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Within-host competition selects for plasmid-encoded toxin–antitoxin systems

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Toxin–antitoxin (TA) systems are commonly found on bacterial plasmids. The antitoxin inhibits toxin activity unless the system is lost from the cell. Then the shorter lived antitoxin degrades and the cell becomes susceptible to the toxin. Selection for plasmid-encoded TA systems was initially thought to result from their reducing the number of plasmid-free cells arising during growth in monoculture. However, modelling and experiments have shown that this mechanism can only explain the success of plasmid TA systems under a restricted set of conditions. Previously, we have proposed and tested an alternative model explaining the success of plasmid TA systems as a consequence of competition occurring between plasmids during co-infection of bacterial hosts. Here, we test a further prediction of this model, that competition between plasmids will lead to the biased accumulation of TA systems on plasmids relative to chromosomes. Transposon-encoded TA systems were added to populations of plasmid-containing cells, such that TA systems could insert into either plasmids or chromosomes. These populations were enriched for transposon-containing cells and then incubated in environments that did, or did not, allow effective within-host plasmid competition to occur. Changes in the ratio of plasmid- to chromosome-encoded TA systems were monitored. In agreement with our model, we found that plasmid-encoded TA systems had a competitive advantage, but only when host cells were sensitive to the effect of TA systems. This result demonstrates that within-host competition between plasmids can select for TA systems.

Keywords: plasmid; toxin–antitoxin system; within-host competition

1. INTRODUCTION

Toxin–antitoxin (TA) systems are found on many plasmids (Gerdes *et al.* 2005; Guglielmini *et al.* 2008). They consist of a tightly linked TA pair. If a plasmid encoding a TA system (TA+) is not inherited by a daughter cell following cell division, the activity of the antitoxin declines, allowing toxin action and consequent cell growth inhibition or death (Jensen *et al.* 1995). Selection for TA+ plasmids was originally hypothesized to arise directly from the inhibition of plasmid-free segregants, freeing resources that could be exploited by remaining plasmid-containing cells (Gerdes *et al.* 1986, 2005; Hayes 2003; Brendler *et al.* 2004). Theoretical work has examined the conditions required for this selection (Mongold 1998; Mochizuki *et al.* 2006). A key finding has been the requirement of within-cell plasmid competition between TA+ and TA− plasmids to provide an advantage to TA+ plasmids. Consistent with this prediction, experimental work has found that TA systems do not provide a competitive advantage in well-mixed environments, where resources freed by cell killing can be shared by all cells, unless competing TA+ and TA− plasmids can co-infect host cells (Cooper & Heinemann 2000).

Several authors have presented models which predict that the different fates of TA+ and TA− plasmids following co-infection of a cell can provide an advantage for TA+ plasmids (Heinemann 1998; Mongold 1998; Cooper & Heinemann 2005; Mochizuki *et al.* 2006). Incompatibility between two plasmids, resulting from the use of the same type of replication control, will sort the plasmids into separate lineages (Nordstrom & Austin 1989). Cells inheriting the TA+ plasmid remain viable, owing to continued production of the antitoxin. By contrast, cells inheriting only the TA− plasmid are exposed to the toxin as the concentration of the antitoxin declines. This exposure is predicted to inhibit the growth of the cell and the resident TA− plasmid, causing a corresponding increase in the relative fitness of TA+ plasmids. Experimental work has supported this prediction, finding that the asymmetry in the outcome of plasmid segregation provided an advantage to TA+ plasmids when co-infection could occur (Naito *et al.* 1995; Cooper & Heinemann 2005). An interesting possibility, emphasized by Mochizuki *et al.* (2006), is that this advantage might depend on environment structure. In a well-mixed environment, TA plasmids would only have an advantage when initially present at a relatively high frequency. In a structured environment, TA plasmids could invade from low initial frequencies.

