid adaptation, and to keep all parameters between the protocols identical except for the choice of host. To start the experiment, five separate colonies of P21_{anc}Rif(pB10) and H2_{anc}Rif(pB10) were inoculated into 5 mL TSB-Tc and incubated overnight. The ten cultures were archived at -80°C , constituting the ancestral strains of the evolution experiment (generation 0), and 4.88 μL of each was transferred to fresh 5 mL TSB-Tc medium and incubated, so that ca. 10 generations were obtained per 24-hr growth cycle ($1/2^{10}$ dilution rate). After 70 generations of serial batch cultivation (7 days), the plasmids underwent a host-switch. This was done by using the ten cultures as donors in conjugations with overnight grown freezer-stock cultures of the appropriate ancestral Nal^R strains as recipients (H2_{anc}Nal for protocols H2 and DH, and P21_{anc}Nal for protocol P21, Fig. 1). The resuspended cells were diluted and plated on TSA-NalTc to obtain approximately 5000 small transconjugant colonies after incubation. The colonies were harvested by applying 1.5 mL of TSB-NalTc onto the plate, suspending the colonies with a spreader and transferring the suspension to a 1.5 mL microcentrifuge tube. The evolution lineages were restarted by transferring 4.88 μL of these suspensions into 5 mL TSB-NalTc. When this procedure was carried out with donor and recipient cultures separately, no visible growth was observed on the plates

and the subsequent liquid media. This ensured that only transconjugants were carried through to the next cycle of the evolution experiment. These ten cultures were grown for 7 days as described above, now in TSB-NalTc, and subsequently the plasmids were transferred by conjugation, now to the appropriate Rif^R ancestral host (Fig. 1). The evolution experiment consisted of six such cycles of 70 generations, including 5 host switches (Fig. 1). When assuming 20 generations of growth from a single cell to a small colony of ca. 10⁶ cells during growth of the transconjugants after each switch, the total amount of generations during this experiment was estimated to be 520 (=6×70+5×20). This may be a conservative estimate since this ignores possible growth of donors and transconjugants respectively before and after plasmid transfer during the 24-hr conjugation procedure, as well as cell death during the stationary phase in every 24-h growth cycle.

Isolation of evolved clones and plasmids: The construction of strains used for analyses is schematically depicted in Fig. 2. At the end of the sixth cycle, all 15 cultures (five replicate lineages for each of the three protocols) were streaked onto TSA-NalTc, and three colonies were picked from each and inoculated into 5 mL TSB-NalTc. These 45 clones thus represented ‘evolved’ hosts harboring ‘evolved’ plasmids (Fig. 2, row 1). Evolved plasmids were named based on the host they evolved in: pP21 and pH2 for plasmids that evolved respectively in hosts P21 and H2, while plasmids evolved in the DH protocol were named pDH. These plasmid names were followed by A-E, representing lineages A-E. Finally the number 1 refers to plasmid 1 of three that were isolated from each lineage. Thus pP21-A1 is a plasmid from clone one in lineage A of protocol P21, and pDH-A1 a plasmid from clone one in lineage A of protocol DH. After overnight incubation, cultures founded from these evolved clones were archived and an aliquot used as donors in conjugations with the appropriate ancestral Rif^R hosts (Fig. 2, row 2). Five plasmids evolved in P21 were also transferred to four other hosts in which they had not evolved: the ancestral *P. putida* H2_{anc}, *P. koreensis* R28, *Escherichia coli* K12 MG1655, and *P. putida* UWC1 (Fig. 2, row 3).

Plasmid stability experiments: Stability experiments were carried out as previously described (DE GELDER *et al.* 2007), except for the use of TSA/TSB medium instead of LB agar/LB (Luria-Bertani). Plasmid loss was routinely assessed by

replicating colonies from TSA onto TSA with and without Tc. To confirm that the loss of Tc resistance corresponded with plasmid loss and not just loss of the *tet* operon (DE GELDER *et al.* 2004), some Tc-sensitive (Tc^S) clones were tested for sensitivity to Sm (Sm^S) by transferring them on TSA-Sm; true segregants should be $Tc^S Sm^S$. This test was done at the end point of stability experiments depicted in Fig. 3 A-B, and Fig. 5, and during the entire stability experiments depicted in Fig. 6 C-D. The fraction of $Tc^S Sm^R$ clones at the end of the stability experiments in P21_{anc} (Fig. 3 A-B, and Fig. 4) ranged from 0 to 6% of the total population. This means that the fraction of plasmid-containing cells depicted in these figures, based only on Tc^S/Tc^R testing, is only slightly underestimated. The fractions represented in Fig. 6, panel C and D, are based on the fraction of $Tc^R Sm^R$ clones and thus represent plasmid containing cells.

Plasmid cost estimation: To estimate plasmid cost values, competition experiments were carried out as previously described (DE GELDER *et al.* 2007), except for the use of TSA/TSB medium instead of LB agar/LB. Total and plasmid-bearing cell counts were determined on TSA and TSA-Tc plates after 1 and 2 days. Parallel control experiments starting with only plasmid-bearing cells were included to verify that no detectable plasmid loss occurred within 2 days.

Cost values for the ancestral plasmid pB10 and plasmids evolved in P21 were also estimated based on the plasmid stability dynamics presented in Fig. 3. We previously showed that a population dynamics model that includes plasmid loss, plasmid cost, and horizontal transfer, ‘the HT model’ adequately described the stability dynamics of ancestral plasmid pB10 in strain P21(pB10) (DE GELDER *et al.* 2007; PONCIANO *et al.* 2007). Here we used the same model and methods to obtain the Maximum Likelihood Estimates (MLEs) of plasmid cost from the stability patterns of the ancestral plasmid and the five plasmids evolved in host P21 (Fig 3). Specifically, for each of the five lineages, a set of three stability series (corresponding to the three plasmids per lineage) was treated as three replicates of the same process. For the ancestral strain, we used the five replicate stability curves to obtain one average value. Parametric Bootstrap (PB) confidence intervals for the HT-model cost estimates were computed as previously described (DE GELDER *et al.* 2004; PONCIANO *et al.* 2007)). The point estimates (MLEs) and PB error bounds (LCL and UCL for lower and upper

confidence interval limits) were compared to the empirical cost estimates and their error bounds (Table 1). Finally, Likelihood Ratio tests for the HT model were carried, where under the null hypothesis, the observed data were binomially distributed with a mean equal to the deterministic model predictions.

