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Evolved plasmid-host interactions reduce plasmid interference cost

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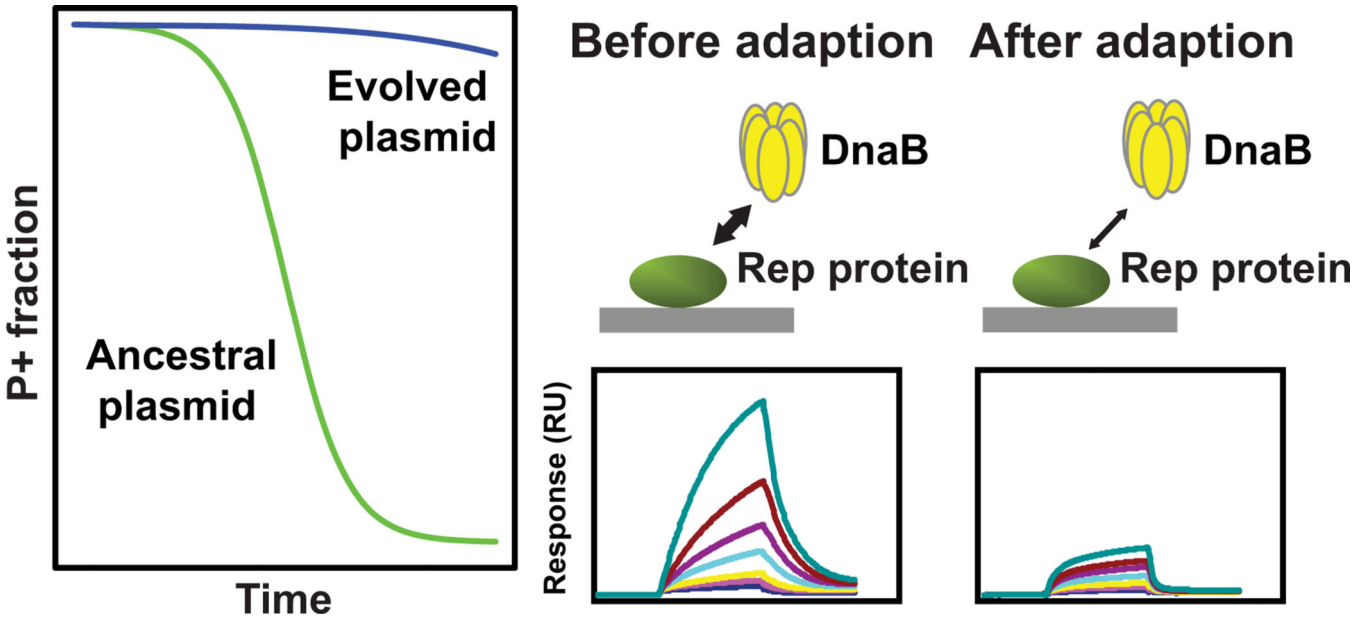
SUMMARY

Antibiotic selection drives adaptation of antibiotic resistance plasmids to new bacterial hosts, but the molecular mechanisms are still poorly understood. We previously showed that a broad-host-range plasmid was poorly maintained in *Shewanella oneidensis*, but rapidly adapted through mutations in the replication initiation gene *trfA1*. Here we examined if these mutations reduced the fitness cost of TrfA1, and whether this was due to changes in interaction with the host's DNA helicase DnaB. The strains expressing evolved TrfA1 variants showed a higher growth rate than those expressing ancestral TrfA1. The evolved TrfA1 variants showed a lower affinity to the helicase than ancestral TrfA1 and were no longer able to activate the helicase at the *oriV* without host DnaA. Moreover, persistence of the ancestral plasmid was increased upon overexpression of DnaB. Finally, the evolved TrfA1 variants generated higher plasmid copy numbers than ancestral TrfA1. The findings suggest that ancestral plasmid instability can at least partly be explained by titration of DnaB by TrfA1. Thus under antibiotic selection resistance plasmids can adapt to a novel bacterial host through partial loss of function mutations that simultaneously increase plasmid copy number and decrease unfavorably high affinity to one of the hosts' essential proteins.

Graphical Abstract

None of the authors have a conflict of interest.

Author contributions: EMT, IK, HY designed the research; HY, KW, WLE, JJ, LMR, GED conducted experiments; HY, WLE analyzed data; HY, EMT wrote the manuscript.



Keywords

Experimental evolution; DNA replication; fitness cost; DNA helicase; plasmid; Rep protein

INTRODUCTION

Horizontal gene transfer can facilitate rapid adaptation of bacterial populations to changes in their environment such as the presence of antibiotics, and plasmids are important players in this evolutionary process (Thomas and Nielsen, 2005; Frost et al., 2005). These mobile genetic elements often encode all the functions required for their replication, stable maintenance and horizontal transfer, in addition to host-beneficial traits such as antibiotic and metal resistance, virulence or catabolism of recalcitrant compounds (Reuter et al., 2014; Conlan et al., 2014; Nojiri et al., 2004; Heuer and Smalla, 2012). However, some plasmids do not carry any identifiable genes that confer a benefit to their host, or they are not under any known positive selection for prolonged periods of time. In theory, even such plasmids can successfully persist as genetic parasites when the following three parameters are balanced: the fitness cost imposed by the plasmid, the segregational loss rate, and the rate of conjugative transfer to plasmid-free cell (Stewart and Levin, 1977; Ponciano et al., 2007; Levin and Stewart, 1980; San Millan et al., 2014). Since plasmids are reservoirs of antibiotic resistance genes, including those that encode resistance to last-resort antibiotics such as colistin (Liu et al., 2015), it is important to understand how temporal selection for plasmid-carrying cells drives plasmid evolution and influences plasmid persistence in the long term.

In the past few decades, experimental evolution studies applied to plasmid-carrying hosts were performed to better understand short-term evolutionary mechanisms of plasmid-host association (Bouma and Lenski, 1988; Modi et al., 1992; De Gelder et al., 2008; Dahlberg and Chao, 2003; San Millan et al., 2014; Harrison et al., 2015). When plasmid-carrying bacteria are grown continuously, mutations that increase their fitness can occur in the host

chromosome, plasmid, or both (Bouma and Lenski, 1988; Modi et al., 1991; Sota et al., 2010; De Gelder et al., 2008; Dahlberg and Chao, 2003; San Millan et al., 2014; Harrison et al., 2015; Loftie-Eaton et al., 2016). However, the detailed molecular mechanisms behind plasmid-host adaption were not clarified in most of these studies. This was due to either lack of available chromosomal sequences of the evolved hosts, or because the studies simply did not go beyond determining DNA sequence or transcriptome changes. Recently a few particular chromosomal mutations were shown to reduce the cost of a non-transmissible or self-transmissible plasmid through changes in gene expression (San Millan et al., 2014; Harrison et al., 2015), but the details of the underlying molecular mechanisms remain to be determined. In our recent study a mini-replicon evolved to reduce its loss rate in diverse hosts by acquiring functions that aid in vertical inheritance, such as a multimer-resolution and toxin-antitoxin systems (Loftie-Eaton et al., 2016). In the coevolved host epistatic interactions with chromosomal mutations were required for improved persistence, but the mechanism behind this epistasis has not yet been revealed.

Some knowledge about the molecular mechanism of plasmid-host adaptation has been obtained from *in vitro* mutagenesis studies. The best example is the adaptation between a *Pseudomonas* plasmid and *E. coli* through mutations in either the plasmid replication initiation protein (RepA) gene (Fernández-Tresguerres et al., 1995) or the host DNA replication initiation protein DnaA (Maestro et al., 2003). Variants obtained in each protein by mutagenesis showed enhanced binding affinity. This typical gain of function mutation most likely reduced the segregational loss rate by increasing plasmid copy number.

When conjugative plasmids have been evolved, impaired transferability as a trade-off for decreased fitness cost was observed in some studies (Turner et al., 1998; Dahlberg and Chao, 2003) but not all (Harrison et al., 2015; De Gelder et al., 2008). A transferability-fitness trade-off was also demonstrated in head-to-head competition assays involving *fin*+ and *fin*− R1 plasmid derivatives (Haft et al., 2009). Although the number of studies is still limited and the molecular details weak, there seems to be a variety of evolutionary pathways towards improved plasmid-host interactions among different plasmid-host systems, and reduction in plasmid cost is often the prime mechanism for improving plasmid persistence.