One aspect of the ecology of TA systems that has not yet been addressed concerns the possibility of a differential benefit to the system depending on whether it is located on a plasmid or a chromosome. TA systems are frequently associated with mobile elements, thus they can be expected to be introduced to both plasmids and chromosomes. Within-host competition can provide a relative advantage to TA+ over TA− plasmids, but the generality of this advantage is not clear. For example, will the same mechanism

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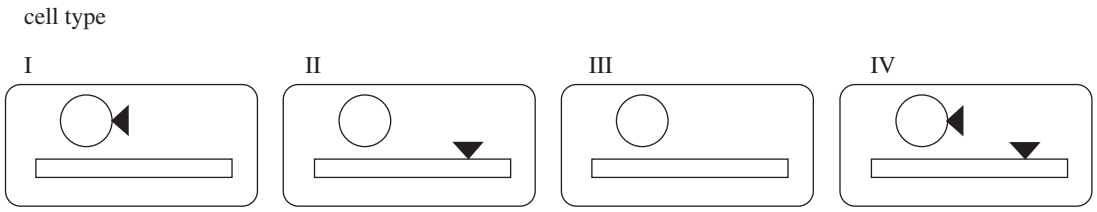

initial ratio in test of prediction				
no. 1:	1	100	100	0
no. 2:	1	100	0	0

Figure 1. Schematic of experimental treatments. TA+ transposons (triangles) were introduced into cells containing a conjugative plasmid (circles). Transposons integrated into plasmids or chromosomes at a ratio determined by their respective sizes. Transposon-containing cells were incubated and competed in environments to test two theoretical predictions. Prediction 1: plasmid co-infection can occur in cells that are not protected by a chromosomal TA system (type III). TA+ plasmids are expected to have an advantage in this environment. Prediction 2: plasmid co-infection almost always occurs in the presence of a chromosomal TA system that renders the cell immune to loss of a TA+ plasmid. This immunity means that TA+ plasmids are not expected to have any advantage.

confer an advantage to plasmid, relative to chromosomal, TA systems? An intuitive answer to this question is complicated by the fact that chromosomal TA systems provide immunity to host killing following loss of a TA+ plasmid (Cooper & Heinemann 2000, 2005; Takahashi *et al.* 2002; De Bast *et al.* 2008). Because chromosomes represent a much larger target size for any incoming mobile TA system, widespread immunity may inhibit the ability of TA+ plasmids to outcompete TA− plasmids.

Here, we develop a model to predict and test the conditions in which plasmid-encoded TA systems have an advantage relative to chromosomal systems. We find that plasmid TA systems do have an advantage, and are able to invade from a low initial frequency, but only when TA-mediated cell death can occur.

(a) *Model predictions*

To provide context for our experiments, we first present results of a simulation model similar to that of Mochizuki *et al.* (2006). This model tracks the relative success of plasmid and chromosomal TA systems in environments with an arbitrary degree of spatial structure. For clarity, we have set off details to the electronic supplementary material. Here, we provide a brief outline of the model together with relevant predictions. An implementation of the model is available from T. Paixão on request.

Our model considers the case when a new TA system enters a population of plasmid-containing cells. We consider three initial cell types: TA− chromosomes with TA+ plasmids, TA+ chromosomes with TA− plasmids and TA− chromosomes with TA− plasmids (corresponding to cell types I, II and III, respectively, in figure 1). The initial proportion of plasmid to chromosomal TA systems (1 : 100) was chosen to reflect their relative target sizes. The model tracks the fate of these cell types, as well as newly arising co-infected cells (cell type IV, figure 1), as cells divide and plasmid transfer and competition occurs. Here, we present predictions assuming a high degree of spatial structure, but qualitative outcomes are robust to this assumption (electronic supplementary material, figure S1). With respect to the relative fitness of plasmid to chromosomal TA systems, the model makes two key predictions.

(i) *Conjugative plasmids can provide an advantage to TA systems*

Figure 2*a* shows the outcome of a simulation-competing plasmid-containing cells with TA systems on either a plasmid or a chromosome when competing against otherwise isogenic TA− cells. We find that plasmid TA systems have a significant advantage in this environment. This advantage could be owing to some aspect of co-infection and within-host competition, or simply to the fact that, unlike chromosomal systems, plasmid-encoded TA systems are able to replicate horizontally as well as vertically. To distinguish between these possibilities, we repeated the simulation substituting a control TA− marker for the plasmid TA+ system. Plasmid-encoded copies of this marker can replicate horizontally, but do not confer any advantage during within-host competition. In this case, we found only a very small advantage to plasmid-encoded copies of this marker during population growth (figure 2*a*). Therefore, horizontal transfer alone was not sufficient to explain the success of plasmid TA systems.

