Selection for plasmid post-segregational killing depends on multiple infection: evidence for the selection of more virulent parasites through parasite-level competition

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Is the virulence of parasites an outcome of optimized infection? Virulence has often been considered an inevitable consequence of parasite reproduction when the cost incurred by the parasite in reducing the fitness of its current host is offset by increased infection of new hosts. More recent models have focused on how competition occurring between parasites during co-infection might effect selection of virulence. For example, if co-infection was common, parasites with higher intrinsic growth rates might be selected, even at the expense of being optimally adapted to infect new hosts. If growth rate is positively correlated with virulence, then competition would select increased virulence. We tested these models using a plasmid-encoded virulence determinant. The virulence determinant did not contribute to the plasmid's reproduction within or between hosts. Despite this, virulent plasmids were more successful than avirulent derivatives during selection in an environment allowing within-host competition. To explain these findings we propose and test a model in which virulent parasites are selected by reducing the reproduction of competitors.

Keywords: conjugative plasmids; post-segregational killing; multiple infection; within-host virulence; between-host virulence

1. INTRODUCTION

Virulence, the negative effect of a parasite on host fitness, is often assumed to be an inevitable consequence of its exploitation of host resources (Levin & Pimentel 1981; Levin & Svanborg Eden 1990; Bull 1994; Frank 1996). Appropriation of host resources is considered a prerequisite for parasite transmission. Thus parasite strategies that increase transmission are expected to also increase virulence. In turn, increased virulence decreases the longevity of the host–parasite association and potential parasite transmission. Between-host models predict that parasites will be selected to optimally exploit this trade-off, thereby maximizing the number of new hosts infected (Anderson & May 1982). Factors affecting this optimum are expected to alter the degree of virulence selected (Lenski & May 1994; Lipsitch *et al.* 1995, 1996). For example, if horizontal transmission of parasites from infected to susceptible hosts is common, the cost of harming the current host is lessened and increased virulence is selected. If most parasite transmission occurs vertically, from an infected host to its offspring, less virulent parasites least affecting host reproduction will be most successful.

Within-host models consider an additional component of a parasite's life history: competition occurring between parasites within a given host. These models predict that infection of a host by multiple parasites will select those parasites having a local or within-host advantage. If viru-

lence influences this advantage, within-host competition will select a level of parasite virulence different from that predicted by consideration of between-host dynamics alone (Levin & Bull 1994; Nowak & May 1994; Van Baalen & Sabelis 1995; Chao *et al.* 2000; Brown *et al.* 2002; Ganusov *et al.* 2002; Schjorring & Koella 2003; Massey *et al.* 2004). Thus, between-host models explain the selection of virulence through optimization of a parasite's relationship with the host, whereas within-host models also consider virulence as an adaptation relevant to direct parasite–parasite competition.

Despite the mathematical arguments in favour of an effect of within-host selection in determining the optimal level of virulence, so far few experimental systems have been developed in which to test predictions of the theory. In this study, we seek to test expectations of between- and within-host models by tracking the dynamics of parasites of different virulence levels in a bacteria–plasmid experimental system in which the degree of co-infection can be manipulated.

(a) *Experimental system*

Plasmids are extra-chromosomal DNA molecules that may be dispensable in some environments and therefore may often behave like parasites because of the metabolic burden they impose on host bacteria (Eberhard 1990; Lenski *et al.* 1994; Boe 1996). Many plasmids encode post-segregational killing (PSK) systems that consist of a tightly linked toxin–antitoxin pair (Loh *et al.* 1989; Gerdes *et al.* 1990; Jensen & Gerdes 1995). If a plasmid encoding a PSK system is not inherited by a daughter cell after cell division, the less stable antitoxin degrades, allowing toxin action and

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Table 1. Bacteria and plasmids used in this study.

