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Non-additive costs and interactions alter the competitive dynamics of co-occurring ecologically distinct plasmids

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Plasmids play an important role in shaping bacterial evolution and adaptation to heterogeneous environments. As modular genetic elements that are often conjugative, the selective pressures that act on plasmid-borne genes are distinct from those that act on the chromosome. Many bacteria are co-infected by multiple plasmids that impart niche-specific phenotypes. Thus, in addition to host–plasmid dynamics, interactions between co-infecting plasmids are likely to be important drivers of plasmid population dynamics, evolution and ecology. *Agrobacterium tumefaciens* is a facultative plant pathogen that commonly harbours two distinct megaplasmids. Virulence depends on the presence of the tumour-inducing (Ti) plasmid, with benefits that are primarily restricted to the disease environment. Here, we demonstrate that a second megaplasmid, the At plasmid, confers a competitive advantage in the rhizosphere. To assess the individual and interactive costs of these plasmids, we generated four isogenic derivatives: plasmidless, pAt only, pTi only and pAtpTi, and performed pairwise competitions under carbon-limiting conditions. These studies reveal a low cost to the virulence plasmid when outside of the disease environment, and a strikingly high cost to the At plasmid. In addition, the costs of pAt and pTi in the same host were significantly lower than predicted based on single plasmid costs, signifying the first demonstration of non-additivity between naturally occurring co-resident plasmids. Based on these empirically demonstrated costs and benefits, we developed a resource–consumer model to generate predictions about the frequencies of these genotypes in relevant environments, showing that non-additivity between co-residing plasmids allows for their stable coexistence across environments.

1. Introduction

Spatial and temporal environmental heterogeneity can select for increased genetic diversity [1–3]. This process can occur quite rapidly for prokaryotes as they can quickly evolve in response to environmental changes [1,4]. Adaptation can result from mutation, recombination and the exchange of genetic material, often via plasmids [5,6]. An individual cell that acquires a plasmid with a novel set of genes potentially has the ability to occupy and obtain resources from an entirely new niche. This is the case for many rhizobia, for which the ability to form symbiotic or pathogenic relationships with plants is almost entirely plasmid-dependent [7,8]. Organisms that experience higher levels of environmental variability exhibit more modular metabolic networks than those living in more static conditions [9]. Similarly, plasmids confer genetic modularity [10] that can accelerate adaptive evolution in a variable environment.

The persistence of plasmids in a bacterial population is complex, determined by a combination of factors including, but not limited to, stability, horizontal transfer, host range and the balance of the costs and benefits conferred on their host cells [11–13]. The beneficial/costly nature of a plasmid-carried trait depends on the environmental conditions, and the population dynamics occurring within the microbial community [14]. For metabolically plastic bacteria that can survive under a wide range of environmental conditions, the costs and benefits conferred by their plasmids will vary according to the environment [14,15]. For example,

facultative pathogens transition between host and non-host environments, each defined by a unique set of resources and conditions. Therefore, the benefits conferred by a specific plasmid may be restricted to the host/non-host environment, whereas in the alternative environment, selection would favour individuals lacking the plasmid [16].

Many bacteria simultaneously harbour multiple plasmids. The distinct genetic composition and segregation of co-occurring plasmids suggests some degree of non-overlapping niche space for these genetic elements [17,18]. Given the likelihood of epistasis [19,20], interactions between multiple plasmids would presumably have large effects on microbial population dynamics. These inter-plasmid interactions may be antagonistic in nature, where different plasmid species compete for resources and stability within a single cell and/or a community of multiple genotypes [21–23]. This inter-plasmid competition could result in higher costs for the individual host cell or the entire population [24,25]. Conversely, synergism may occur such that genes encoded on distinct plasmids increase survival or growth of the cell in combination, whereas independently they are less beneficial [26,27].

For bacteria experiencing spatial and temporal variability in resources, harbouring multiple plasmids, each conferring locally beneficial genes, can mediate adaptation to a dynamic environment [5,6]. A great deal of empirical work has demonstrated that single plasmids evolve to have diminished costs on their host bacteria [28,29]. Nevertheless, despite their prevalence in bacterial populations, there is limited research that focuses on the interactions of co-occurring or coevolving plasmids. There are, however, several examples where multiple plasmids interact to differentially influence host fitness [22,30]. *Bacillus anthracis* contains multiple plasmids that are all required for optimal virulence on mammalian hosts [31]. This is suggestive of a synergistic relationship between plasmids that is realized as an increase in fitness for the plasmids and their host bacteria during infection. Likewise, in *Rhizobium leguminosarum*, strains containing four species-associated plasmids outcompete strains lacking any single plasmid [32]. The context dependence of any plasmid's costs and benefits coupled with the observed heterogeneity of plasmid genotypes across environments suggests that this type of synergy is not stable. As the environment changes, the presence of multiple plasmids in a bacterial community can result in fluctuating epistasis between genetic elements [33], potentially leading to the evolution of complex interactions.

In this study, we examined the individual and interactive costs of two ecologically distinct plasmids, pAt and pTi, of the facultative plant pathogen *Agrobacterium tumefaciens*, invalidating the prediction that the costs of the individual plasmids would be competitor-independent and strictly additive. Furthermore, we show evidence for specific functional interactions and environment-specific plasmid benefits that probably influence bacterial population dynamics. Finally, we demonstrate the ecological significance of these results by expanding upon a Monod model of competition to account for these observed fitness effects.