Statistical analyses: To verify whether the stability of different plasmids within a lineage was statistically different, we used a repeated measures ANOVA to analyze the data per lineage. In the model we took as fixed effects the factors *Plasmid* and *Day*. The *Replicate* was considered a random effect. The model equation is:

$$y_{ijk} = \mu + \alpha_i + \beta_k + \alpha\beta_{ik} + \pi_{j(i)} + \varepsilon_{ijk}$$

where α_i corresponds to the *Plasmid* effect, β_k corresponds to the effect of time (*Day*), $\alpha\beta_{ik}$ is the interaction between *Plasmid* and *Day*, $\pi_{j(i)}$ the random effect due to the j^{th} *Replicate* of *Plasmid* i , and ε the residuals. Only days for which data is available for all assays were included into the analyses. The response variable y_{ijk} is the square root of the fraction of plasmid free cells per day, per replicate, per plasmid. These analyses were implemented in SAS.

Plasmid DNA sequencing: The complete nucleotide sequence was determined for four evolved plasmids: pP21-B1, pP21-D1, pDH-B1, and pDH-D1 (see “Isolation of evolved clones and plasmids” above). The evolved plasmids were first transferred to *E. coli* K12 MG1655 by conjugation. Cultures used for plasmid extraction were grown in LB-Tc10. Plasmid DNA was extracted using the Plasmid Mini Kit (Qiagen) according to manufacturer’s instructions and using the recommendations for low-copy number plasmids. Ca. 95% of each plasmid was sequenced by Macrogen Inc. (South Korea) using pyrosequencing technology with ca. 20X coverage. To close the gaps and to examine every potential mutation as suggested from the pyrosequencing data, additional sequence determination was done in-house using a 3730 DNA Analyzer (Applied Biosystems). PCR and sequencing primers were designed using Primer3. PCR was performed using AccuPrime Pfx DNA Polymerase (Invitrogen) according to manufacturer’s instructions. Applied Biosystems Big Dye Terminator v3.1 Cycle Sequencing Kit was used for the sequencing reactions, from which the DNA was purified using DyeEx 96-kit or 2.0 (Qiagen). Sequence data were analyzed using NCBI Blast and ContigExpress (Vector NTI).

RESULTS

Stability of evolved plasmids in their ancestral hosts: Our first goal was to determine if plasmids had adapted to their hosts by improving their stability, after being maintained under selection for ca. 500 generations. Therefore, we tested the stability of evolved plasmids in the ancestral hosts in the absence of selection. The plasmids evolved in *S. maltophilia* P21, named pP21, were much more stable in the ancestral host P21_{anc} than the ancestral plasmid pB10 (Fig. 3, left column: P21-A – P21-E). Thus genetic changes that affect segregative loss rate, plasmid cost, and/or conjugative transfer, must have occurred in these plasmids. Moreover, all fifteen plasmids, three from each of the five independently evolved replicate lineages, showed strikingly similar plasmid stability dynamics. The data demonstrate that a plasmid can drastically improve its stability in an initially unfavorable host within ca. 500 generations.

In order to examine if plasmid adaptation to unfavorable host P21 would be different when the plasmid was regularly switched between two hosts than when it is maintained in one host, we compared the stability of plasmids evolved in P21 with those evolved alternatingly in hosts P21 and H2 (named plasmids pDH). Because of the expected variation between and within lineages, triplicate stability assays were performed for each of the three plasmids per lineage. While the plasmids evolved under this DH protocol also showed increased stability in host P21_{anc} compared to pB10, in some of the lineages the stability patterns displayed much more variation and some plasmids were less stable than those evolved in host P21 alone (Fig. 3 DH panels versus P21 panels). Moreover, within three lineages (B,D,E) there was a statistically significant effect of the plasmid replicate ($p < 0.05$) on the stability pattern (see p-values in Fig. 3), suggesting the presence of distinct plasmid mutants in the population. Overall, these results show that switching the plasmid between two unfavorable hosts can hamper plasmid adaptation in some populations.

The plasmids that evolved in host *P. putida* H2 (named pH2) did not show improved stability in the ancestral host H2_{anc} compared to the ancestral plasmid pB10 (Fig. 4). This shows that during 500 generations no genetic changes in the plasmid had been selected that measurably increased plasmid stability in the ancestor. Similarly, the

stability of the pDH plasmids in H2_{anc} was also not different from that of pB10. However, the clones of evolved H2 strains, H2_{ev}(pH2) showed highly increased plasmid stability (Fig. 4, horizontal dashed line). Thus within 70 generations the host adapted to the plasmid, and/or plasmids and hosts co-evolved. **Detailed analysis of this (co-) evolutionary process was beyond the scope of this study, but could reveal new pathways of plasmid host range shifts.**

Cost of evolved plasmids: Plasmid cost is one of the factors that determine plasmid stability patterns, because it affects the rate at which plasmid segregants sweep through the population in the absence of selection. To determine whether the increased stability of the plasmids evolved under the P21 and DH protocols was in part due to a decrease in plasmid cost, competition experiments between plasmid-carrying and plasmid-free ancestral P21 strains were performed in antibiotic-free medium (Table 1). Since the competition was done for only 20 generations, plasmid loss was not yet significant (Fig. 3) and did not confound the results. The average cost of the ancestral plasmid to the ancestral host, tested in five replicate clones, was 46%, an extremely high value. In contrast, all evolved plasmids showed a significantly lower cost to the ancestral host ($p < 0.0001$). Only for one of the five lineages **(B), the cost of plasmids evolved in the DH protocol (pDH-B) was significantly higher ($p = 0.008$) than that of the plasmids from the corresponding P21-B lineage.** However, when pooling all the data from each protocol together over lineages, the cost of all plasmids isolated from the DH protocol was significantly higher than the cost of all plasmids evolved in P21 ($p = 0.024$). Thus during long-term association of plasmid pB10 with single host P21, the plasmid reduced its cost to that host significantly, and this cost reduction was also significant but slightly less pronounced when the plasmid was regularly switched between two distinct hosts.