strain or plasmid	abbreviation	relevant phenotype
strains		
JHC510	W ⁻	does not support plasmid horizontal transmission
JHC514a	W ⁺	does support plasmid horizontal transmission
TC105	W ⁺ PSK ^{par}	Gm ^r , ParDE (psk ⁺)
TC106	W ⁺ PSK ^{h/s}	Gm ^r , Hok/Sok (psk ⁺)
TC107	W ⁺ Nx ^r	Nalidixic acid resistant
plasmids		
pTP101	pPSK-Cm	Cm ^r , Km ^r
pTP102	pPSK-Gm	Gm ^r , Km ^r
pTP103	pPSK ^{par}	Gm ^r , ParDE (psk ⁺), Km ^r
pTP104	pPSK ^{h/s}	Gm ^r , Hok/Sok (psk ⁺), Km ^r

consequent cell death. PSK-mediated killing of plasmid-free segregants effectively ensures that a higher proportion of cells within a population remain plasmid-containing than would otherwise be the case (Gerdes *et al.* 1986; Sia *et al.* 1995). Indeed, it is widely held that this apparent contribution to plasmid vertical transmission has been the selection responsible for the success of plasmid-borne PSK systems (Gerdes *et al.* 1986; Nordstrom & Austin 1989; Naito *et al.* 1995). However, recent observations have suggested an alternative possibility. The finding that psk⁺ plasmids can effectively exclude psk⁻ competitors from host cells hints that within-host competition may play a role in the success of psk⁺ plasmids (Kusano *et al.* 1995; Naito *et al.* 1998; Nakayama & Kobayashi 1998; Cooper & Heinemann 2000a).

In this study, we seek to test the ability of between- and within-host models to explain the success of two PSK systems, *parDE* (Sia *et al.* 1995) and *hok/sok* (Gerdes *et al.* 1986) (these two systems are examples of the two families of PSK systems, proteic and RNA-based, respectively). We use two bacterial strains that are isogenic except in their ability to suppress a premature stop (amber) codon. The plasmids used in this study contain an amber mutation in the *traC* gene, which is essential for plasmid horizontal transmission. Therefore in the non-suppressing host strain (JHC510) plasmids can only replicate vertically along with cell division and any changes in the relative frequencies of plasmids can only occur by their effect on the fitness of the host. In the suppressing strain (JHC514a) the TraC protein is fully translated and the plasmid replicates by horizontal transfer. In this case, plasmid competition is mediated additionally by dynamics occurring within host cells after transfer of a plasmid to a host cell containing a plasmid of the competing type. Thus a comparison of the relative success of psk⁺ and psk⁻ plasmid types in the two hosts allows the effect of within-host competition on plasmid success to be determined. A key advantage of this bacteria-plasmid system is that the degree of within-host competition can be manipulated genetically while keeping other aspects of the environment constant.

2. MATERIAL AND METHODS

(a) Bacteria and plasmids

JHC510 and JHC514a (and derivatives) were used as the host bacteria in this study. These strains are isogenic except in their ability to suppress amber nonsense mutations as discussed in § 1. (Hereafter JHC510 and JHC514a are abbreviated to W⁻ and W⁺,

respectively, denoting their ability to support plasmid within-host competition after transfer between host backgrounds.) TC105–106 differ from W⁺ by the unmapped insertion of mini-Tn10 elements as detailed in table 1. TC107 is a nalidixic acid (Nx) resistant mutant of W⁺. Hereafter, TC105–107 are designated W⁺PSK^{par}, W⁺PSK^{h/s} and W⁺PSK^{Nx}, respectively, to denote the presence of added chromosomal PSK and resistance determinants. Unless otherwise noted, competitions were performed in the W⁺ background. pTP101–104 differ from the progenitor plasmid, Jp145, by the unmapped insertion of mini-Tn10 elements as detailed in table 1. Hereafter, pTP101–104 are designated pPSK-Cm, pPSK-Gm, pPSK^{par} and pPSK^{h/s}, respectively, to denote their PSK phenotype and antibiotic resistance. The different antibiotic resistance profiles allowed us to determine the number of cells within a population that contained each plasmid type by screening on medium supplemented with the appropriate antibiotic.

For convenient reference, the relevant phenotype and abbreviation of strains and plasmids used in this study are given in table 1. Further details and methods of construction are supplied in electronic Appendix A.

(b) Media

Antibiotics and chemicals, added exclusively to Luria–Bertani–Herskowitz medium (Herskowitz & Signer 1970) for culturing at 37 °C, were used at the following concentrations: chloramphenicol (Cm), 20 µg ml⁻¹; gentamicin (Gm), 5 µg ml⁻¹; Nx, 60 µg ml⁻¹; kanamycin (Km), 40 µg ml⁻¹.