(ii) *Success of plasmid TA systems requires death of competing plasmids*

To test our expectation that death of cells and TA− plasmids following displacement of competing TA+ plasmids was responsible for the advantage of plasmid-encoded TA systems, we repeated the simulation above, except omitting the subpopulation of TA− cells. Here, almost all cells initially had a chromosomal TA system and were, therefore, immune to the action of the toxin. In this environment, plasmid-encoded TA systems had only a small advantage relative to chromosomal systems (figure 2*b*). Therefore, competition between TA+ and TA− plasmids in sensitive cells was necessary for an advantage of plasmid-encoded TA systems.

(b) *Experimental system*

To test our prediction that plasmid-encoded TA systems will be more successful than chromosomal systems when sensitive cells were present in the environment, we introduced a TA+ transposable element into a population of cells containing a conjugative plasmid. The element was introduced into cells on a suicide vector such that it

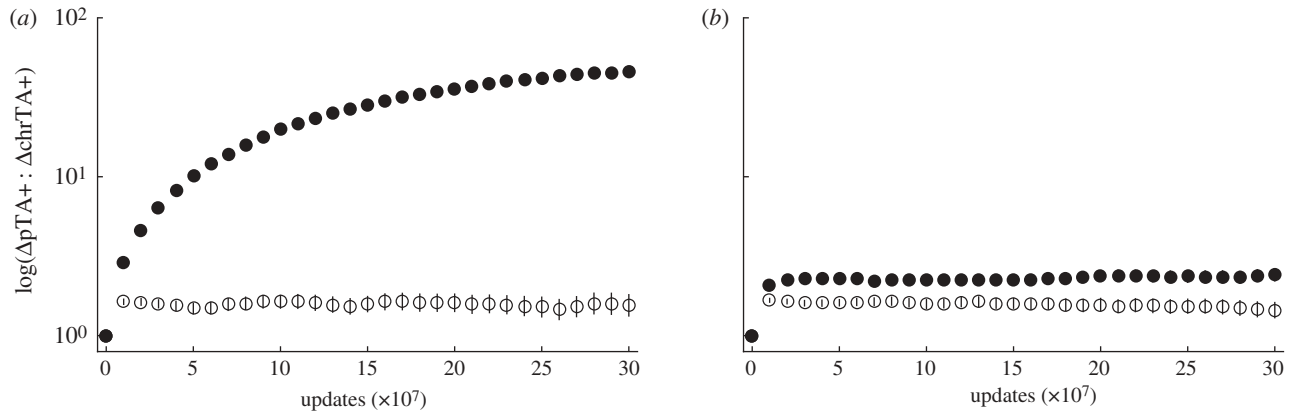

Figure 2. Simulation showing the effect of a subpopulation of toxin-sensitive cells in determining the success of plasmid-encoded TA systems. (*a*) Competition tracking the ratio of plasmid : chromosome TA systems when a subpopulation of TA− cells (no chromosomal or plasmid TA system) is present (corresponding to prediction 1 in text and figure 1). (*b*) Competition tracking the ratio of plasmid : chromosome TA systems when a subpopulation of TA− cells is *not* present (corresponding to prediction 2 in text and figure 1). Each point represents an average of 100 independent simulations and error bars are the s.e.m. Simulations here were performed enforcing strictly local interactions for plasmid transfer and cell competition. Filled circles, TA+ systems; hollow circles, control TA− competitions; 10^4 updates correspond to approximately one population generation.

was only stably maintained in cells in which it transposed into the resident plasmid or chromosome. The outcome of this process was the generation of TA+ : TA− and TA− : TA+ plasmid : chromosome subpopulations at a ratio of approximately 1 : 100 (see §3).

We monitored the change in the ratio of plasmid- to chromosome-encoded TA systems during population growth in environments corresponding to those considered in our two theoretical predictions. In the first, cells containing a TA system were mixed with plasmid-containing cells that were sensitive to the action of the TA system. Here, co-infection of cells with TA+ and TA− plasmids frequently occurred in cells that did not contain a chromosomal TA system and the ratio of plasmid- to chromosomal-encoded TA systems was predicted to increase over time (figure 2*a*). In the second, all cells initially contained either a plasmid or chromosomal TA system. Co-infection of TA+ and TA− plasmids usually occurred in the presence of a chromosomal TA system, which is the majority subpopulation. Here, our model predicts that plasmid TA systems will have a much smaller advantage over their chromosomal counterparts (electronic supplementary material, figure S3).