To understand the adaptation mechanisms of broad host range plasmids and their novel hosts, we previously evolved a non-self-transferrable derivative of IncP-1β plasmid pBP136, namely pMS0506, in a *Shewanella oneidensis* MR-1 host in which the plasmid was poorly maintained (Sota et al., 2010). The mutations that improved plasmid persistence occurred in the 5'-end of the replication initiation protein gene *trfA1*, which encodes one of the two replication initiation proteins conserved in most IncP-1 plasmids (Fig. 1). The evolved plasmids carrying in-frame mutations within *trfA1* showed a ca. 1.5-fold increase in plasmid copy number compared to the ancestral plasmid, whereas the plasmid copy number of frame-shift mutants remained unchanged. In the evolving populations, multiple *trfA* alleles carrying a frame-shift mutation in the 5'-end arose in the early stages of evolution, but *trfA* alleles with an in-frame mutation followed and subsequently swept through the populations (Hughes et al., 2012). Therefore, there are at least two ways by which the plasmid was able to adapt to this host: either through a mutation in the TrfA1 N-terminus that resulted in

simple loss of TrfA1 function, or through in-frame mutations in *trfA1* whose effects remained elusive.

During initiation of replication, the initiator proteins of iteron-containing plasmids like those of the IncP-1 group need to interact with host proteins such as DnaA, DnaB, sliding clamp, and protein chaperones (DnaKJ/GrpE, ClpB, ClpX, ClpA) (Lu et al., 1998; Konieczny et al., 1997; Konieczny and Helinski, 1997a; Wickner et al., 1994; Konieczny and Liberek, 2002; Wickner et al., 1992; Zhong et al., 2003; Kongsuwan et al., 2006; Wawrzycka et al., 2015). The mutations that occurred in the *trfA1* gene during evolution of pMS0506 in *S. oneidensis* were concentrated in the region coding for the predicted second helix within the TrfA1 N-terminus region (Hughes et al., 2012). This region corresponds to the N-terminal part of TrfA-44 of RK2 (TrfA1 ortholog), which was shown to interact with the DnaB helicase of *Pseudomonas aeruginosa* (Jiang et al., 2003; Zhong et al., 2003). Therefore we hypothesize (1) that the initial instability of mini-IncP-1 plasmid pMS0506 can be explained by a negative fitness effect of the TrfA1 protein itself due to the interaction of its N-terminus with DnaB, and (2) that the mechanism of plasmid adaptation to *S. oneidensis* centers on a decrease in the DnaB-binding function of the TrfA1 N-terminus. Here we address these hypotheses using *in vivo* and *in vitro* experiments.

RESULTS

Negative fitness effect of replication protein TrfA1

It was previously shown that the ancestral host *S. oneidensis* MR-1 had a higher exponential growth rate when carrying evolved pMS0506 variants with *trfA1* mutations than when containing the ancestral plasmid (Sota et al., 2010). This is likely due to the evolved plasmids imposing a lower fitness cost than ancestral pMS0506. Here, we first tested our hypothesis that the observed mutations in *trfA1* affect the fitness cost of the TrfA1 protein itself. To do so we cloned the ancestral *trfA1* gene, the evolved in-frame deletion variants (Δ5, Δ43, Δ77), the variants with point mutations (R31P, A25T) or *trfA2* (Fig. 1c) behind the constitutive *lac* promoter in vector pBBR1-MCS2. The growth rates of MR-1 carrying the constructs with evolved genes and *trfA2* were compared to that of MR-1 carrying the ancestral TrfA1. The *S. oneidensis* clone producing TrfA1 showed a lower growth rate than all the clones producing TrfA1 variants or TrfA2 (Fig. 2A). These findings indicate that all five in-frame mutations in the TrfA1 N-terminus ameliorated the fitness cost of the TrfA1 protein itself.

To determine whether the observed fitness advantage of the in-frame mutations in *S. oneidensis* was general across species we conducted additional growth rate analyses for the Gammaproteobacteria *Pseudomonas putida, E. coli*, and the Betaproteobacterium *Cupriavidus pinatubonensis* containing the pBBR1-MCS2 vector with the ancestral or evolved *trfA* genes. The evolved TrfA proteins and TrfA2 imposed a lower cost than TrfA1 on *P. putida* (Fig. 2C), but there were no measurable differences in *E. coli* (Fig. 2B) and *C. pinatubonensis* (Fig. 2D). Thus, the negative fitness effect of the TrfA1 N-terminus, and amelioration of its cost through mutations in this region was not unique to strain MR-1. However, the extent of the difference in cost depends on the host.

To exclude that the positive fitness effects of the TrfA1 mutations in pMS0506 were due to the changes in the TrfA1 and TrfA2 protein expression levels, we determined the TrfA levels produced from pMS0506 variants in MR-1 by Western blotting (Fig. S1). In all instances the expression levels were similar or even higher for the TrfA1 variants and TrfA2 than for the ancestral TrfA1. Thus, improved host fitness was not due to the reduction of protein expression levels.

It was recently shown that a plasmid replication protein can induce an SOS response (San Millan *et al.*, 2015). If TrfA1-mediated cellular stress would lead to DNA damage and thereby induce SOS response, cell elongation should be observed (Dwyer et al., 2012; Huisman et al., 1984). To test this we measured the length of cells in exponential phase using phase contrast microscopy (Fig. S2). The mean length of cells producing TrfA1 tended to be longer than that of cells producing evolved TrfA1 variant Δ43 or TrfA2. This was true for strains carrying the actual IncP-1 replicons and strains containing the cloned *trfA* genes. Furthermore, the coefficient of variation (CV) was generally larger in ancestral TrfA1-producing cells, partly due to the presence of unusually long cells, which were never observed in cells containing evolved TrfA1 or TrfA2 (Fig. S2). This suggests that the nature of the N-terminus of the ancestral TrfA1 may cause DNA damage in a small fraction of the MR-1 population through mechanisms other than plasmid replication.

Effect of TrfA1 N-terminus mutations on the helicase activation activity

It was previously demonstrated *in vitro* for some hosts that the TrfA-44 protein of RK2 (an ortholog of TrfA1) can activate the DnaB helicase at the plasmid's origin of vegetative replication (*oriV*) without the aid of host DnaA (Jiang *et al.*, 2003), and that the second helix in the N-terminus of TrfA-44_{RK2} is essential for this process (Zhong et al., 2003). Since all mutations in the TrfA1 protein of pMS0506 were in this N-terminus we hypothesized that the plasmid adapted to *S. oneidensis* by decreasing the DnaB-binding function of its TrfA1 N-terminus. We first asked whether TrfA1 of pMS0506 also possesses DnaB-loading and activation activity using an FI* assay (also called helicase unwinding assay). When ancestral TrfA1 protein and DnaB of *Shewanella oneidensis* ($DnaB_{son}$) were provided, supercoiled substrate DNA containing *oriV* was unwound (Fig. 3). This indicates that TrfA1 was able to load and activate $DnaB_{son}$ at the *oriV* without the aid of DnaA (Fig. 3). This TrfA1 activity was similar to that observed on DnaB of *Pseudomonas aeruginosa* ($DnaB_{pae}$), whereas for activation of $DnaB_{eco}$ from *E. coli*, host $DnaA_{eco}$ was needed (Fig. 3). Strikingly, the evolved TrfA1 variants were unable to activate $DnaB_{son}$ and $DnaB_{pae}$ *in vitro* in the absence of DnaA, thus making replication of the evolved plasmids DnaA dependent.

Effect of TrfA1 N-terminus mutations on DnaB binding

As our results suggested that the mutations cause a change in the binding affinity between TrfA1 and DnaB, we further tested this by conducting surface plasmon resonance (SPR) analysis. TrfA1 or its variants were immobilized to the sensor chip and $DnaB_{son}$ was provided at different concentrations (Fig. 4). TrfA1 bound $DnaB_{son}$ more efficiently than the TrfA1 variants or TrfA2. The reduced binding affinity between the TrfA1 variants and $DnaB_{son}$ was also confirmed by ELISA tests (Fig. S4). All these results together indicate that mutations in the replication initiation protein impaired interaction between TrfA1 N-

terminus and the host-encoded DnaB. Our results are thus consistent with the findings from Zhong *et al.* (Zhong et al., 2003) that the second helix of TrfA-44 $_{RK2}$ was essential for the *in vitro* activation of DnaB at the *ori V* in the absence of DnaA, and our hypothesis that decreasing this function would improve plasmid persistence in *S. oneidensis* was supported.

We previously found that the evolved pMS0506 variants cannot establish replication in *P. aeruginosa* (Sota et al., 2010). In the case of RK2 replication, the $DnaB_{pae}$-TrfA-44 N-terminus interaction was shown to be required for plasmid replication in *P. aeruginosa* (Jiang et al., 2003). These previous observations suggest that TrfA1 variants cannot sufficiently interact with $DnaB_{pae}$. Here, the ancestral TrfA1 was indeed able to bind $DnaB_{pae}$, but TrfA1 variants barely did (Fig. 4). This result is consistent with the analysis of DnaB-loading activity and evolved plasmid phenotypes, in that such weak binding explains the inability of the evolved plasmids to load DnaB and thus replicate in *P. aeruginosa*.