(c) Effect of post-segregational killing on host fitness

To measure the effect of a plasmid-borne PSK system on the relative fitness of host bacteria, W⁻ cells carrying one of the Gm-resistant plasmids (pPSK-Gm, pPSK^{par} or pPSK^{h/s}) were competed separately against a reference strain containing pPSK-Cm. W⁻ cells were used to allow the effect of the PSK systems to be measured without the complication of plasmid transfer between competing cells. The two competing strains were separately grown to mid-exponential phase, mixed 1 : 1 at a dilution of 1 : 100, and competed for 24 h. The relative frequencies of the two competing plasmids were determined by first plating cells to Km-supplemented medium (on which all plasmid-containing bacteria could grow) and then replica plating colonies arising on this plate to medium supplemented with Gm (on which only cells containing pPSK-Gm, pPSK^{par} or pPSK^{h/s} plasmids could grow). Let the initial densities of the two competitors be $N_1(0)$ and $N_2(0)$, and their densities after 24 h competition be $N_1(1)$ and $N_2(1)$, respectively. The time-average rate of

increase is then calculated as $M_i = \ln[N_i(1)/N_i(0)]/1$ day. The fitness of one strain relative to the other was expressed as the ratio of their rates of increase (Lenski *et al.* 1991). Fitness assays were performed with 10-fold replication unless reported otherwise.

(d) Horizontal transmission

Donor and recipient bacteria were co-incubated for 3 h and then plated onto media supplemented with antibiotics to differentially select donor, recipient and plasmid-infected recipient (transconjugant) bacteria. Conjugation frequency was measured as the number of transconjugants divided by the product of the densities of donor and recipient bacteria. Assays were performed with 10-fold replication.

(e) Competition experiments

To assess the effect of PSK on plasmid success during within-host competition, W^+ host bacteria carrying pPSK^{−Gm}, pPSK^{par} or pPSK^{h/s} were mixed at 1 : 1 with reference pPSK^{−Cm} containing bacteria and added at a dilution of 1 : 100 to fresh medium. Cultures were incubated without shaking except for a daily 20 s agitation using an orbital vortex shaker. To measure the initial frequency of competing plasmids, a sample was removed at day 0 and plated on medium supplemented with either Cm or Gm. In competitions in which all bacteria were resistant to Gm, the number of pPSK^{−Cm}-infected bacteria was estimated as the difference between all plasmid-containing bacteria (Km^r) and those containing the reference pPSK^{−Cm} plasmid (Cm^r). Further aliquots were periodically removed and plated similarly to follow changes in plasmid frequency throughout the competition. The final samples of some competitions were examined for changes in plasmid antibiotic resistance markers. In no cases were changes to the ancestral genotype observed. Competitions were performed with 10-fold replication unless specified otherwise in the text. This same methodology was used in competition experiments done to test the specific predictions of the mathematical model developed in § 3.

3. RESULTS

(a) Post-segregational killing has a cost

W^- host bacteria infected by pPSK^{−Cm} and either pPSK^{par} or pPSK^{h/s} were competed to assess the effect of psk⁺ and psk[−] plasmids on host fitness. The W^- strain only supports vertical reproduction of the plasmids, so this assay measures the effect of the PSK systems on the fitness of the host cell without the complication of plasmid transfer between host cells. A significant cost was associated with both PSK systems (mean relative fitness and one-tailed *t*-test: 0.974, $p < 0.01$ and 0.970, $p = 0.041$ for pPSK^{par} and pPSK^{h/s}, respectively). Control competitions between pPSK^{−Cm} and pPSK^{−Gm} showed that the competition was not affected by the different resistance markers (relative fitness and two-tailed *t*-test: 0.992, $p = 0.466$). These results indicate that the plasmid-borne *hok/sok* and *parDE* PSK systems imposed a fitness cost to host bacteria and therefore confirm that PSK is a virulence determinant.

(b) Post-segregational killing has no measurable effect on horizontal transmission

The mean conjugation rates of pPSK^{−Gm}, pPSK^{par} and pPSK^{h/s} to plasmid-free recipient bacteria were 1.37×10^{-8} , 1.16×10^{-8} and 1.44×10^{-8} , respectively. These rates did not differ from one another (one-way ANOVA: $F_{2,27} = 0.0254$, $p = 0.875$). Therefore, the PSK

systems did not contribute to the ability of plasmids to transmit horizontally to new hosts.