To validate the drastic decrease in plasmid cost after adaptation to host P21, as determined by competition assays, we also estimated the cost of the ancestral and pP21 plasmids based on the stability curves shown in Fig. 3 (P21-A – P21-E), using a mechanistic time series model (DE GELDER *et al.* 2007; PONCIANO *et al.* 2007). Very much like the competition-derived plasmid cost values, the model-derived cost estimates were much lower for the evolved plasmids than for the ancestral plasmid.

Moreover, there was no statistically significant difference between both types of cost estimates (Table 1). The Likelihood Ratio tests results provided strong support for the deterministic horizontal transfer (HT) model for three of the evolved plasmids (P21-B, D, and E), but not for the ancestral plasmid and P21-A and P21-C (data not shown). While rejection of this deterministic null model in three of the six data sets suggests that the predicted mean trend and/or the variance are not well explained (DE GELDER *et al.* 2004), the results strengthen our conclusion that a plasmid can drastically improve its long-term stability by reducing its cost.

Plasmid host range expansion: In order to determine whether the plasmids that evolved in a single host, *S. maltophilia* P21, merely shifted or truly expanded their host range, plasmid stability was tested in four 'naïve' hosts, which had not previously carried plasmid pB10 (Fig. 2, row 3). In two of these hosts, *P. putida* H2 and *P. koreensis* R28, the ancestral plasmid pB10 was known to be unstable, whereas in the other two, *E. coli* K12 MG1655 and *P. putida* UWC1, it is very stable (DE GELDER *et al.* 2007; PONCIANO *et al.* 2007). Surprisingly, the evolved plasmids were much more stable than pB10 in the unfavorable host R28 (Fig. 5, panel A). In contrast, the stability of these plasmids in strain H2 did not differ from that of pB10 (Fig. 5, panel B). This shows that the adaptive changes in the evolved plasmids that were responsible for increased stability in host P21, also improved stability in naïve host R28, but not in H2. In the two 'favorable' hosts the evolved plasmids were at least as stable as pB10 (Fig. 5, panels C and D). Based on these results, these plasmids did not undergo an obvious evolutionary trade-off typical for a host range shift, but truly expanded the range of hosts in which they can be stably maintained.

Mutations in evolved plasmids: To determine the molecular basis of the observed improvement in plasmid cost and stability, four plasmids were completely sequenced: pP21-B1, pP21-D1, pDH-B1 and pDH-D1. These four plasmids were chosen because pDH-B1 and pDH-D1 showed the least improvement in stability compared to all other evolved plasmids (lowest stability curves in Fig. 3, DH-B and DH-D). Thus they differed most from their counterpart plasmids evolved in P21 alone, pP21-B and pP21-D. Because there was no noticeable difference in stability and cost among these pP21 plasmids, one was randomly chosen from each of the two lineages (and

named pP21-B1 and pP21-D1, respectively). Interestingly, all four plasmids shared the same genetic change: a point mutation in *trbC* that resulted in an amino acid change from valine to alanine in the TrbC protein at amino acid 95, which is located in the trans-membrane region. The *trbC* gene codes for a putative prepilin, which is involved in mating pair formation. This mutation was the only one in the entire 64.5-kb genomes of the two plasmids evolved in P21, and thus must be the cause of the observed increase in plasmid stability and cost. The mutated plasmids can still transfer from P21 to various hosts (strains P21, H2, R28, K12, UWC1), but a difference in transfer frequencies (transconjugant/recipient fractions after overnight mating on LB agar) was observed as follows. When K12 was the recipient and P21Rif the donor, the frequency for pP21-D1 was ca. 10-fold lower than for pB10. However, when P21Nal was the recipient strain, either in matings with P21Rif or *E. coli* K12 as donor, evolved plasmid pP21-D1 transferred at a ca. 1,000-fold higher frequency than pB10 (data not shown). This increased transfer frequency may help explain how this mutant plasmid rapidly swept through the population, since it would outcompete pB10 in the conjugative transfer during every host switch.

The sequence data for the two pDH plasmids showed a second genetic change, a duplication of the *orfE*-like integron gene cassette, resulting in respectively two and three copies of this cassette in pDH-B1 and pDH-D1. To confirm this interesting gene cassette amplification, PCR was performed using primers, 5'CS and 3'CS, that specifically anneal to the 5'- and 3'-conserved sequences of the class 1 integrons (LÉVESQUE *et al.* 1994; SZCZEPANOWSKI *et al.* 2004). The PCR products of pB10, pDH-B1 and pDH-D1 were ca. 1.2 kb, 1.7 kb, and 2.0 kb respectively, which corresponded to the sizes expected for one, two or three copies of the *orfE*-like cassette. Since the genes in the integron are under the control of a general promoter, we examined if this gene duplication affected expression of *oxa-2*, an integron gene that is located downstream of *orfE* and encodes resistance to β -lactam antibiotics. Amoxicillin resistance of K12(pDH-B1) and K12(pDH-D1) was tested in LB-Amx, and both strains had lost the resistance. It is currently not known if and how this genetic change in the integron contributed to improved plasmid cost and stability in hosts H2 and/or P21, but loss of expression of the *oxa-2* gene is one possible explanation. The lower stability of

these two pDH plasmids in P21_{anc}, compared to plasmids P21-B1 and pP21-D1, suggests that the mutation was detrimental to stability in host P21.