It should be noted that the TrfA1 variants and TrfA2 were still able to bind $DnaB_{son}$ from *S. oneidensis*, and *E. coli* to some extent (Fig. 4), and were still able to replicate in these hosts (Sota et al., 2010). These results suggest that the TrfA2 domain itself interact with $DnaB_{eco}$ and $DnaB_{son}$, but little with $DnaB_{pae}$.

DnaB overproduction improves plasmid persistence

Based on the results above we postulate that the improved persistence of the evolved pMS0506 variants was due to amelioration of the plasmid fitness cost caused by relaxing the binding of TrfA1 to $DnaB_{son}$. The fitness cost of the ancestral plasmid could either arise from reducing the concentration of free DnaB in the cell (titration model), or from inhibiting essential cellular functions, such as chromosomal DNA replication by formation of a TrfA1-DnaB complex (inhibition model) (Fig. 5). If the titration model is true, overproduction of DnaB should reduce the negative fitness effect of the ancestral TrfA1, and thus improve persistence of the ancestral plasmid in *S. oneidensis* MR-1. If the latter model is more likely, overproduction of DnaB may increase the amount of TrfA-DnaB complex and thereby increase plasmid cost and negatively affect plasmid persistence. These models are not necessarily mutually exclusive. We determined the effect of DnaB overproduction on the persistence of ancestral plasmid pMS0506 and evolved plasmid pEvo-Sh1 by comparing two *S. oneidensis* MR-1 strains wherein we chromosomally inserted a mini-Tn*7* transposon either with or without an extra copy of *dnaB* inducible by IPTG ($dnaB^+$ vs $dnaB^-$). Different concentrations of IPTG were used (0 μM, 10 μM, 100 μM; average curves are shown in Fig. 6; individual curves in Fig. S4 and Fig. S5). When IPTG was added at 10 μM and 100 μM, the ancestral plasmid showed significantly different persistence dynamics in the $dnaB^+$ strain than in the $dnaB^-$ strain (ΔBIC was −24.8 for 10 μM, and −119.9 for 100 μM; statistical analysis results are summarized in Table S3). In a set of follow-up experiments we even observed a significant difference between the $dnaB^+$ strain and the $dnaB^-$ strain at 0 μM IPTG (ΔBIC: −240.8; Fig. S5). For the three paired assays the quasi extinction time of the ancestral plasmid was higher in the $dnaB^+$ strain than when no additional DnaB was expressed, (Table S4), indicating that DnaB overproduction improves plasmid persistence.

To assess which underlying parameters differentiated the persistence dynamics of the evolved and ancestral plasmid, we estimated the fitness cost, loss rate, and initial plasmid-

free cell fraction by model fitting. Although not significant due to too much variation, the maximum likelihood estimates (MLE) of the fitness cost of pMS0506 were lower when *dnaB* was induced. This is consistent with improved persistence and supports our titration model, (Table S4). Moreover, in the *dnaB*+ strain the inferred fitness cost of pMS0506 (0.043) was not different from that of pEvo-Sh1 (0.043) at 100 μM IPTG (Table. S4). However, pEvo-Sh1 was still more stable than pMS0506 even at the highest DnaB induction levels (Fig. 6A, 6B and Fig S6). It should be noted that in the presence of 100 uM IPTG the *dnaB* mRNA levels were >800-fold higher in the *dnaB*$^{+}$ strain according to qPCR (Fig. 6C). We were unable to draw conclusions on comparisons of the plasmid loss rates because the spread on the parameter estimates was too large (Table S4). Finally, the MLE of the initial plasmid-free cell fraction was significantly higher for pMS0506 (0.073) than pEvo-Sh1 (0.012) at 100 μM IPTG, even though the plasmid-containing cells were grown in the presence of antibiotics to prevent plasmid loss before time point 0 (Fig. S6). Together these results suggest that the titration of DnaB by TrfA1 and the resulting interference cost partially but not entirely explains the poor persistence of pMS0506 in host MR-1.

Effect of TrfA1 mutations on plasmid copy number is host specific

In *S. oneidensis* MR-1, a higher plasmid copy number has been observed for the evolved pMS0506 variants with in-frame *trfA1* mutations compared to ancestral pMS0506, yet there was no difference for the evolved plasmids with frame-shift mutations in *trfA1* (thus expressing only TrfA2) (Sota et al., 2010). Such an increase in plasmid copy number can be caused either by TrfA-host factor interactions or by TrfA-plasmid factor interactions (interaction between TrfA molecules or between TrfA and *oriV*). If the first mechanism were true, the increase in copy number could be specific to host *S. oneidensis* MR-1. To test this, we analyzed the effect of the in-frame *trfA1* mutations on plasmid copy number in both *S. oneidensis* and *E. coli*. A copy number reporter plasmid carrying *oriV* was supplied to the strains, which carried either an ancestral or evolved *trfA1* gene in the chromosome (see 'plasmid and strain construction method' section in the supporting information for details). The copy number of the reporter plasmid relative to the chromosome was then determined by qPCR. In *S. oneidensis* MR-1 the copy number was significantly higher for the evolved TrfA1 variants than for either of the three other strains, expressing either TrfA1, TrfA2, or both (the latter three being not significantly different from each other) (Fig. 7A). In contrast, in *E. coli* the plasmid copy number was highest for the ancestral TrfA1, and the evolved TrfA1 variants generated a similar or slightly lower copy number (Fig. 7B). This suggests that the increase in plasmid copy number due to *trfA1* in-frame mutations was specific to host MR-1 and that the plasmid copy number shift is thus likely due to the TrfA1-host interactions. These results suggest that the ability of TrfA1 to generate a higher plasmid copy number than TrfA2 was suppressed in S*. oneidensis* MR-1, possibly due to tight binding to DnaB, and that the in-frame mutations counteracted this. In summary, antibiotic selection for improved plasmid persistence favored a decrease in the ability of TrfA1 N-terminus to bind DnaB, thereby decreasing the interference cost imposed by TrfA1 and increasing plasmid copy number for the *trfA* variants with in-frame mutations.

DISCUSSION

Experimental evolution of plasmid-host interactions

When a plasmid can replicate in a bacterial host, its persistence in the population is determined by various plasmid-encoded functions such as copy number control, partitioning, multimer-resolution, post-segregational-killing, and conjugation. These are expected to improve plasmid persistence by reducing segregational loss rate or increase the frequency of plasmid uptake after plasmid loss. Moreover, any of the plasmid-encoded genes may affect the fitness cost of plasmid carriage, which in turn influences plasmid persistence in the presence of plasmid-free cells. Selection for faster growing plasmid-carrying cells has been shown to drive the evolution of plasmid-encoded functions by either gain of function mutations (Maestro et al., 2002; Maestro et al., 2003; De Gelder et al 2008), acquisition of functions such as toxin-antitoxin and plasmid multimer-resolution systems (Loftie-Eaton et al., 2016), or loss of function mutations (Dahlberg and Chao, 2003). Recently putative loss of function mutations in chromosomal genes was also shown to reduce the fitness cost of plasmids (San Millan et al., 2014; Harrison et al., 2015). In long-term experimental evolution studies with *E. coli* (without plasmids), fitness gain was accomplished not only by simple gain or loss of function of a gene, but also by fine-tuning of protein expression levels (Philippe et al., 2009; Woods et al., 2006; Herring et al., 2006). However, the molecular mechanisms that explain the beneficial mutations observed during plasmid-host coevolution are still poorly understood. In this study, we examined how protein interactions changed during long-term host-plasmid interactions. We found that mutations in the plasmid replication initiation protein TrfA1 improved plasmid persistence by reducing the protein's ability to bind with the host DNA helicase, DnaB. We propose several mechanistic models for how this decreased binding could affect the persistence of the plasmid in the absence of antibiotics.

In the course of adaptation of pMS0506 to the host *S. oneidensis*, both frame-shift mutations and in-frame mutations occurred in the 5'-end of *trfA1* within a plasmid population (Hughes et al., 2012). Frame-shift mutations were the majority in the initial stage of evolution, but in-frame mutations eventually swept through the populations. This implies that the characteristics of in-frame and frame-shift mutants were distinct. While in-frame mutants showed slightly higher plasmid copy number than frame-shift mutants, we were not able to detect significant differences in fitness effects between them on the basis of growth rate analysis (Sota et al., 2010). In this study, we found that the in-frame mutations improved the fitness of the host that carried only the *trfA1* locus cloned into a plasmid vector (Fig. 2), suggesting that the cost of TrfA1 itself can explain the poor persistence of pMS0506 (Fig. 6), and that a decrease in that cost was the primary target of selection. This is consistent with a recent experimental evolution study that focused on the interaction of a non-transferrable plasmid with its *Pseudomonas aeruginosa* host (San Millan et al., 2015). The fitness cost of plasmid carriage was due to enhanced expression of the replication initiation protein, which was in turn caused by the presence of a chromosomally encoded UvrD family DNA helicase, as the only mutation in one of the evolved clones was a nonsense mutation in this helicase gene (San Millan et al., 2015). In our model system the plasmid mutations in *trfA1* alone could explain the improved plasmid cost and persistence (Sota et al., 2010).