2. MATERIAL AND METHODS

(a) *Bacteria and plasmids*

Jp145, a derivative of the F plasmid conferring kanamycin resistance (Km^r), was used as the progenitor plasmid throughout the experiment (Heinemann *et al.* 1996). Jp145 can replicate vertically, through inheritance by daughter cells during cell division, and horizontally, by conjugation. In the environmental conditions we used, co-incubation of cells containing different plasmid types resulted in frequent co-infection because the strength of plasmid surface exclusion is reduced during the stationary phase (Cooper & Heinemann 2005). JHC514a was used as the host strain in all competition experiments (Heinemann *et al.* 1996). This strain is *recA*$^-$, preventing possible complications that could arise from plasmid–chromosome recombination. A spontaneous nalidixic acid-resistant (Nx^r) derivative, TC107, was isolated from JHC514a for use in the plasmid localization assay.

Construction of mini Tn*10* transposons containing the *hok/sok* or *parDE* TA systems and a gentamicin-resistance determinant (Gm^r) on a conjugative suicide vector unable to replicate in JHC514a has been described previously (Alexeyev & Shokolenko 1995; Cooper & Heinemann 2000). These systems are representatives of two TA families; *hok/sok* is RNA-based and *parDE* is protein-based. Two control TA− transposons, conferring Gm^r or chloramphenicol (Cm^r) resistance, were also used. The mini-Tn*10* transposons contain a mutation that reduces insertion site bias (Kleckner *et al.* 1991). Our method therefore reflects the situation where a mobile element encoding a TA system initially infects a population, creating a heterogeneous population of cells. Introduction of transposons to recipient bacteria was carried out using a donor : recipient ratio of 1 : 10. Combined with a relatively low transposition rate, this protocol is very unlikely to result in any recipient cells containing more than one inserted transposon.

(b) *Media*

Liquid and solid media were supplemented with antibiotics at concentrations used previously (Cooper & Heinemann 2000). Cells were grown with shaking overnight at 37°C in Luria-Bertani-Herskowitz (LBH) medium supplemented with antibiotics as appropriate to maintain plasmids, diluted 100-fold in fresh antibiotic-free media and grown with shaking to mid-log phase prior to all competition experiments.

(c) *Competition experiment*

Cells were inoculated into fresh LBH medium in combinations described in §3 and incubated at 37°C for 10 days. Incubation was static except for a brief vortex each day to allow for new cell–cell contacts. In these conditions, cells reached a maximum density of approximately 3×10^9 cfu ml^{-1}, declining approximately 10-fold after 10 days incubation. Jp145 encodes native TA systems, but these were common to all plasmids in our treatments and thus do not differentially affect plasmid competition. We therefore refer to the progenitor plasmid as TA−. All competition experiments were performed with fourfold replication.

(d) *Fitness costs and plasmid transfer rates*

Fitness costs of *Hok/Sok* and *ParDE* TA systems were estimated by mixing at 1 : 1 control TA− Cm^r plasmid-containing cells with either TA+ Gm^r *hok/sok*, TA+ Gm^r *parDE* or control TA− Gm^r plasmid-containing cells. Mixes were competed in the same environment used for the competition experiments except that plasmids were introduced in JHC510, a derivative of JHC514a that does not support plasmid transfer (Heinemann *et al.* 1996). We report selection rate constants (r) to account for cell death occurring during competition (Travisano *et al.* 1995). We did not find any significant effect of TA systems on fitness (two-tailed t-test comparing relative fitness of TA− Cm^r versus: TA− Gm^r $r = -0.013$, $p = 0.667$; TA+ *hok/sok* $r = -0.026$, $p = 0.167$; TA+ *parDE* $r = -0.022$, $p = 0.196$). The lack of net population growth in the competition environment complicates the estimation of plasmid transfer rate (Simonsen *et al.* 1990). To establish that plasmid transfer does occur at a significant rate, we performed a control experiment in which donor (JHC514a (TA− Cm^r)) and recipient (JHC510-NX^r (TA− Gm^r)) were co-incubated at a ratio of 1 : 1 for 10 days in the competition environment. Transconjugants were identified by being able to grow on medium supplemented with Cm and Nx. We found that 8 per cent ($n = 12$, s.e.m. 0.8%) of recipients had the donor plasmid after this time.