(c) Post-segregational killing can be an advantage during competition

The results presented above do not support the hypothesis that PSK has been selected by increasing plasmid between-host transmission. For this reason we sought to test an alternative mechanism for the selection of plasmid-borne PSK systems. We have previously noted that PSK can enhance the ability of a plasmid to establish in a host bacterium already occupied by a competing psk[−] plasmid (Cooper & Heinemann 2000a). To test whether this advantage could be sufficient to explain the success of psk⁺ plasmids, competitions between psk⁺ and psk[−] plasmids were performed under conditions where co-infection of hosts was possible. During this competition no uninfected bacteria were present, therefore plasmid transfer occurred only between already-infected hosts. In competitions between pPSK^{−Cm} × pPSK^{par} or pPSK^{−Cm} × pPSK^{h/s}, the psk⁺ plasmids increased in frequency relative to the psk[−] plasmids (slope = 0.166, $p < 0.001$; slope = 0.157, $p < 0.001$, respectively; figure 1a). Again, the different antibiotic resistance markers were selectively neutral in the competition environment (pPSK^{−Cm} × pPSK^{−Gm}, slope = −0.042, $p = 0.0974$). To determine the contribution of within-host competition to these results, the competitions were repeated in the W^- host background. In these competitions, psk⁺ plasmids demonstrated no advantage (pPSK^{−Cm} × pPSK^{par}, slope = −0.005, $p = 0.84$; pPSK^{−Cm} × pPSK^{h/s}, slope = −0.081, $p < 0.01$; figure 1b). Thus, within-host competition was necessary for the success of psk⁺ plasmids in the competition environment.

(d) New insights from mathematical modelling

The results presented above demonstrate that within-host competition is necessary for the success of psk⁺ plasmids. To gain some insight into the mechanism underlying this success we sought to formally model the processes occurring during plasmid co-infection and within-host competition. Although the effect of PSK on plasmid reproduction has been modelled before, the effect of within-host competition was not considered (Mongold 1992). Our model had to incorporate three observations: (i) bacteria infected by psk⁺ plasmids grow more slowly than those infected by psk[−] plasmids; (ii) psk⁺ plasmids do not transmit horizontally more frequently than psk[−] plasmids; and (iii) psk⁺ plasmid success in the competition environment is dependent on horizontal transmission.

In this model, horizontal transfer results in occasional co-infection of cells by both psk⁺ and psk[−] plasmids. Replication incompatibility between the two plasmid types results in frequent mis-segregation (Nordstrom & Austin 1989). In daughter cells inheriting only the psk[−] plasmid, antitoxin degradation or dilution causes cell death. By contrast, daughter cells inheriting only the psk⁺ plasmid remain viable. Therefore, the result of co-infection of a host by psk⁺ and psk[−] plasmids will be the biased replication of the psk⁺ plasmid (figure 2). The model differs from most previous within-host models in that success of the more virulent parasite derives from its ability to effectively halt the vertical transmission of its competitor, rather than by an increased within-host growth rate. The experiments

Figure 1. Competition between psk^+ and psk^- plasmids. (a) W^+ host bacteria carrying $pPSK^{par}$ or $pPSK^{h/s}$ were separately co-incubated with W^+ ($pPSK^{-Cm}$) as described in § 2. Competition between $pPSK^{-Cm}$ and $pPSK^{-Gm}$ plasmids was performed similarly to assess the effect of the different antibiotic resistance markers on plasmid success. (b) The competition was repeated in the W^- background, which does not support plasmid horizontal transfer. Results shown are the mean of at least three independent experiments. Linear regression lines are shown. All results are plotted on log-linear plots to allow regression lines to best fit data. Open circles, $pPSK^{-Cm} \times pPSK^{-Gm}$; triangles, $pPSK^{-Cm} \times pPSK^{par}$; filled circles, $pPSK^{-Cm} \times pPSK^{h/s}$.

described below test the ability of the model to predict novel aspects of competition between psk^+ and psk^- plasmids. Full details of the model and experiments pertaining to parameter estimation are presented in electronic Appendix A.

(e) Does the model describe the dynamics of plasmid competition?