Origin of fitness cost of replication protein

We demonstrated that the reduced cost of evolved TrfA1 variants was concomitant with reduced binding affinity to the helicase $DnaB_{son}$ (Fig. 4). This resulted in loss of DnaA-independent DnaB-activation activity *in vitro*, and most likely explains previously observed loss of replication activity of the evolved pMS0506 variants in *P. aeruginosa* (Sota et al., 2010). This suggests that the tight binding of the ancestral replication initiator TrfA1 to $DnaB_{son}$ is the cause of the high plasmid cost imposed by TrfA1 on *S. oneidensis*, leading to poor plasmid persistence. We propose two models that can explain this cost of tight binding. One is a simple titration model in which TrfA1 titrated free DnaB available for chromosome replication. The other is the replication fork arrest, or inhibition model, where the plasmid replication protein blocks progression of DNA helicase on chromosome and plasmids (Fig. 5). This may result in generating single strand DNA that is prone to DNA cleavage and induces SOS response. Interestingly, San Millan *et al.* (San Millan et al., 2015) observed induction of an SOS response in the presence of a chromosome encoded UvrD helicase and a plasmid replication protein. During SOS response, the induced protein SulA inhibits cell division, resulting in elongated cells (Huisman et al., 1984). We found that the fraction of elongated cells was larger in the *S. oneidensis* population that expressed the ancestral TrfA1 than in populations with TrfA2 or evolved TrfA1 variants (Fig. S2). While the means of cell lengths were not always significantly different, the spread in cell length was significantly higher in TrfA1 expressing cells. Thus, SOS response due to strand break after replication fork arrest may be induced in a subset of TrfA1-producing cell populations.

Overexpression of DnaB improved persistence of pMS0506. However, the persistence was not improved to the level of the evolved plasmid tested (Fig. 6), implying that not only the amount of free DnaB but also the TrfA1-DnaB complex negatively affected plasmid persistence. Even when DnaB was overproduced *in vivo* at the highest level, only the initial plasmid-free cell fraction was significantly different between ancestral and evolved plasmid, and not the plasmid cost (Table S4). According to our simulation, the difference in the estimated cost alone cannot account for the 6% of difference in initial plasmid free fraction between pMS0506 and pEvo-Sh1 at time point 0. Thus we speculate that a TrfA1-DnaB complex had a negative effect on plasmid segregation, and not just on fitness cost. Occurrence of partially replicated plasmid molecules caused by replication fork arrest would lead to aberrant plasmid segregation in the presence of a partitioning system, and lead to lower than normal plasmid copy number. Consistent with this the copy number in *S. oneidensis* MR-1 of pMS0506 and its minimal replicon was lower than their respective evolved variants (Fig. 7, see the discussion below). Therefore, although the effect on fitness is unclear, the speculated events in the fork arrest model may occur in the host carrying the ancestral plasmid.

The in-frame mutations in trfA1 are partial loss of function mutations

The change in plasmid characteristics other than its reduced fitness cost (Fig. 2) was a slightly elevated plasmid copy number in *S. oneidensis* MR-1 (Fig. 6). We previously found that in *E. coli, P. putida, C. pinatubonensis*, and *Sphingobium japonicum* pMS0506, which encodes both TrfA1 and TrfA2, can generate higher plasmid copy numbers than its *trfA1* frame-shift mutants that no longer produce TrfA (Yano et al., 2012). However this was not

the case in *S. oneidensis* (Sota et al., 2010). Moreover, the positive effect of the *trfA* in-frame mutations on plasmid copy number was observed only in host *S. oneidensis* MR-1 (Fig. 6). It is thus reasonable to think that the typical characteristic of TrfA1 to elevate plasmid copy number is suppressed in *S. oneidensis* MR-1, possibly due to the tight binding between the TrfA1-N terminus and DnaB. It therefore follows that the in-frame mutations in *trfA1* are essentially partial-loss of function mutations that were selected to simultaneously ameliorate the cost of TrfA1 and slightly increase the plasmid copy number. The TrbC point mutation we previously observed in adaption of IncP-1 plasmid pB10 to *Stenotrophomonas maltophilia* had a similar dual function, as it simultaneously reduced the cost and increased the transferability of the plasmid (De Gelder et al., 2008). Is currently difficult to explain why the DnaB-TrfA1 interactions have a negative effect on host fitness and plasmid copy number in *S. oneidensis* MR-1 and not in other hosts tested. No conclusions can be drawn so far based on the DnaB sequence alone, because of the high number of amino-acid substitutions (see alignment in Fig. S7).

Significance of TrfA1

The question remains why broad host range plasmids possess such a costly TrfA1 protein. According to our growth assay (Fig. 2), not encoding TrfA1 may be beneficial for plasmids to persist in *P. putida*. This was supported by experimental data that used the self-transmissible plasmid pBP136Km, from which pMS0506 was derived (Yano et al., 2012) (Fig. S3). IncP-1 plasmids are unique among iteron-containing plasmids in that most of them encode two forms of replication initiation protein, namely TrfA1 (TrfA-44 in RK2) and TrfA2 (TrfA-33 in RK2). The TrfA2 region is equivalent to Rep proteins from other iteron-containing plasmids such as R6K, P1, F, and pPS10, while TrfA1 has an extended N-terminus in addition to the TrfA2 domain (Zhong et al., 2003). It has been shown that the TrfA1 N-terminus has two distinct intrinsic functions: DnaA-independent DnaB activation (Zhong et al., 2003), and increasing plasmid copy number (Yano et al., 2012). The former function allows replication of RK2 in a *Pseudomonas aeruginosa* strain where DnaA-TrfA-33 interaction is not functional (Caspi et al., 2001; Jiang et al., 2003). This trend seems to be the same in IncP-1β plasmid pBP136 (Fig. 3). The plasmid copy number function on the other hand helps plasmid establishment in recipient cells, thereby contributing to plasmid transmission in bacterial communities where inter-species transfer is common (Klumper et al., 2014). Both TrfA1 functions are important to support promiscuity of IncP-1 plasmids, but the instability of pMS0506 and plasmid copy number suppression in *S. oneidensis* suggests that the former function can interfere with the latter. DnaA-independent DnaB activation was lost during specialization to *S. oneidensis* and the plasmid replication became dependent on host DnaA (Fig. 3). Thus, the role of the DnaA-independent DnaB-activation function of TrfA1/TrfA-44 N-terminus in the host range expansion needs to be reevaluated, because *P. aeruginosa* is by far the only bacterial species where IncP-1 plasmids can benefit from that function. Furthermore our results may explain why most iteron-containing plasmids use DnaA for helicase recruitment.

Concluding remarks

Several experimental evolution studies have shown that plasmids can adapt to their hosts (Sota et al., 2010; De Gelder et al., 2008), hosts adapt to their plasmids (San Millan et al.,

2015; Harrison et al., 2015), and plasmids and hosts can coevolve (Loftie-Eaton et al., 2016). Beneficial mutations that occurred in *S. oneidensis* (pMS0506) during experimental evolution were diverse (Hughes et al., 2012), yet here we showed that the genotypes that swept through the populations were likely selected based on more than one functional improvement, i.e. decreasing interference cost and increasing plasmid copy number (Fig. 2, and Fig. 7). The selection was host-specific, thus pointing to a mechanism for plasmid-host specialization. There have been three plasmid-host experimental evolution studies now wherein the host's DNA helicases and host-plasmid interactions were highlighted as a cause of high plasmid cost (San Millan et al., 2015; Sota et al., 2010; Loftie-Eaton et al., 2016). Future studies on the molecular mechanisms behind these interactions are needed to provide a clear picture of the factors that can limit plasmid persistence. Understanding these factors and possibly determining common mechanisms across bacterial species and plasmids is critical in our attempts to limit the rapid spread of antibiotic resistance among bacterial pathogens.

EXPERIMENTAL PROCEDURES

Bacterial strains and plasmids

The bacterial strains used were as follows: *E. coli* EC100 (Epicentre, Madison, WI, USA); BL 21(DE3) (EMD Millipore, Billerica, Massachusetts, USA); BW25113 (Baba et al., 2006); *Shewanella oneidensis* MR-1 (ATCC700550); *Pseudomonas putida* KT2440 (Bagdasarian et al., 1981); *Cupriavidus pinatubonensis* JMP228 (Amy et al., 1985). Strains EC100 and BL21 (DE3) were used as DNA cloning host and protein expression host for affinity protein purification, respectively. Strains BW25113, MR-1, KT2440, and JMP228 were used to assess fitness effects of TrfA proteins or plasmid persistence. We constructed MR-1 derivatives carrying *trfA* or an additional copy of *dnaB* in the chromosome using the method described in the Supporting Information. A complete list of strains and plasmids used in this study is shown in Table S1.