(e) *TA system location assay*

We used a simple genetic assay to track the ratio of TA+ : TA− plasmids during competitions. The basis of this assay was to sample a representative subset of plasmids present in a competition population by transferring them to a secondary recipient strain. The fraction of TA-encoding plasmids in this subset was determined from the fraction of plasmids also conferring resistance to Gm, which was linked to the TA system. To do this, throughout competition experiments, aliquots of cells were removed and mated with TC107 Nx^r recipients for 2 h in Luria-Bertani (LB) medium. Recipient cells were added in 10-fold excess to reduce the chance of multiple plasmid transfer to a single recipient cell. Following incubation, cells were plated on LB plates supplemented with Nx and Km to select transconjugants. Transconjugants were of two sorts: those containing progenitor plasmids that did not encode a TA system (conferring Km^r only), and those that did encode a TA+ transposon (conferring Km^r and Gm^r). The frequency of transposon-encoding plasmids was calculated as the number of Gm^r transposon-containing transconjugants divided by the total number of transconjugants. To estimate the ratio of TA+ : TA− chromosomes, we used replica plating to estimate the ratio of Gm^r: Gm^s cells. This measure provides an upper limit to the true ratio because all cells containing a TA+ plasmid will also be Gm^r. Thus, the ratio of Gm^r: Gm^s cells will overestimate the frequency of chromosomal TA systems if cells that contain only a plasmid TA system are common. In fact, in simulations, we predict that such cells are present at a frequency of less than approximately 2 per cent in the competition population (electronic supplementary material, figure S3). Moreover, we note that considering these cells as having TA+ chromosomes is conservative, tending to reduce the relative advantage we calculate for plasmid- compared with chromosome-encoded TA systems.

3. RESULTS

To estimate the relative success of plasmid- and chromosome-encoded TA systems in an environment where plasmids competed through co-infection of host cells, we introduced TA+ transposons into a population of conjugative plasmid-containing cells. Transposons could insert into either the plasmid or the chromosome of any individual cell. The initial ratio of plasmid- to chromosome-encoded TA systems was 0.015 ($\pm$0.011; 95% confidence interval), which was consistent with a simple expectation based on the relative target size of the two target genomes (plasmid : chromosome, approx. 100 kb : approx. 4.6 Mb = approx. 0.022).

To start the competition experiment, the TA+ populations generated above were mixed with an equal number of plasmid-containing cells that did not encode a relevant (*parDE* or *hok/sok*) TA system. After 10 days of competition, cells having plasmid-encoded TA systems had increased in frequency approximately 30-fold relative to their chromosomally encoded counterparts. This increase represents a highly significant fitness advantage for the plasmid-encoded TA systems (*parDE*: $F_{1,18} = 34.09$, $p < 0.001$; *hok/sok*: $F_{1,18} = 21.33$, $p < 0.001$; figure 3*a*). Thus, in agreement with our model, plasmid TA systems have an advantage over chromosomal systems in an environment in which TA+ plasmids were advantaged during within-host competition. This advantage was dependent on the TA system and was not simply a consequence of plasmid horizontal transfer. In a control competition with an otherwise identical TA− transposon, no advantage to plasmid localization was seen ($F_{1,17} = 1.19$, $p = 0.29$; figure 3*a*).

To test our prediction that the mechanism underlying the success of plasmid-encoded TA systems depends on the death of cells inheriting only competing TA− plasmids, we repeated the competition described above, except without adding any TA− transposon-containing cells. In this environment, all cells contained either a plasmid or a chromosomal TA+ transposon. The vast majority of these transposons (approx. 98%) were originally present on chromosomes, where they protected cells against the effect of the toxin following loss of a TA+ plasmid. Because within-host competition between TA+ and progenitor TA− plasmids will usually occur in cells that are immune to the toxin, the competition model predicts that plasmid localization will not confer any advantage to TA systems in this environment (figure 1*b* and electronic supplementary material, figure S3). Consistent with this prediction, the proportion of plasmid-encoded TA systems did not increase during competition (*parDE*: $F_{1,17} = 2.78$, $p = 0.11$; *hok/sok*: $F_{1,18} = 0.84$, $p = 0.37$; TA− transposon control: $F_{1,17} = 0.68$, $p = 0.42$; figure 3*b*). Therefore, the success of plasmid-encoded TA systems did depend on the death of cells in which the TA+ plasmid was displaced following co-infection by a competing TA− plasmid.