Experimental data from figure 1a were used to calibrate the model and to estimate parameters not easily measured experimentally. The ratio of plasmid vertical to horizontal transmission (Ψ) needed to be set at 0.1 and transfer between infected hosts (γ') was set at 9.5×10^{-11} . This latter value is somewhat higher than that measured experimentally (1.77×10^{-11}). A possible explanation for this difference is that experimentally we can only measure plasmid *transmission*, the frequency of inheritance of an incoming plasmid in offspring bacteria, whereas the simulation models plasmid *transfer*, the frequency of transfer to a recipient regardless of whether the incoming plasmid is inherited (Heinemann 1991). Other parameters were set at experimentally derived values (see figure 3a and electronic Appendix A). Experimental results were compared with model prediction using ANCOVA tests. Model predictions were not significantly different from observations (model versus $pPSK^{-Cm} \times pPSK^{h/s}$: $p = 0.798$; model versus $pPSK^{-Cm} \times pPSK^{par}$: $p = 0.445$). Thus, the model as presented and parameterized reconciled psk^+ plasmid success with within-host competition. A sensitivity

analysis (figure 3b) confirmed that the model was qualitatively robust to the particular parameter values chosen.

(f) Unique model predictions

The mathematical model developed above allows us to formalize several unique predictions of the competition hypothesis explanation of psk^+ plasmid success (Cooper & Heinemann 2000a). Tests of three key predictions are described below.

(i) Prediction 1

A psk^+ plasmid can displace a competing psk^- plasmid from a lineage. Although frequency of transfer into bacteria already containing a plasmid is identical for both psk^+ and psk^- plasmids (Cooper & Heinemann 2000a), only psk^+ plasmids will establish within the background in which the competing plasmid was initially introduced. We tested this prediction by performing competitions as before but with one plasmid carried by a specially marked (Nx^r) host. This marker made it possible to monitor plasmid transmission occurring between host backgrounds throughout the competition. Repeating the experiment, but swapping the host background in which each plasmid was initially introduced, provided a control to assess the effect of the Nx^r marker. As predicted by the competition model, relatively more psk^+ plasmids were able to establish in hosts initially infected by psk^- plasmids than vice versa ($pPSK^{-Cm} \times pPSK^{par}$ slope = 0.167, $p < 0.001$; $pPSK^{-Cm} \times pPSK^{h/s}$ slope = 0.185, $p < 0.001$; figure 4a). Much less difference was seen between psk^- plasmids differing only by antibiotic resistance markers ($pPSK^{-Cm} \times pPSK^{-Gm}$ slope = 0.060, $p = 0.1448$). Reconstruction experiments performed as previously (Cooper & Heinemann 2000b) eliminated the possibility that plasmid transmission occurred on selection plates (data not shown).

Figure 4b shows a comparison between model simulation and these empirical results. The predicted outcome was not significantly different from the observed outcome of competition between $pPSK^{-Cm} \times pPSK^{par}$ or $pPSK^{-Cm} \times pPSK^{h/s}$ (ANCOVA: $p = 0.7025$ and $p = 0.5235$, respectively).

(ii) Prediction 2

If success of psk^+ plasmids depends on the death of psk^- plasmid-containing daughter cells after division of co-infected parents (figure 2), then immunizing psk^- segregants against the toxin will rescue them from this fate and neutralize any advantage of plasmid-borne PSK. Host bacteria were immunized to the PSK toxin by the insertion of an identical PSK system into the bacterial chromosome. These immunized hosts carrying $pPSK^{-Cm}$ were then competed with W^+ hosts carrying the psk^+ plasmids. As shown in figure 5a, the success of psk^+ plasmids was neutralized by the chromosomally supplied antitoxin ($W^+PSK^{par}(pPSK^{-Cm}) \times W^+(pPSK^{par})$, slope = -0.0189, $p = 0.429$; $W^+PSK^{h/s}(pPSK^{-Cm}) \times W^+(pPSK^{h/s})$; slope = -0.0114, $p = 0.699$).

Figure 5b shows a comparison between model simulation and these empirical results. In both comparisons, psk^+ plasmid-containing cells had slightly higher fitness than predicted. However, in neither case were these differences

Figure 2. Competition model explanation for the success of psk^+ plasmids. After the horizontal transfer of a plasmid to a bacterium containing a plasmid of opposite PSK type, replication incompatibility forces the plasmids to segregate to separate daughter cells. In those bacteria losing the psk^+ plasmid (diagonal stripes), loss of the antitoxin renders descendants vulnerable to the effect of the toxin. psk^- plasmids (horizontal stripes) present in these bacteria are also 'killed'. Dominance of psk^+ plasmids is therefore not a result of the inability of psk^- plasmids to transfer to, or stabilize in, bacteria containing a psk^+ plasmid, but of the death of those bacteria in which displacement does occur.

significant (ANCOVA: $W^+ \text{PSK}_{\text{par}}(\text{pPSK}^-_{\text{Cm}}) \times W^+$ (pPSK^{par}), $p = 0.076$; $W^+ \text{PSK}^{\text{h/s}}(\text{pPSK}^-_{\text{Cm}}) \times W^+$ ($\text{pPSK}^{\text{h/s}}$), $p = 0.065$, respectively).