E. coli, S. oneidensis, and *P. putida* strains were grown in Luria-Bertani (LB) medium. *C. pinatubonensis* JMP228 was grown in 1/10-TSB medium (one-tenth dilution of Bacto tryptic soy broth, [BD Bioscience, Sparks, MD, USA]). All strains were incubated at 30°C except for DNA-cloning experiments.

Plasmid pMS0506 is a non-transferable deletion derivative of pBP136, a plasmid originally discovered in *Bordetella pertussis* (Kamachi et al., 2006). The evolved pMS0506 variants, pEvo-Sh1, pEvo-Sh3, pEvo-Sh5, pEVo-Sh11, pEvo-Sh13, pEvo-Sh14, pEvo-Sh15 were obtained after growing strain *S. oneidensis* MR-1 carrying pMS0506 for 1000 generations in the presence of antibiotic selection for the plasmid (Sota et al., 2010). All evolved plasmids encode the shorter replication initiation protein TrfA2, and pEvo-Sh1, pEvo-Sh3, pEvo-Sh13, pEvo-Sh11 and pEvo-Sh5 additionally encode TrfA1 variant Δ43, Δ77, Δ5, A25T, and R31P, respectively (Fig.1). To address the fitness effects of the expressed wild-type *trfA* gene and its variants, each gene was cloned into pBBR1-MCS2 (Kovach et al., 1995). The pBBR1-MCS2 derivatives were designated, pHY987 (wild-type TrfA1), pHY988 (TrfA2), pHY1010 (R31P), pHY1011 (A25T), pHY1012 (Δ43), pHY1014 (Δ5), pHY1015 (Δ77).

Growth rate analysis

The strains carrying pMS0506 derivatives or pBBR1-MCS2 derivatives were initially streaked on LBA with kanamycin. Single colonies (n=8) were inoculated into 5 ml LB containing Km, and then incubated for 24 hours. The cultures were diluted 100-fold in fresh medium, and 100 ul was transferred to each well of a 96-well microtiter plate. The plate was incubated for 24 hours with agitation at 30°C in BIO-TEK Power Wave HT (BioTek instruments Inc., Winooski, VT, USA). OD_{600nm} was recorded every 10 min. The maximum growth rate of each strain was determined using smoothing function of the Grofit package of R (Kahm et al., 2010). Maximum growth rate (μ) was used for multiple comparisons (ANOVA followed by Dunnett's test) among samples.

Plasmid persistence

The strains carrying pMS0506 or pEvo-Sh1 were first grown in LB with antibiotics for 24 hours, and the culture was subsequently diluted 1024-fold in fresh medium without antibiotics and incubated for 24 hours. This serial batch culture transfer was repeated 10 times. Under this protocol, each 24-h period resulted in 10 generations. Each day the cultures were diluted and spread on non-selective plates. The next day, 52 randomly selected colonies were replicated on selective and non-selective LB agar to determine the number of colonies retaining a plasmid. Where necessary the data were fit to a mathematical segregation and selection model to estimate the initial plasmid-free cell fraction (0), fitness cost (σ) and segregational loss rate (λ) of the plasmids as described by (Ponciano et al., 2007) using a custom software package written for R (Loftie-Eaton et al., 2016). The β0, σ and λ parameters were then used to estimate the time in days till only one 1% ($T_{1\%}$) of the bacteria retained the plasmid. In addition the statistical significance of the difference between two persistence dynamics was evaluated using Bayesian Information Criterion (BIC) as described by us recently (Loftie-Eaton et al., 2016). When comparing two persistence dynamics, negative ΔBIC values (smaller than −2) suggest significant differences, with more negative values pointing to larger differences.

Phase contrast microscopy

Cells from exponential phase cultures (OD_{600nm} = 0.4–0.6 in 1 cm gap cuvette) were suspended in PBS (pH 7.0), then prepared as wet mounts on glass slides. The cells were observed using a Nikon/Andor Spinning Disk confocal microscope with 100× magnification. Images were captured and analyzed using Andor Zyla sCMOS camera and NIS-elements acquisition software, respectively. Cell debris and chained cells were manually removed from the analysis.

Proteins

N-terminally histidine-tagged TrfA proteins were expressed from pET11a derivatives, pHY915 (TrfA1), pHY916 (R31P), pHY917 (A25T), pHY919 (Δ43), and pHY921 (TrfA2). In these proteins, the M124L mutation was introduced to eliminate co-expression of TrfA2 (for details, see supplemental materials). It was previously shown that two point mutations (G254D/S267L) make TrfA-33_{RK2} monomeric and constitutively active in iteron-binding (Blasina et al., 1996). The equivalent mutations (Q279D/S292L) were also introduced into

TrfA1 and its variants. Thus, purified TrfA1 and its variant commonly contained the mutations M124L/Q279D/S292L, and TrfA2 contained Q279D/S292L. C-terminally histidine-tagged *S. oneidensis* DnaB ($DnaB_{son}$) was expressed from pET22b derivative pHY1032. These proteins and *E. coli* DnaA, DnaB, DnaC, and *P. aeruginosa* DnaB (referred to as $DnaB_{eco}$, $DnaC_{eco}$, $DnaB_{pae}$ respectively) were overexpressed in *E. coli* BL21(DE3) and purified using affinity chromatography as described previously (Blasina et al., 1996; Caspi et al., 2000; Caspi et al., 2001).

Western blotting

To quantitate TrfA1 production levels *in S. oneidensis* MR-1, we conducted Western blotting for whole cell lysates made from strain MR-1 carrying pMS0506 or its derivatives, using purified TrfA1, TrfA2, Polyclonal anti-TrfA2 antibodies from rabbit, IRDye 800CW goat anti-rabbit IgG (Rockland, Gilbertsville, PA). Densitometry analysis was performed with an Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE), as described in detail in our previous work (Yano et al., 2012).

Quantification of dnaB transcripts by RT-qPCR

To estimate *dnaB* transcription levels, we performed RT-qPCR on total RNA purified from three replicate stationary cultures of strain HY759 and HY1041, using the PureLink RNA Mini Kit and DNA-*free* Kit (Life Technologies, Carlsbad, CA, USA). Reaction mixtures were made using the SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (ThermoScientific, Waltham, MA, USA), and qPCR was conducted using a one-step RT-qPCR protocol on the ABI 7900HT thermal cycler (Life Technologies). The mRNA copy number was estimated for *dnaB* and *gap* (Glyceraldehyde-3-phosphate dehydrogenase gene) based on standard curves. The *dnaB* to *gap* ratio was determined for each total RNA, and compared between experimental conditions.

Helicase unwinding assay

The helicase unwinding assay [Form I* (FI*) formation assay] was performed as described by (Konieczny and Helinski, 1997b). Reactions contained supercoiled plasmid pMS0506 DNA (1.4 nM), appropriate His-6-TrfA proteins (TrfA1, TrfA1 R31P, TrfA1 A25T, TrfA1 Δ43 or TrfA2) (180 nM), HU (110 nM), SSB (115 nM), DNA gyrase (13 nM) and DnaB helicases (200 nM) from *E. coli, S. oneidensis* or *P. aeruginosa*, as noted. Additionally, reactions with *E. coli* DnaB protein also contained DnaA (7.5 nM) and DnaC (1.1 μM) proteins. The samples were electrophoresed at 25 V for 22 h, and the gel was stained with ethidium bromide.

Surface plasmon resonance analysis

Standard SPR analyses using a BIAcore 2000 were performed essentially as described in the manufacturer's manual. Binding of TrfA proteins by DnaB helicases from *E. coli, S. oneidensis* or *P. aeruginosa* was studied using a CM5 Sensor Chip. TrfA proteins (TrfA1, TrfA1 R31P, TrfA1 A25T, TrfA1 Δ43 and TrfA2) were immobilized on a Sensor Chip surface. Increasing amounts of DnaB proteins (7, 15, 30, 60, 125, 250, and 500 nM) were flown over the Sensor Chip surface in running buffer HBS-EP (150 mM NaCl, 10 mM

HEPES pH 7.4, 3 mM EDTA, 0.005% Surfactant P20). In all experiments the buffer flow was set to 15 μl/min with all injections at a volume of 30 μl. The results are presented as sensorgrams obtained after subtraction of the background response signal from control experiments with buffer injections.