4. DISCUSSION

Previously, we demonstrated that TA systems could confer an advantage to plasmids in an environment where co-infection of cells by competing plasmids occurred (Cooper & Heinemann 2000, 2005). This advantage was shown to result from the death of daughter

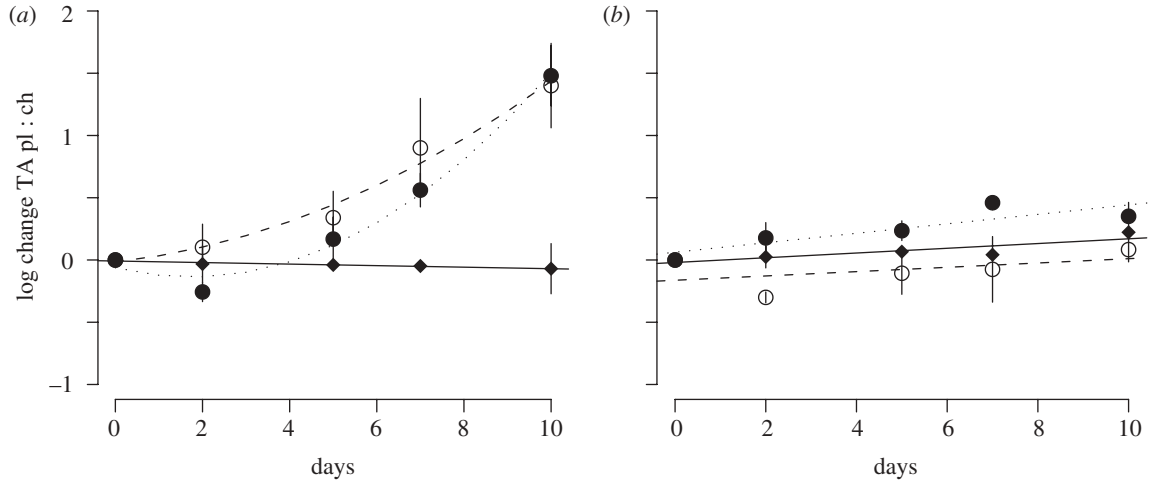

Figure 3. Effect of co-infection and within-host competition on the success of plasmid and chromosomally encoded TA systems. (*a*) Equal numbers of plasmid-containing cells containing randomly inserted TA+ or TA− transposons were competed. TA+ plasmid-mediated cell death can occur in this environment. (*b*) All cells contain either a plasmid- or chromosome-encoded TA system. Results shown are the average (and 95% confidence interval) of four independent experiments. Dashed line and filled circles, *parDE* TA system; dotted line and hollow circles, *hok*/*sok* TA system; solid line with filled diamonds, control TA− transposon.

cells that inherit only the TA− plasmid following co-infection by TA+ and TA− plasmids and was formalized as the competition model (Heinemann 1998; Cooper & Heinemann 2005). The results presented here extend this work to show that the same mechanism which selects TA+ plasmids over TA− plasmids can also select plasmid-encoded TA systems over chromosomally encoded TA systems.