(iii) Prediction 3

Success of psk^+ plasmids is frequency dependent. If psk^+ plasmids succeed through a relative advantage, caused by the 'death' of psk^- plasmids after loss of the psk^+ plasmid from co-infected bacteria, rather than by an intrinsically higher reproductive rate, then there should be a positive correlation between psk^+ plasmid success and increasing initial psk^+ plasmid frequency. Consistent with competition model prediction, initial psk^+ plasmid frequencies of 0.1, 0.5 and 0.9 of total plasmid-containing bacteria correlated positively (but not significantly) with the success of psk^+ plasmids ($\text{pPSK}^-_{\text{Cm}} \times \text{pPSK}^{\text{par}}$, slope = 0.576, $p = 0.604$; $\text{pPSK}^-_{\text{Cm}} \times \text{pPSK}^{\text{h/s}}$, slope = 1.077, $p = 0.072$). No effect of initial frequency was seen on the outcome of competition between $\text{pPSK}^-_{\text{Cm}} \times \text{pPSK}^-_{\text{Gm}}$ (slope = -0.0652, $p = 0.977$; figure 6a).

Figure 6b shows a comparison between model prediction and these experimental results. Again, we used ANCOVA to compare the predictions of the mathematical model to the experimental outcomes. The frequency dependence of competition between $\text{pPSK}^-_{\text{Cm}} \times \text{pPSK}^{\text{par}}$ was not significantly different from the model prediction ($p = 0.13$). However, the effect of initial frequency on final success of the psk^+ plasmid in competition between $\text{pPSK}^-_{\text{Cm}} \times \text{pPSK}^{\text{h/s}}$ was significantly different from the model prediction ($p = 0.0363$). To further examine this difference we performed t -tests to separately contrast the slope and y -intercept of the predicted and observed regression lines. In neither competition was the

slope of the experimental regression line different from the model prediction (model versus pPSK^{par} and model versus $\text{pPSK}^{\text{h/s}}$, $p = 0.426$ and $p = 0.499$, respectively). The y -intercept of the experimentally derived regression line was significantly different from the model prediction in competition between $\text{pPSK}^-_{\text{Cm}} \times \text{pPSK}^{\text{h/s}}$, but not between $\text{pPSK}^-_{\text{Cm}} \times \text{pPSK}^{\text{par}}$ ($p = 0.029$ and $p = 0.298$, respectively).

4. DISCUSSION

In this study, we have tested the ability of between- and within-host models of virulence evolution to account for the apparent success of plasmid-borne PSK systems. We found no evidence to support the expectation of the between-host view that PSK systems increase the ability of a plasmid to infect new hosts. Instead, our findings suggest that the selection of PSK may be best explained by the within-host success of psk^+ plasmids.

The basis of our competition hypothesis explanation for the success of psk^+ plasmids is that vertical reproduction of a psk^- plasmid is inhibited when in the same host as a psk^+ plasmid (Naito *et al.* 1995, 1998; Cooper & Heinemann 2000a; figure 2). After a plasmid transfers to a bacterium already infected by a plasmid of the opposite PSK type, replication incompatibility forces the plasmids to segregate between daughter cells. Daughters retaining the psk^+ plasmid are protected by ongoing antitoxin expression. Daughters inheriting only the psk^- plasmid are killed by the unopposed toxin. Success of psk^+ plasmids is thus predicted to be a result of the death of those bacteria that lose the psk^+ plasmid rather than the inability of psk^- plasmids to transmit between hosts already infected by a psk^+ plasmid.