Plasmid copy number analysis

E. coli strains and *S. oneidensis* strains were tagged with mini-Tn*7* carrying the *tac* promoter, *trfA*, and *lacIq* (see Supporting Information). Then, the constructed strains were transformed with pHY872 that carries $ori V_{pBP136}$, but not *trfA* (Fig 1B). The strains harboring pHY872 were grown up to stationary phase. Total DNA was extracted and linearized by EcoRI digestion, ethanol precipitated, and diluted to 5 to 10 ng/ul. This DNA was used as template for qPCR, which was performed using an ABI 7900HT thermal cycler, Fast SYBR Green Master Mix (Life technologies), primer sets, tetAF and tetAR for pHY872, Ecoli_atpF and Ecoli_atpR for *E. coliatpB* in the *oriC* region, atpF and atpR for MR-1 *atpB* (Table S2). Vectors pHY873 or pHY924 digests were used as copy number control. The *tetA* to *atpB* ratio was used to represent plasmid copy number.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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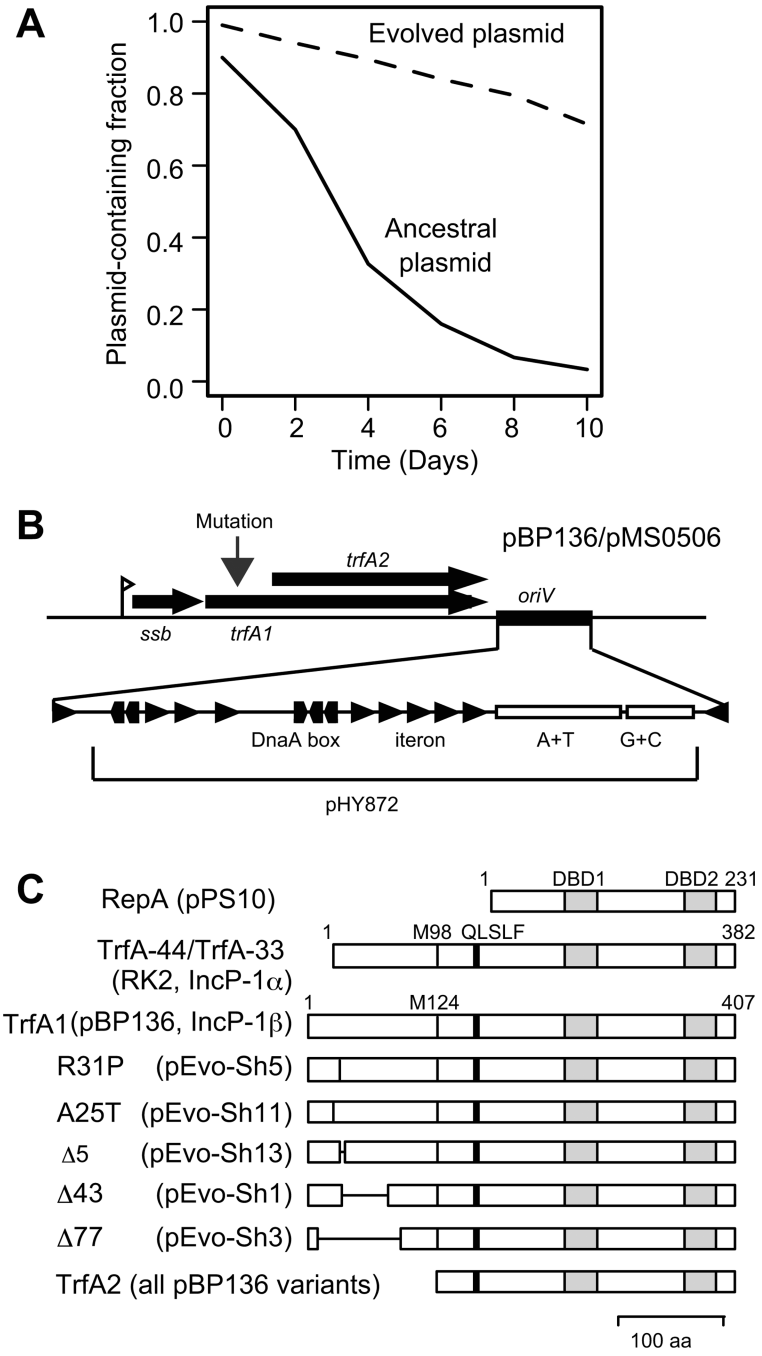

Fig 1. Improvement of plasmid persistence after plasmid adaptation to *S. oneidensis*

(A) Representative plasmid persistence curves in the absence of selection for the plasmid. Curves were redrawn based on data from our previous study (Sota et al., 2010). (B) Genes and sites around the *trfA* locus in wild-type pBP136 and its derived mini-replicon pMS0506, used in this study. Triangles indicate iterons and pentagons indicate DnaA-boxes. (C) Locations of TrfA1 mutations and conserved motif in Rep proteins. DBD1 and DBD2 both indicate iteron-binding motifs, M98 and M124 are start methionines for TrfA-33 and TrfA2,

respectively. QLSLF is a motif associated with binding to sliding clamp (Kongsuwan et al., 2006).

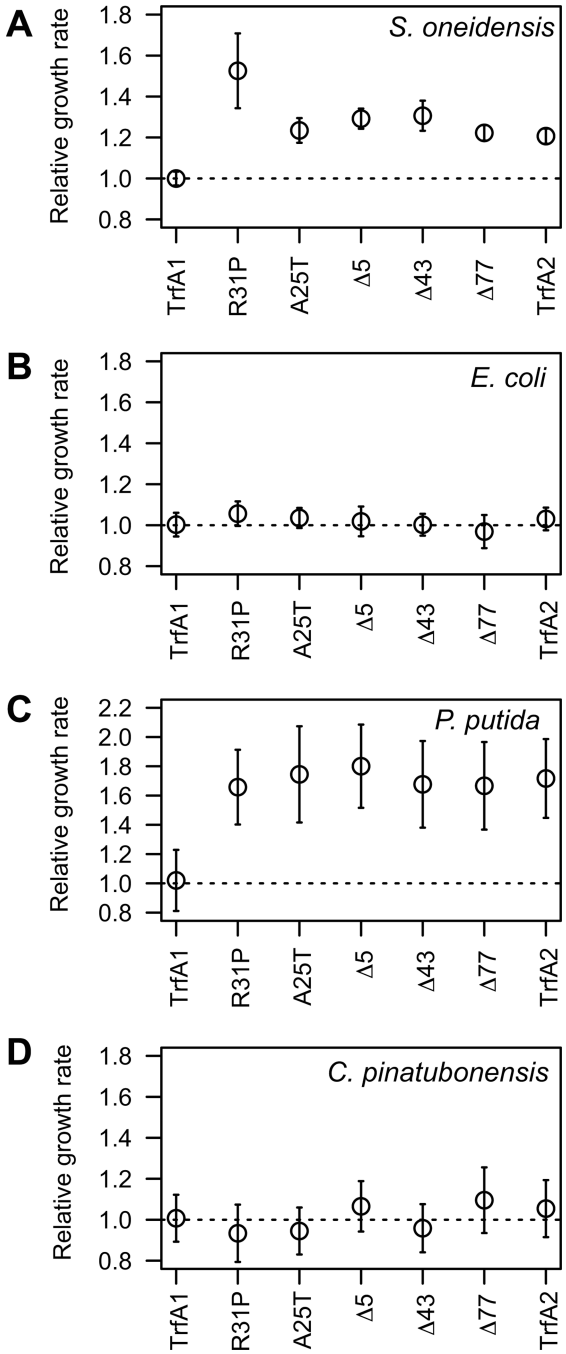

Fig 2. Effect of TrfA1 mutations on maximum growth rates

Relative maximum growth rates of bacterial cells carrying a cloned *trfA* gene are shown. A: *S. oneidensis*; B. *E. coli*; C, *P. putida*; D, *C. pinatubonensis*. Open circles represent means and error bars standard deviations. The M124L mutation was introduced into *trfA1* and all its variants. For *S. oneidensis* and *P. putida*, the strain carrying wild-type *trfA1* had a significantly lower growth rate than the same strain with *trfA1* variants ($P < 0.001$, in multiple comparisons in Dunnett's test), but this was not the case for the other two hosts.