In conclusion, the clear example of parallel evolution of the *trbC* mutation, which was the only mutation in plasmid evolved in host P21, strongly suggests that this mutation was responsible for the improved plasmid cost and stability, and was under very strong selection in both the P21 and DH evolution protocols. The molecular analysis also showed that one or two genetic changes in the genome of a BHR plasmid can expand its long-term host range.

DISCUSSION

Despite the threat of drug resistance and virulence plasmids to human, animal, and plant health, we do not understand if and how the host range of a plasmid evolves over time. In this study we examined if a BHR multi-drug resistance plasmid, the IncP-1 plasmid pB10, could adapt to two unfavorable hosts through evolutionary changes in its genome. Adaptive evolution of plasmid pB10 during ca. 500 generations in host P21 resulted in improved stability and decreased cost in this host. Plasmids that were maintained alternatingly in both hosts, also showed improved stability and cost in ancestral host P21_{anc}, but rapid adaptation was hindered by regular host switching. In contrast, during that same evolutionary time, the same plasmid did not adapt to the second host, H2, but the host had adapted to the plasmid. Plasmids adapted to host P21 were also more or equally stable in other naive hosts compared to ancestral plasmid. This suggests for the first time that a BHR plasmid can even further expand its long-term host range. DNA sequence analysis of evolved plasmids indicates that one genetic change in a 64.5-kb genome caused the drastic improvement in plasmid cost and stability. This work provides new insights into the adaptability of BHR plasmids to initially unfavorable hosts, and in the possible mechanisms of evolution of their long-term host range.

There was a striking similarity in the stability dynamics and fitness cost values of the evolved plasmids that were randomly picked from the five independently evolved lineages of *S. maltophilia* P21(pB10) (Fig 3 and Table 1). Although phenotypic convergence could have arisen through different adaptive walks in genotype space,

DNA sequence analysis of evolved plasmids suggests parallel evolution, since a single point mutation in the *trbC* gene independently swept through at least two of the five P21 lineages. In a previous study we demonstrated that the high plasmid cost in host P21 was the main reason for high instability of pB10 in this host (DE GELDER *et al.* 2007). Our results here show that a drastic improvement in stability of the evolved plasmid was accompanied by a significant decrease in the cost of the plasmid to the host. The cost, calculated based on competition experiments decreased from 46% for pB10 to 9-12% for the plasmids evolved in P21. These results were corroborated by the plasmid cost estimates based on stability patterns, obtained with the 'horizontal transfer model' developed earlier (DE GELDER *et al.* 2007; PONCIANO *et al.* 2007) (a decrease in cost from 54% to 5%). The single mutation in the prepilin protein (TrbC) in two independently evolved plasmids pP21-B1 and pP21-D1, strongly suggests that this amino acid change must be responsible for the improvement in cost and stability. Future studies will have to determine how a change from Val to Ala in the TrbC protein can explain such a drastic effect on plasmid cost and stability in this host. The higher frequency of transfer of evolved plasmids like P21-D1 into host P21 in filter matings compared to the poor transferability of the ancestral plasmid, may in part be responsible for the higher stability in liquid medium. However it is unlikely to explain the improved cost as determined by competition experiments in liquid medium, because IncP-1 plasmid transfer in liquids is typically too low to confound the competition experiment (BRADLEY *et al.* 1980). Moreover, the mathematical model used to estimate plasmid cost would have to be wrong in its prediction of the parameter values corresponding to the underlying causes of plasmid stability. In spite of the uncertainty about the molecular mechanism of improved plasmid cost and stability, our results suggest that a single plasmid-encoded mutation explains the drastically improved plasmid stability in independently evolved lineages of *S. maltophilia* P21(pB10).

We further demonstrated for the first time that regular horizontal transfer of a plasmid between distinct unfavorable hosts can have a negative effect on plasmid adaptation to a novel host. For three of the five DH lineages (DH-B, DH-D, DH-E), three randomly chosen plasmids per population showed significant differences in stability dynamics. These results indicate the co-existence of and competition between

subpopulations that harbored genetically distinct plasmids with different stability characteristics. We therefore conclude that clonal interference in three of the five lineages **slowed down fixation of plasmid-encoded stability-enhancing** mutations in these large populations, as previously suggested in theoretical models and experimental studies of bacteria (DE VISSER and ROZEN 2006; GERRISH and LENSKI 1998). These findings suggest that ‘generalist’ plasmids, which frequently transfer between distinct hosts in natural communities would not adapt as fast to any host as ‘specialist’ plasmids that remain in one host. **This is consistent with theory that specialist species, which have narrower niche breadths than generalists, are able to evolve faster than generalists (WHITLOCK 1996).** In spite of this constraint on the evolution of generalist plasmids, BHR plasmids like those of the IncP-1 group seem to compete successfully with NHR, specialist plasmids, as they are found in many diverse hosts **world-wide (SCHLÜTER *et al.* 2007).** However, as we recently showed (DE GELDER *et al.* 2007), these plasmids are not necessarily stable in all hosts within their replication range. Thus they may not be very well adapted to a subset of hosts but nevertheless persist due to their high horizontal transfer (infection) rate (BERGSTROM *et al.* 2000).