To test whether the mechanism outlined above can explain the success of psk^+ plasmids we performed two experiments. In the first, psk^+ and psk^- plasmid-containing cells were incubated together in an environment designed to maximize co-infection. Even without a transfer advantage, psk^+ plasmids still dominated (figure 1a). That competition was repeated using host cells that could not support plasmid horizontal transmission. In this environment within-host competition is absent because plasmids of the different PSK types never encounter one another in the same cell and, as expected by the competition model, no significant change in the ratio of psk^+ to psk^- plasmids was observed (figure 1b).

Additional experiments confirmed two out of three predictions derived from a mathematical formalization of the competition model. Experimental test of the third prediction, that the success of psk^+ plasmids in competition with psk^- competitors will be frequency dependent, differed from the model prediction (figure 6b). The model predicted that psk^+ plasmids must achieve a threshold frequency of ca. 0.135, whereas experimental results indicate an advantage to psk^+ plasmids even when rare. The reason for this discrepancy is not clear, although perhaps the most probable explanation is that measurement of model parameters was not strictly transitive between measured and competition environments. For example, the effect of the relatively slower psk^+ plasmid vertical reproduction, apparent in the growth rate assay and in the W^- competition assay (figure 1b), may have been lessened in

Figure 3. Comparison of theoretical model simulation and experimental observation. For model simulation the initial frequency of psk^+ plasmids was set at 0.5, the ratio of vertical to horizontal reproduction was set at 0.1 and the rate constant of plasmid transfer to already-infected bacteria was set at 9.5×10^{-11} . Other parameters were set at the experimentally determined values of: conjugation frequency = 1.3×10^{-8} , missegregation = 5×10^{-4} and psk^+ fitness = 0.96. The psk fitness was set to 0.98. Total viable bacteria density was constant at 2×10^8 colony-forming units ml^{-1} . (a) Mean value at each time point and linear regression lines are shown. Open circles, simulation outcome; triangles, $pPSK^{-Cm} \times pPSK^{par}$; filled circles, $pPSK^{-Cm} \times pPSK^{h/s}$. (b) Sensitivity analysis. Initial parameter values were allowed to vary randomly as follows over 5000 simulations: $\gamma' = 7 \times 10^{-11} - 1.2 \times 10^{-10}$, $\Psi = 0.08-0.12$, initial frequency of psk^+ plasmid-containing bacteria = 0.45–0.55. Mean and 95% confidence intervals are shown. Full details of the model are given in electronic Appendix A.

the competition environment if conjugation itself slowed cell division (Clark & Warren 1979; Turner *et al.* 1998).

Most models that attempt to predict the effect of within-host competition on selection of parasite virulence assume that success is caused by a higher within-host growth rate. Because virulence is seen as a consequence of a higher parasite growth rate, within-host competition is expected to select higher parasite virulence (Levin & Bull 1994; Nowak & May 1994; Van Baalen & Sabelis 1995; Mosquera & Adler 1998). Significantly, the findings reported here contradict that view. We find that PSK did not contribute to plasmid growth rate. Instead, PSK systems advantage plasmids only relatively, by slowing the effective growth rate of competing psk^- plasmids. This mechanism corresponds to the ecological notion of interference competition, whereby competitive success results from physical interference rather than the differential use of resources *per se* (Ricklefs 1990). We note that this mechanism of selection can explain the multiple PSK systems often found on plasmids. Although in the case of PSK systems this competition does select more virulent parasites, there is no *a priori* reason why this must be the case.

Figure 4. Effect of PSK on plasmid invasion. (a) Ability of psk^+ plasmids to establish in bacteria initially infected with psk^- plasmids, relative to establishment of psk^- plasmids in bacteria initially occupied by psk^+ plasmids. W hosts carrying $pPSK^{par}$ or $pPSK^{h/s}$ plasmids were co-incubated with $W^+Nx^+(pPSK^{-Cm})$. Relative establishment in bacteria initially occupied by the competing plasmid (transmission) is expressed as the ratio of psk^+ plasmids in the W^+Nx^+ background to psk^- plasmids in the W^+ background. The experiment was repeated introducing plasmids in the alternate host background to control for any effect of the Nx^+ marker. Results shown are the average of four independent experiments (two replicates of each alternative initial host background). Linear regression lines were constrained to go through the origin (at the outset all plasmids were in their initial background). Open circles, $pPSK^{-Cm} \times pPSK^{-Gm}$; triangles, $pPSK^{-Cm} \times pPSK^{par}$; black circles, $pPSK^{-Cm} \times pPSK^{h/s}$. (b) Comparison of experimental results with those generated by the mathematical model. Parameters simulated were those described in figure 3. Solid and dashed lines are linear regression lines of $pPSK^{-Cm} \times pPSK^{h/s}$ and $pPSK^{-Cm} \times pPSK^{par}$, respectively. Circles, simulation outcome.