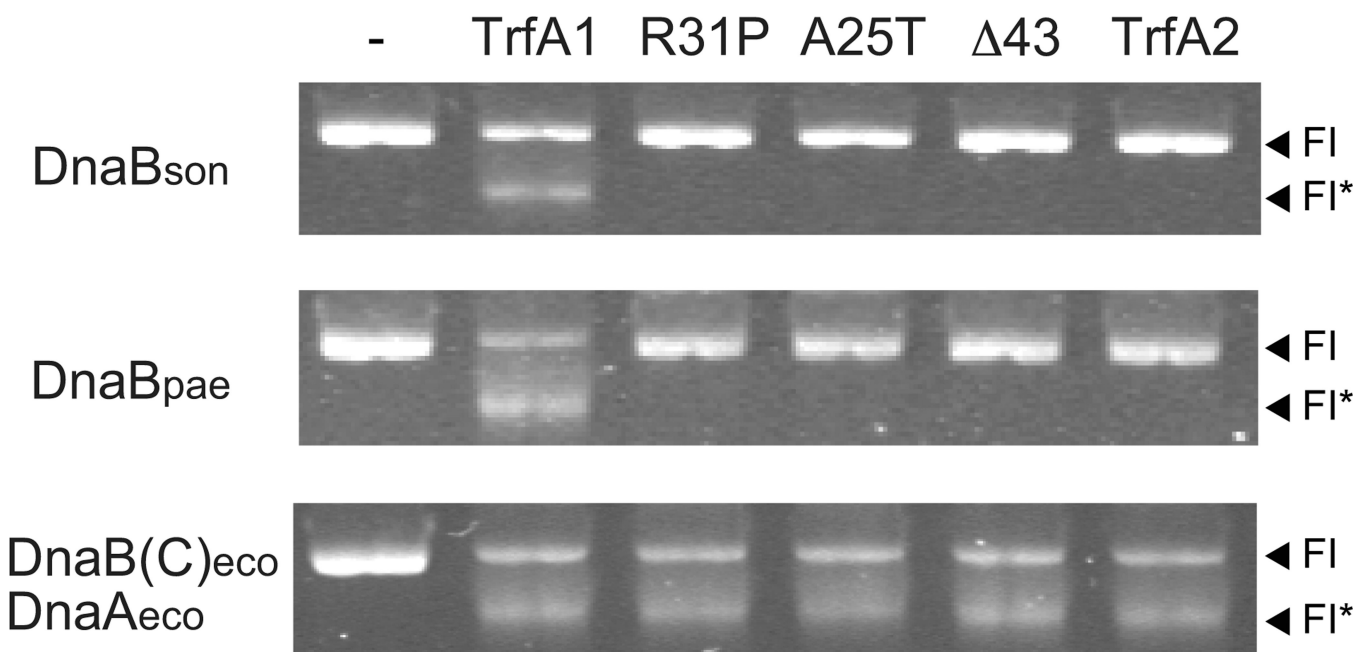

Fig 3. DnaB-loading and activation activity of TrfA1 and its variants

FI indicates the position of supercoiled substrate, pMS0506, containing *oriV*. FI* indicates the position of unwound substrate, and is indicative of DnaB-loading and activation activity. The 1st lane (left) indicates a control reaction without TrfA protein. To make constitutively monomeric TrfA, Q279D/S292L mutations were introduced into all TrfA proteins. A M124L mutation was also introduced into all TrfA1 variants to avoid production of TrfA2.

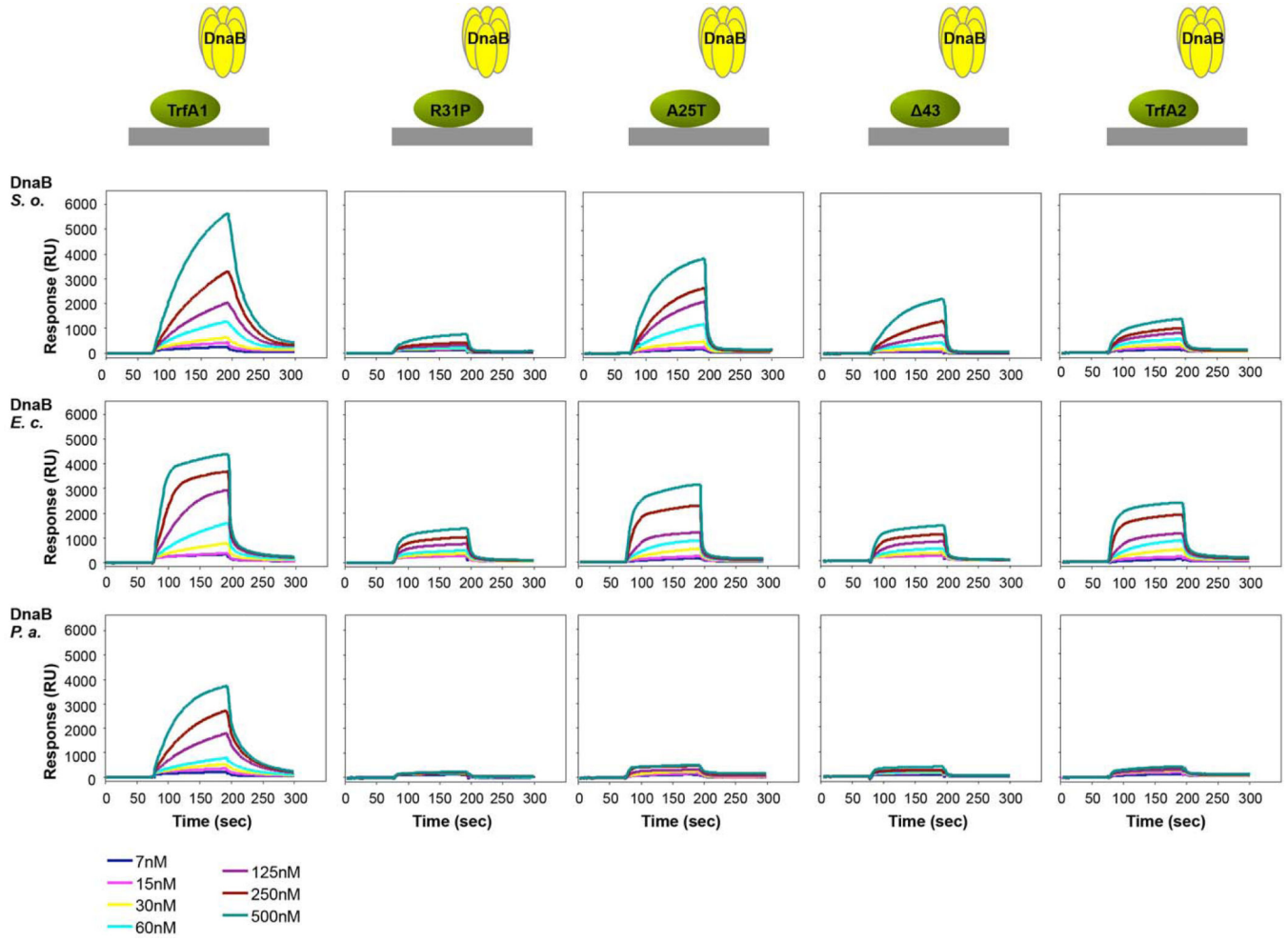

Fig 4. Analysis of DnaB helicases interactions with TrfA proteins

Interactions of DnaB helicase from *S. oneidensis, E. coli* or *P. putida* with monomeric forms of TrfA1, R31P, A25T, Δ43 and TrfA2 proteins were analyzed with Surface Plasmon Resonance. TrfA proteins were immobilized on the surface of CM5 sensorchip. Increasing amounts of DnaB proteins (7, 15, 30, 60, 125, 250, and 500 nM) were flown over the sensor chip. TrfA proteins contain the M124L/Q279D/S292L mutations.