There are at least three explanations **for the higher variability in stability patterns of plasmids evolved under the DH protocol compared to those evolved** in single host P21. First, under that regime plasmid pB10 was maintained in host P21 for only 50% of the evolutionary time compared to when evolved in P21 only, while it spent the other 50% of the time in host H2. Therefore, mutations that were adaptive in P21 but neutral in H2, might have been occasionally lost by drift during propagation in H2, or may not have swept through the populations yet at the end of our experiment. Second, beneficial mutations selected for in P21 might have been under negative selection while in host H2 due to negative effects on plasmid stability or cost in that host **(an example of antagonistic pleiotropy in heterogeneous environments).** They would thus have a low chance of surviving the next severe bottleneck when switched back to P21. Since plasmids adapted to P21 showed the same stability patterns in host H2 as the ancestral plasmid (Fig. 5A), it is more likely that the plasmid mutations were neutral and not deleterious for stability in host H2. Third, **antagonistic pleiotropy may have played a role because** mutations selected during the cycle in host H2 may have been deleterious to

host P21. Our results support this **last hypothesis since** the sequenced plasmids pDH-B1 and pDH-D1, which have multiple copies of *orfE* in the integron in addition to the *TrbC* mutation, showed the lowest stability in P21_{anc} (represented by the lower stability curves in Fig. 3, DH-B and DH-D). Moreover, this genetic change was only observed in the two sequenced plasmids that were maintained in both hosts, and not in the two pP21 plasmids. In addition, the complete sequence of one plasmid evolved in host H2 in a previous study (HEUER *et al.* 2007) also contains a change in *orfE*, i.e. an almost complete deletion of the ORF (Williams and Top, unpublished). More data are needed to understand the effect of the integron rearrangement, but it could **represent an example of antagonistic pleiotropy with respect to plasmid stability**.

Our results obtained for switching pB10 between **hosts** P21 and H2 **are not necessarily** representative of all adaptive scenarios during plasmid host-switching in general. For example, if strains P21 and R28 would have been chosen as plasmid hosts for this experiment, plasmid adaptation during the DH protocol might have been very similar to that in the single host protocols, as mutations in P21 **also clearly improved plasmid stability** in R28 (Fig. 5, **and see discussion below**). **Thus with respect to molecular plasmid-host interactions that affect plasmid stability, those two hosts may represent more similar environments for the plasmid**. Overall, we can conclude that plasmid adaptation to one unfavorable host may be hampered when the plasmid is regularly switched over evolutionary time between genetically distinct unfavorable hosts compared to when it is maintained in only one host.

The plasmids **that adapted to host P21 by improving their stability and cost**, showed equal or improved stability in four naïve hosts. This finding suggests that they truly expanded their long-term host range. The most intriguing result is the improved stability of pP21 plasmids in the naïve host R28, which suggests that the *trbC* mutation must benefit plasmid stability in more than one host. In the absence of selective pressure, these plasmid variants with expanded host range might become more dominant in the bacterial community as they are now stable in a wider range of hosts, thus representing more successful mobile elements. We are not aware of any similar findings in the literature, but at least two studies corroborate our results. DIONISIO *et al.* (2005) grew *E. coli* K12 with the narrow-host-range (NHR) drug resistance plasmid R1

for 420 generations. Whereas the ancestral plasmid conferred a high fitness cost to the *E. coli* host, evolved plasmids conferred a fitness advantage not only to this host, but also to a naïve *Salmonella enterica* strain. Thus, while stability was not examined in that study, the improved fitness in a naïve host would probably lead to longer plasmid persistence in the absence of selection (antibiotics) compared to ancestral plasmid R1. The molecular basis of the fitness increase was not described. In addition, *in vitro* mutagenesis studies have shown that the replication range of the NHR *P. syringae* plasmid pPS10 can be expanded to more than one host by a single genetic change in the plasmid RepA protein (FERNANDEZ-TRESGUERRES *et al.* 1995; MAESTRO *et al.* 2003). Further studies are needed to improve our insight into the tempo and mechanisms of plasmid host range shift or expansion.

In contrast to the very clear plasmid adaptation to host P21, plasmids that evolved in host H2 did not show any **detectable improvement in stability** in the ancestral host. However, evolved H2 strains maintained their plasmids for at least 200 generations (Fig. 4). **This suggests that host evolution (or plasmid-host co-evolution)** has occurred in these lineages within one cycle since **the last host-switching event**, thus during 70 generations. **Given this short time period, the underlying mechanism is most likely a single genetic change in the host chromosome.** The poor stability in H2_{anc} of plasmids evolved in this host is contradictory to our previous finding that genetic changes in pB10 resulted in drastic improvement of plasmid cost and stability in host H2 (HEUER *et al.* 2007) (Williams and Top, unpublished data). However, the experiment was done in different medium and under different bottleneck and host-switching regimes. Not only was the daily dilution rate for the batch cultures in this study higher **than previously** (1/1000 versus 1/256), but host-switching by conjugative plasmid transfer every 70 **generations (versus every 100 previously) was done using a different procedure than before. Both changes resulted in stricter bottlenecks in the current study. Thus, our** results show that the potential of a BHR plasmid to adapt to unfavorable hosts depends on the host (P21 versus H2 in this study), as well as on the environmental conditions or population dynamics (H2 in this study versus Heuer *et al.* (2007)). Moreover, although preliminary, our **study suggests** that a single host mutation may allow stable maintenance of a previously unstable plasmid. **This corroborates our**

previous finding that subtle differences in host genotypes among strains of the same species can affect plasmid stability (DE GELDER *et al.* 2007; SOTA and TOP 2008). It is also in agreement with the observation that a single point mutation in DnaA of *E. coli* allowed replication of the NHR *P. syringae* plasmid pPS10 in this host (MAESTRO *et al.* 2002). Future analysis of the evolved H2 hosts will improve our currently poor understanding of the role of plasmid-host interactions in plasmid stability.

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FIGURE LEGENDS

Fig. 1: Experimental design of the three experimental evolution protocols

Plasmid evolution in single host *P. putida* H2 (protocol H2), in single host *S. maltophilia* P21 (protocol P21), and alternatingly in a double host protocol (DH).