Thus, we see no reason to assume that co-infection and the ensuing within-host competition must lead to an increase in parasite virulence.

Several other authors have recently reported finding that the effect of within-host competition depends crucially on the nature of parasite–parasite and parasite–host interactions. Interactions identified to select for a reduced optimum level of virulence following within-host competition include the trade-offs between competitive ability and parasite growth (Chao *et al.* 2000) and a requirement for cooperation between parasites to achieve virulence (Brown, S. P. *et al.* 2002; West & Buckling 2002). Here, we demonstrate a third possibility: virulence can be selected indirectly as a consequence of selection for mechanisms mediating parasite–parasite interference competition. One previous study has observed interference competition occurring between co-infecting parasites (Massey *et al.* 2004). However, in that study, competition occurred between different parasite species and therefore did not

Figure 5. Observed and predicted effect of a chromosomally borne PSK system on psk^+ versus psk^- plasmid competition. (a) W^+ hosts carrying $pPSK^{par}$ or $pPSK^{h/s}$ plasmids were co-incubated with $W^+PSK^{par}(pPSK-Cm)$ or $W^+PSK^{h/s}(pPSK-Cm)$. Relative success of the competing plasmid types is expressed as the ratio of psk^+ plasmid-infected bacteria to psk^- plasmid-infected bacteria. Results shown are the average. Lines show linear regressions. Triangles, $W^+PSK^{par}(pPSK-Cm) \times W^+(pPSK^{par})$; circles, $W^+PSK^{h/s}(pPSK-Cm) \times W^+(pPSK^{h/s})$. (b) Comparison of experimental results with those generated through model simulation. Parameters simulated are those described in figure 3. Dashed and solid lines are linear regression lines of $W^+PSK^{par}(pPSK-Cm) \times W^+(pPSK^{par})$ and $W^+PSK^{h/s}(pPSK-Cm) \times W^+(pPSK^{h/s})$, respectively. Circles, simulation outcome.

address its influence on selection of virulence within a particular parasite lineage.

Our findings are relevant to hypotheses seeking to predict the ecology and selective pressures relevant to other genetic systems that accumulate on horizontally mobile elements (e.g. plasmids, bacteriophage and pathogenicity islands; Heinemann & Silby 2003). For example, many virulence and antibiotic resistance determinants are encoded by plasmids (Eberhard 1990; Boyd *et al.* 2001; Amabile-Cuevas & Heinemann 2004). These determinants may be considered *de facto* PSK systems in that in certain environments they render loss of an otherwise dispensable plasmid lethal. By analogy, it might be impossible to explain why genes of these types are on plasmids without knowledge of their influence on parasite-parasite level competition. For example, in some instances bacterial virulence may be an adaptation selected by aiding the success, not of the organism, but of the elements encoding the trait. If so, there is little hope that the ecology and evolution of such traits will be explicable solely by consideration of the effect of the trait at the organism level (Boyd *et al.* 2001; Heinemann & Silby 2003). Furthermore, the ability of parasites such as plasmids to reproduce horizontally and thus compete under conditions not allowing host reproduction (Heinemann *et al.* 1996; Cooper & Heinemann 2000b), might further broaden the environmental conditions able to select for parasite virulence.

Figure 6. Effect of initial frequency on the fitness of psk^+ plasmids. (a) Plasmid fitness is measured as change in the ratio (relative to initial) of competitor: pPSK-Cm plasmids after 10 days co-incubation in the competition environment. Results reported are the average and standard deviation of four independent experiments. Least square regression lines are shown. Open circles, $pPSK-Cm \times pPSK-Gm$; triangles, $pPSK-Cm \times pPSK^{par}$; filled circles, $pPSK-Cm \times pPSK^{h/s}$. (b) Comparison of empirical observation with model prediction. Parameters used in model simulation are as for figure 3. Dashed and solid lines are linear regression lines of $pPSK-Cm \times pPSK^{par}$ and $pPSK-Cm \times pPSK^{h/s}$, respectively. Circles, simulation outcome.

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