The repertoire of 'accessory' plasmid genes—genes that are not essential for plasmid maintenance—remains poorly characterized, but is thought to be enriched for genes that encode potentially costly traits that can provide a sporadic advantage to host cells, for example, resistance to antibiotics or the ability to use or detoxify rare compounds (Eberhard 1989; Frost *et al.* 2005). It has been suggested that plasmid localization could be of advantage to these genes by allowing them to interact with a greater variety of host cells, from which the combination best adapted to a particular environment could be selected (Eberhard 1989). In this view, accessory genes are selected through conferring an advantage to the host cell. Supporting this proposal, in one of the few evolutionary models examining the existence conditions for conjugative plasmids, Bergstrom *et al.* (2000) found that the ability to combine established and newly arising beneficial genes could select for plasmids encoding genes that increased the fitness of their host cell. By contrast, the competition model presented here represents a mechanism by which plasmid-encoded genes are selected by directly increasing plasmid fitness during within-host competition. Several laboratory experiments have demonstrated that this type of competition can influence the outcome of plasmid and virus evolution (Bull & Molineaux 1992; Turner & Chao 1998). Moreover, findings of antagonistic interactions between horizontally transmitted elements suggest that within-host competition plays an important role in their evolution in natural environments (Molineux & Spence 1984; Pecota & Wood 1996; Engelberg-Kulka *et al.* 1998).

If TA systems are selected for their role in increasing plasmid competitiveness during within-host competition, we can ask: what does the widespread presence of TA+ plasmids tell us about the selective conditions that plasmids encounter? Maintenance of multiple TA systems is hard to explain by summing their contribution to plasmid stability alone. However, if they are selected to increase the success of plasmids in within host-competition, then a diverse collection of TA systems increases the chances that one plasmid will have one that a competitor does not. From this perspective, the presence of multiple TA systems can be understood as a reflection of the important role co-infection, and the resulting within-host competition, plays in plasmid evolution.

Our finding of a plasmid-specific selective mechanism for TA systems is somewhat surprising in light of recent findings that they are present on the chromosomes of many bacteria (Pandey & Gerdes 2005; Guglielmini *et al.* 2008). Several adaptive explanations have been offered to explain this observation (reviewed in Hayes 2003; Gerdes *et al.* 2005; Magnuson 2007). We emphasize that our results and the proposed selective mechanism do not exclude the possibility of other forces that might select for chromosomal copies of TA systems, but that were either overwhelmed or were not present in our competition experiments. Moreover, we can imagine at least two ways in which the model presented here may contribute to selection of chromosomal TA systems.

First, although our results show that within-host competition can select for TA+ plasmids, the underlying model can be applied to any horizontally mobile element that competes with other elements for maintenance within host cells. For example, homologous recombination can mediate competition between resident and incoming stretches of DNA (Kusano *et al.* 1995; Handa *et al.* 2001; Sadykov *et al.* 2003; Mochizuki *et al.* 2006). Regions of DNA encoding TA-like restriction-modification systems can displace those that do not, but not vice versa (Kusano *et al.* 1995; Handa *et al.* 2001). If chromosomal TA systems are selected, at least in part, through their ability to mediate competition between incoming and resident regions of DNA, we would expect that bacterial species with higher rates of

horizontal gene transfer (HGT) would tend to have higher numbers of TA systems. To examine this possibility, we tested for a correlation between estimates of genome-wide HGT (Nakamura *et al.* 2004) and number of chromosomal TA systems across a sample of 88 completely sequenced bacteria (Pandey & Gerdes 2005). Consistent with TA systems playing a role in genomic stability, we found a significant positive correlation between these variables (Pearson: $r = 0.296$, $p = 0.005$; Spearman: $\rho = 0.654$, $p \ll 0.001$). We note that other explanations could contribute to this correlation. For example, to the extent that chromosomal TA systems are associated with mobile elements, higher HGT may provide greater opportunity for genomic 'infection' by TA systems.

Second, chromosomal TA systems might be selected by providing immunity to host bacteria that would otherwise be killed following loss of a TA+ plasmid (Brendler *et al.* 2004; Cooper & Heinemann 2005; De Bast *et al.* 2008). In a recent study, De Bast *et al.* (2008) demonstrated that this immunity could increase the fitness of host strains when they initially carried unstable TA+ plasmids. An interesting consequence of this kind of selection is the potential for an 'arms-race' between plasmid-encoded TA systems and cognate chromosomal antitoxins.

In summary, we have shown that, as predicted by theoretical models, within-host competition can create selection for TA systems located on plasmids and that this selection causes their biased accumulation on plasmids relative to bacterial genomes. The presence of multiple TA systems on plasmid genomes suggests within-host competition may play an important role in their ecology and evolution. If so, an appreciation of this role will be necessary to understand how they, and the traits they encode, will evolve.

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