All protocols were carried out using five independent serial batch cultures (lineages A-E), represented by the 5 horizontal lines in the first cycle (only one line is drawn in later cycles for clarity): - - - , H2, — , P21. These lineages were started from five separate colonies of the ancestral host with ancestral plasmid (H2_{anc}Rif(pB10) or P21_{anc}Rif(pB10), both rifampicin resistant). After 70 generations (7 days) of serial batch culture, the plasmids (designated pB10' to indicate putative plasmid mutations) from each lineage were transferred to the nalidixic acid resistant (Nal) ancestral host (H2_{anc}Nal or P21_{anc}Nal) by conjugative transfer (dotted line arrows). The DH protocol was initiated at this point by transferring plasmids from the five P21 lineages to the alternate host H2, thus generating lineages DH-A through DH-E. This first cycle was repeated five more times, whereby the plasmids were always transferred back into the ancestral host with the reciprocal resistance. Evolved populations at the end of cycle 6 were marked by 'ev' in subscript behind the strain name.

Fig. 2: Construction of strains used for plasmid stability analysis

Plasmid and strain designations are described in Material and Methods and in the legend of Fig. 1. All ancestral hosts used to analyze evolved plasmids were Rif resistant, but for clarity, Rif is omitted from all strain designations.

Fig. 3: Stability of plasmids evolved in *S. maltophilia* P21 (pP21) and under DH protocol (pDH) in naïve ancestral host P21_{anc}.

In each P21 panel (lineages A-E), the dashed line represents the stability of the ancestral plasmid in the ancestral host (one stability assay for each of the five independent ancestral cultures, A-E). In these P21 panels, solid lines represent results from single stability assays for three randomly chosen plasmids per lineage. In panels DH (lineages A-E) solid lines represent the results of triplicate stability assays for three randomly chosen plasmids per lineage; errors bars represent standard deviations. ▲, ■, ◆: plasmids 1, 2, 3. Since the five DH lineages were founded from generation 70 of each of the five P21 lineages (Fig. 1), ancestral plasmids for P21 and DH protocols were the same, and their stability data (dashed lines) are not repeated in the DH panels. P-values <0.05 (panels B, D, E) suggest that the stability dynamics were significantly different between plasmid isolates.

Fig. 4 Stability of plasmids evolved in *P. putida* H2 (pH2) and under DH protocol (pDH), in ancestral and co-evolved host H2.

●, average stability of the ancestral plasmid in the ancestral host (one assay for each of the five independent ancestral cultures); ■ and ◆, average stability of the evolved plasmids pH2 in the ancestral host (H2_{anc}) and in the co-evolved host (H2_{ev}), respectively; ▲, average stability of the plasmids evolved in DH protocol (pDH) in the ancestral host H2_{anc}. For all evolved plasmids, data represent averages from five evolved plasmids, one from each of the five independent lineages. Error bars represent standard deviations.

Fig. 5: Stability of plasmids pP21 (evolved in host P21) compared to stability of the ancestral plasmid pB10 in four naïve hosts: two unfavorable hosts (A, *P. koreensis* R28; B, *P. putida* H2), and two favorable hosts (C, *E. coli* K12; D, *P. putida* UWC1). Data for plasmid pB10 are averages from five replicate stability assays; data for plasmids pP21 represent averages from five evolved plasmids, one per lineages (same five plasmids that were tested in host P21_{anc}, Fig. 3). Error bars represent standard deviations. In panels C and D open instead of filled squares are used for pP21 plasmids to show both symbols. Note that the Y-axis of panel A is on a logarithmic scale. In *E. coli* and *P. putida*, both ancestral and evolved plasmids were stable for at least 200 and 300 generations (data not shown).