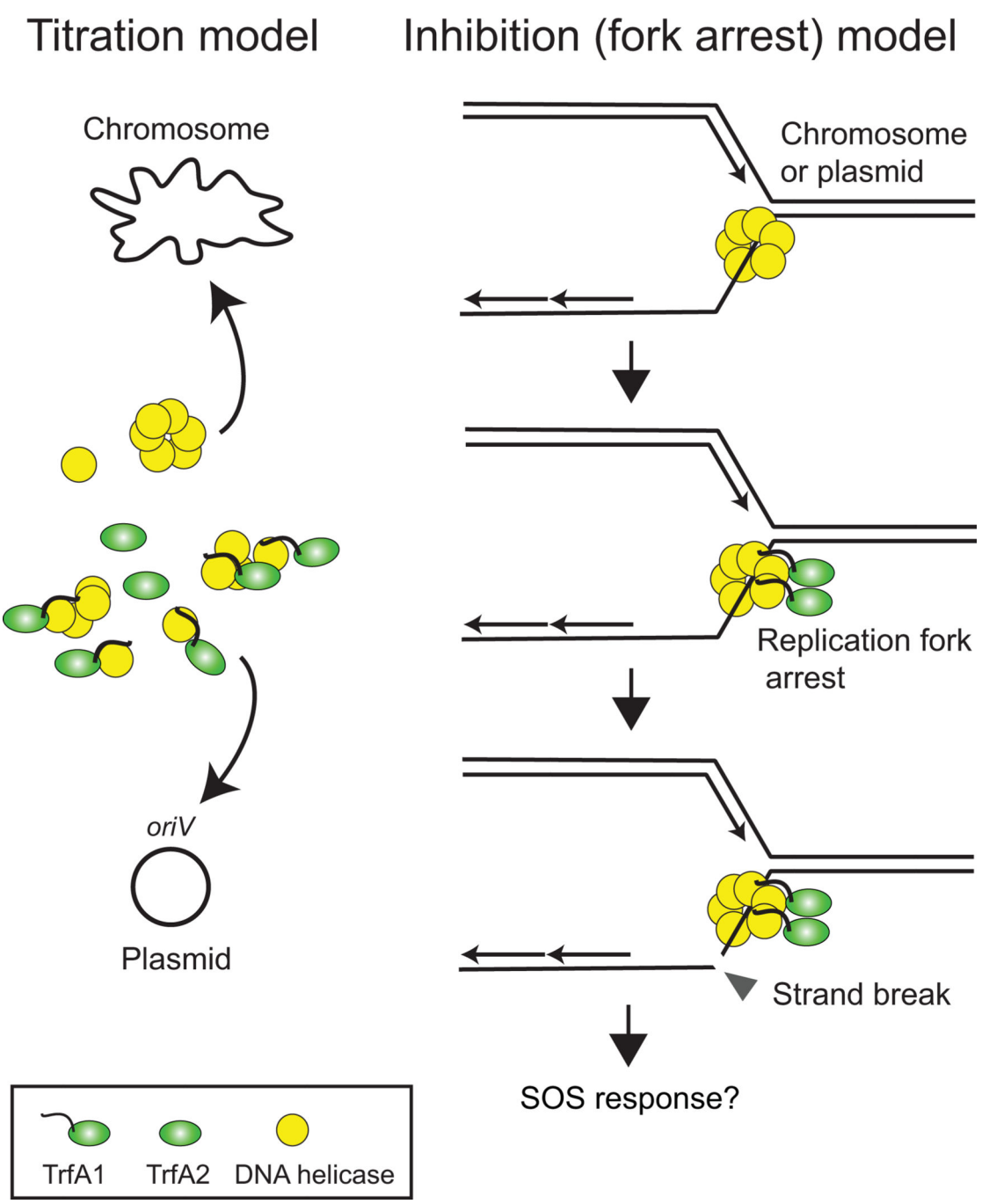

Fig.5. Two models for the possible cause of plasmid interference cost associated with the replication initiation protein

Left: Titration model. TrfA1, which shows high affinity to DnaB DNA helicase, reduces the amount of free DnaB available for chromosomal replication and repair. Right: Inhibition model. The TrfA1-DnaB complex occurring at the replication fork arrests replication or repair, leading to exposure of single strand DNA followed by strand break. This may occur on both the plasmid and chromosome.

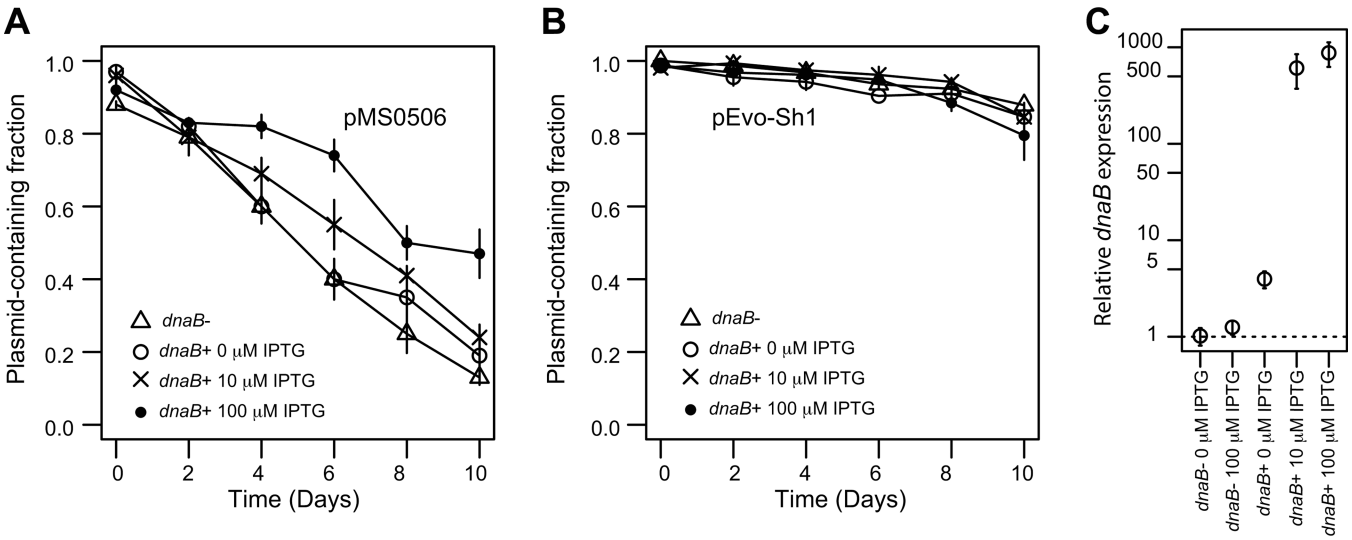

Fig 6. Effect of DnaB overproduction on plasmid persistence in *S. oneidensis*

The data points indicate means and standard deviations from triplicate assays. (A) Persistence of pMS0506. In this data set, the *dnaB*$^{+}$ strain with 10 μM IPTG, and 100 μM IPTG showed improved persistence compared to the *dnaB*$^{-}$ strain (See ΔBIC test in Table S3, and quasi-extinction time in Table S4). In a second set of experiments for 0 μM IPTG condition, a significant difference was observed between *dnaB*$^{+}$ and *dnaB*$^{-}$ strains (Fig. S5). (B) Persistence of pEvo-Sh1. (C) Relative *dnaB* mRNA levels determined by qPCR; the mean value for the *dnaB*$^{-}$ strain, 0 μM IPTG, was represented as 1 in this plot. Error bars indicate SD. The host used were *S. oneidensis* HY1014 (chr::mini-Tn7-*tac*$_{p}$-*dnaB; dnaB*$^{+}$), and *S. oneidensis* HY0759 (chr::mini-Tn*7; dnaB*$^{-}$). For the *dnaB*$^{-}$ strain, only IPTC 100 μM conditions were shown for simplicity.

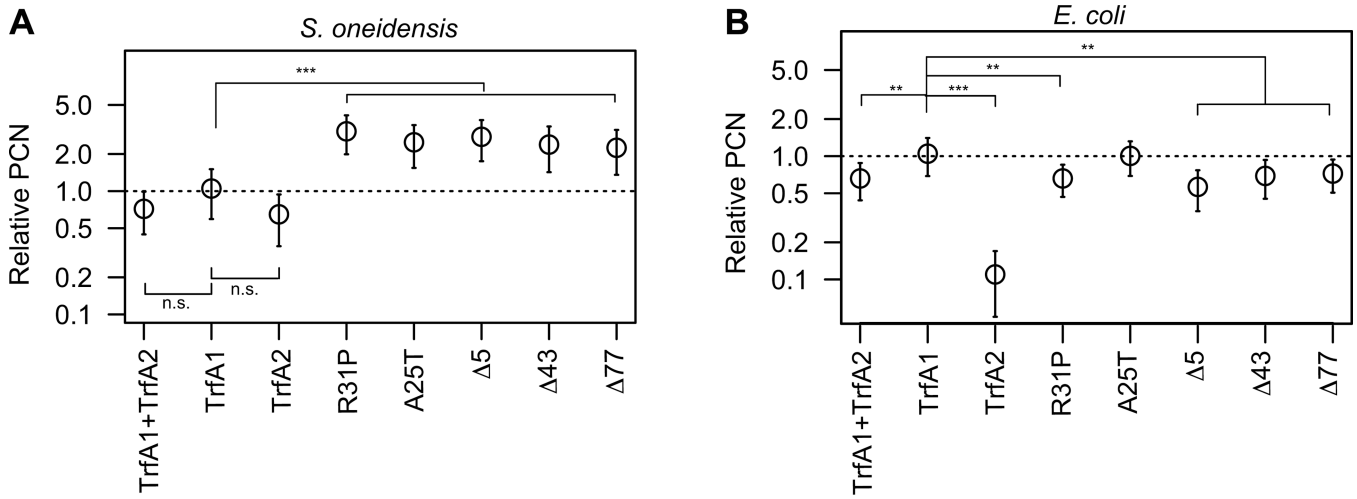

Fig 7. Effect of TrfA1 mutations on plasmid copy number

(A) Relative plasmid copy number (PCN) in *S. oneidensis*. The copy number of pHY872 (Fig.1) for the TrfA1 condition is represented as 1. The data are shown on a logarithmic scale. (B) Relative PCN in *E. coli*. TrfA proteins were expressed from the chromosome (see materials and methods for details) 'TrfA1+TrfA2' indicates expression of two proteins from the wild-type *trfA1* gene. The M124L mutation was introduced into TrfA1 and TrfA1 variants. In (B), the data for TrfA1+TrfA2, TrfA1, and TrfA2 were previously published (Yano et al., 2012). The *trfA*-tagged strains used were HY0321, HY0323, HY0325, HY0329, HY0333, HY0337, HY0341, HY0345 for panel (A), and HY0414, HY0390, HY0391, HY0392, HY0395, HY0408, HY0409, HY0475 for panel (B). Comparisons were made using the post-hoc Tukey test for the data before normalization to relative PCN (**, P <0.01; ***, P <0.001; n.s., not significant). Data are obtained from at least four independently grown cultures for each clone.