Figure 1

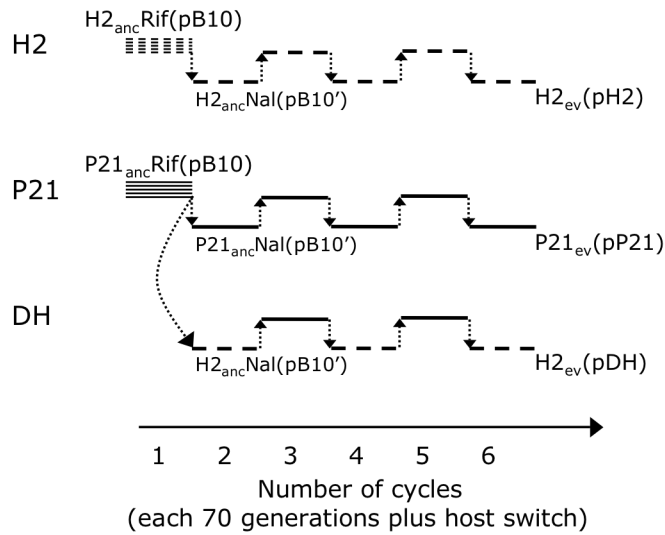


Figure 2

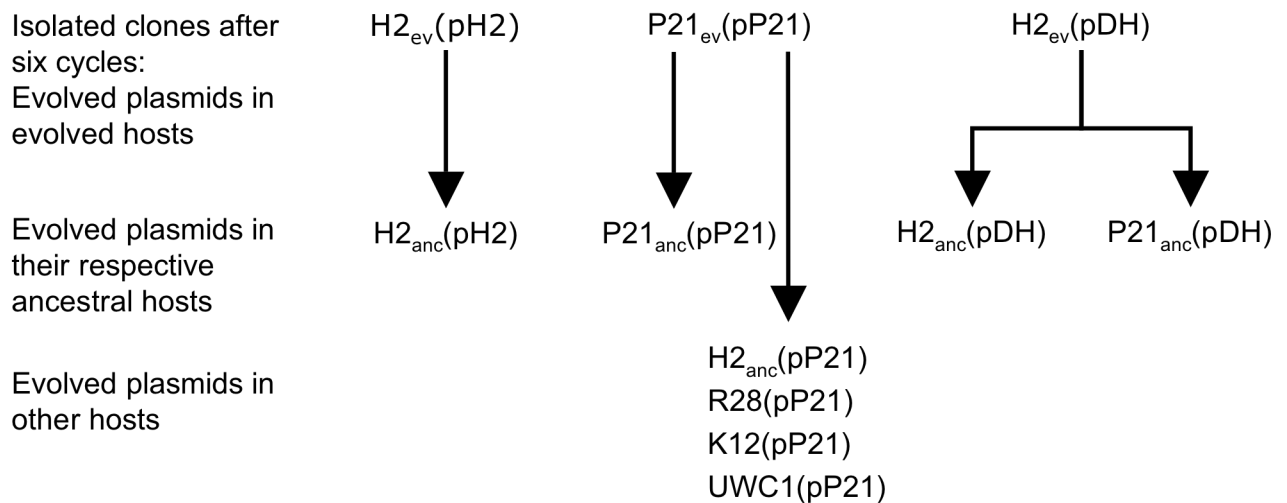


Figure 3

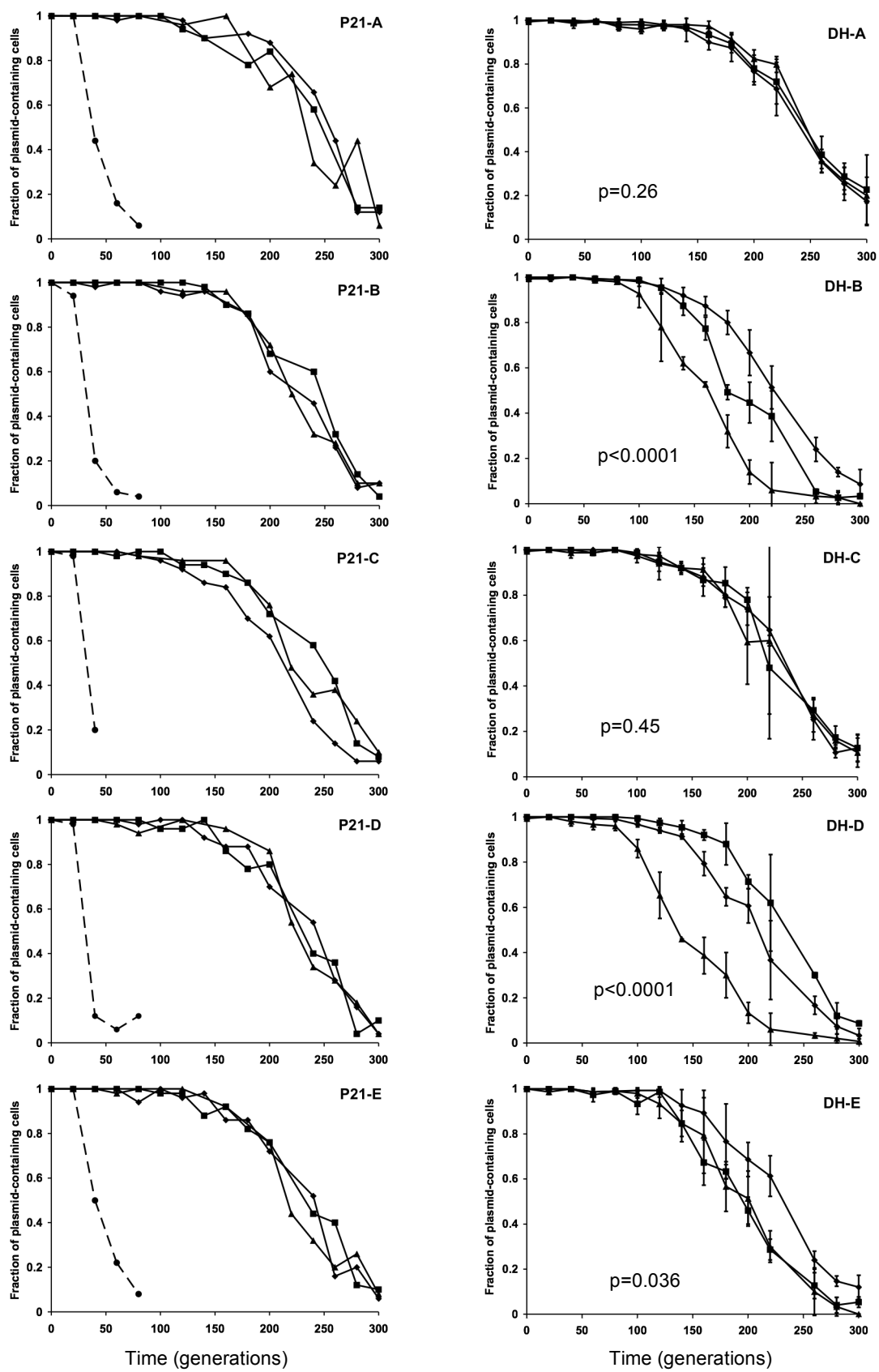


Figure 4

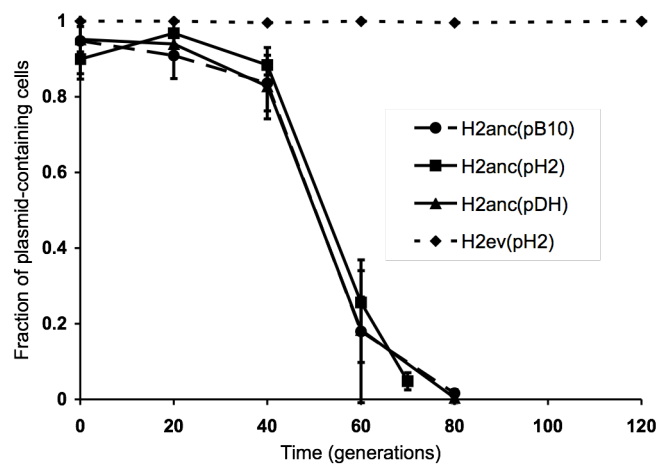


Figure 5

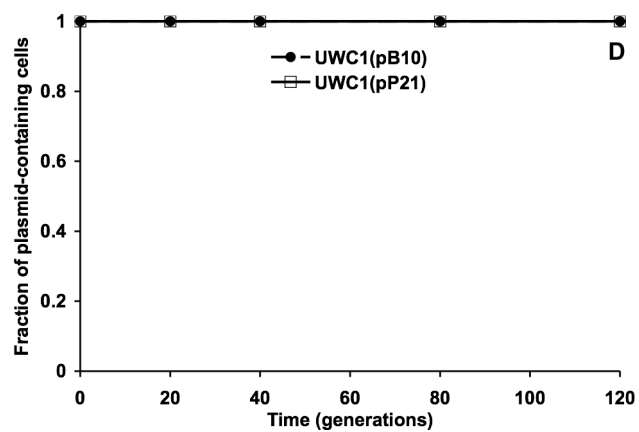
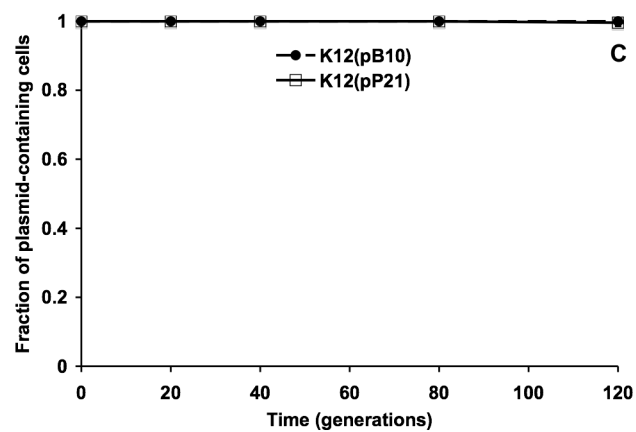
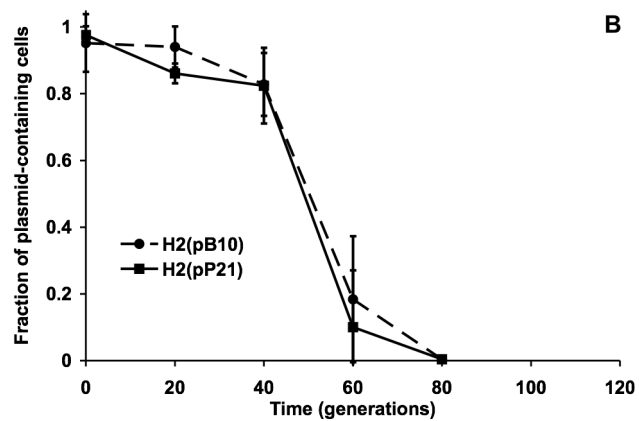
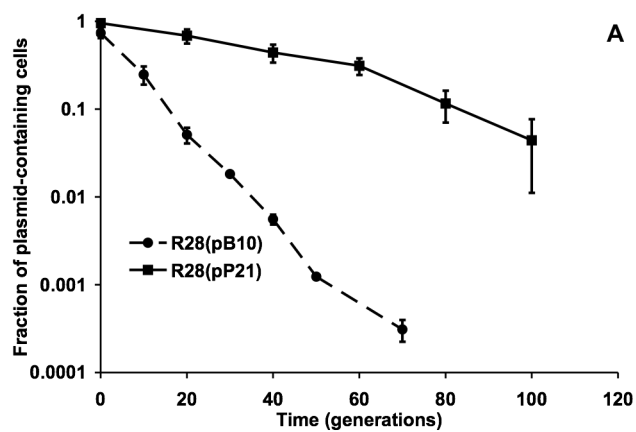


Table 1: Plasmid cost (expressed as % decrease in host fitness) of ancestral and evolved plasmids in ancestral host P21_{anc.}

Lineage	Cost of pB10	Cost of pP21	Model derived cost of pP21	Cost of pDH
	Mean (LCL, UCL)	Mean (LCL, UCL)	MLE (LCL, UCL)	MLE (LCL, UCL).
A	43.2% (37.2%, 49.2%) [†]	10.3% (6.8%, 13.7%)	4.8% (4.06%, 6.85%)	13.6% (8.9%, 18.4%)
B	50.7% (46.5%, 55.0%)	9.1% (6.8%, 11.5%)	4.9% (4.48%, 7.90%)	14.1% (10.9%, 17.3%)
C	42.0% (40.5%, 43.5%) [†]	10.2% (6.9%, 13.5%)	4.3% (3.94%, 7.56%)	10.6% (4.6%, 16.6%)
D	49.7% (41.9%, 57.6%)	12.5% (7.0%, 18.0%)	4.8% (4.42%, 5.31%)	12.3% (6.3%, 19.3%) [†]
E	45.0% (39.4%, 50.6%)	10.1% (3.9%, 16.2%) [†]	5.0% (4.27%, 8.56%)	13.3% (7.9%, 18.8%) [†]
Average	46.1% (44.5%, 47.8%)	10.4% (9.9%, 11.0%)		12.8% (12.2%, 13.4%)

Plasmid cost was determined in two ways. First, cost was measured through competition experiments between the plasmid-bearing and plasmid-free ancestral P21 strain (columns 2, 3, 5). The plasmids used correspond to plasmid ‘1’ in all of the Fig. 3 panels. For each plasmid, the mean plasmid cost was calculated from five replicates (except for data with [†]: only four replicates). To compare the data with model-derived estimates, the lower and upper limit (LCL, UCL) of the 95% confidence interval are presented. The average cost of ancestral and evolved plasmids was calculated from the means of all lineages. Second, the cost of pP21 plasmids was also estimated based on the stability data (Fig. 3) and the

deterministic 'HT' model, as in Ponciano *et al.* (2007) (column 4). 'MLE' denotes 'maximum likelihood estimate', and LCL, UCL the lower and upper limits of Parametric Bootstrap confidence intervals. For the ancestral plasmid, the MLE of the model derived cost of the plasmid (based on one stability pattern for each of the five ancestral plasmid replicates (A-E) was 54.6% (33.3%,126%).