(a) Study system

The tumour-inducing (Ti) plasmid of *A. tumefaciens* (0.21 Mb) is required for pathogenesis and the induction of crown gall tumourigenesis. Disease occurs when Ti plasmid-bearing cells encounter a certain set of conditions generated by wounded plant tissue which stimulate the expression of virulence genes that drive the replication, cross-kingdom horizontal transfer and chromosomal integration of a large segment of the Ti plasmid (the transferred or T-DNA) into the plant host genome [34]. This transfer is mediated by a Ti plasmid-encoded type IV secretion system (T4SS), a complex and energetically costly structure [16,35]. The integrated T-DNA encodes genes for the production of low-molecular weight compounds called opines, which serve as sources of nitrogen, carbon and sometimes phosphorus for Ti plasmid-containing cells, as the plasmid also carries opine catabolic genes [36]. Additionally, the conditions of this disease environment are necessary signals for induction of Ti plasmid conjugation [37]. Thus, the metabolic costs of the Ti plasmid are directly coupled to the catabolic benefits, and restricted to the disease environment.

The At plasmid is a second megaplasmid of *A. tumefaciens*. In *A. tumefaciens* strain C58, this plasmid's size (pAtC58) is 0.54 Mb, over twice as large as pTiC58, but is not required for pathogenesis. It does however carry genes predicted to have a wide variety of functions, ranging from chemotaxis to DNA damage repair and catabolism. Two particular set of genes, *soc* and *blc*, confer the ability to catabolize plant-released deoxyfructosyl glutamine (DFG) and γ-butyrolactones (GBLs), respectively, as sole carbon and nitrogen sources [38,39], suggesting that pAtC58 provides a benefit for cells growing in the rhizosphere. The At plasmid also encodes its own conjugation machinery, the expression of which has been demonstrated to be constitutive [40]. Although there is little data regarding the frequency and distribution of the At and Ti plasmids in nature, many of the strains isolated from crown gall tumours harbour both plasmids.

2. Methods

(a) Strains, plasmids and growth conditions

All strains and plasmids used in this study are described in the electronic supplementary material, tables S1 and S2. Antibiotics and reagents were purchased from VWR (Radnor, PA), Sigma–Aldrich (St. Louis, MO), Fisher Scientific (Pittsburgh, PA) and New England Biolabs (Ipswich, MA). We obtained oligonucleotide primers from Integrated DNA Technologies (Coralville, IA) and used Omega Biotek E.Z.N.A miniprep and gel extraction kits (Norcross, GA) for isolation of nucleic acids. Plasmids were transferred into *A. tumefaciens* strains via either conjugation or electroporation using standard approaches [41]. Unless otherwise stated, *A. tumefaciens* strains were grown in AT minimal medium supplemented with 0.5% glucose (w/v) and 15 mM $(NH_4)^2SO_4$ [42] and incubated at 28°C in a rotary aerator or on 1.5% agar plates. Antibiotics were used at the following concentrations: 2.5 mg ml^{-1} streptomycin (Sm) and 250 µg ml^{-1} spectinomycin (Sp). Induction broth for activating virulence gene expression was prepared using the recipe described in Morton & Fuqua [43].

(b) Generating isogenic strains

Isogenic strains were generated by independent curing of both the Ti and At plasmids from wild-type *A. tumefaciens* C58 using approaches described previously [41,44] and in the electronic supplementary material. Subsequently, both plasmids were reintroduced by conjugation into the plasmidless derivative, independently and together. Derivatives of each strain carrying the *aadA* marker (conferring resistance to streptomycin and spectinomycin, Sm^R/Sp^R) were generated by allelic replacement of the *tetRA* locus [41]. To distinguish between competing strains, the *aadA*

marker was removed from each strain to generate antibiotic-sensitive derivatives (ERM74, ERM75 and ERM89).

(c) Measuring the costs of harbouring the At and Ti plasmids

In order to determine the fitness costs associated with bearing the At and Ti plasmids, both together and apart, isogenic strains were competed in carbon-limited ATGN medium as described in by Platt *et al*. [16]. The experiment was set up with a full-factorial design, so that each strain was competed against every other strain. All competitions were inoculated with approximately 6×10^6 cells at an approximately 1 : 1 ratio. To control for the costs associated with the *aadA* marker (conferring Sm^R and Sp^R), the strain carrying the cassette was reversed for half of the competitions. At the beginning of each competition, strains were used to inoculate 2 ml of ATGN medium and incubated at 28°C for 24 h until reaching mid-exponential phase. Individual strains were subsequently washed in 1× AT buffer three times to remove excess glucose from the starting medium. The cultures were each normalized to an OD_{600} of approximate 0.2, at which point they were mixed 1 : 1 into 2 ml carbon-limiting medium to a final OD_{600} of 0.01. The competition cultures were then incubated at 28°C for 24 h, after which all mixed cultures were subcultured 1 : 100 into 2 ml of carbon-limited medium and incubated as before. This passaging was repeated the same way for a total of seven passages. At the first, second, and seventh passages, a dilution series of each mixed culture was plated onto ATGN with and without streptomycin and spectinomycin, so that the frequencies of each strain could be estimated. Although, based on experiments in the laboratory, conjugation is unlikely to occur in shaken liquid culture, samples (10–20 isolates) of both the sensitive and resistant populations were screened for the presence/absence of the Ti and At plasmids. We found no evidence that horizontal transfer or segregational loss of either plasmid was taking place during the experiment. For each competition experiment, there were four replicates of each competition pair, and this experiment was repeated four times on separate occasions.

(d) Calculating relative fitness

Relative fitness was calculated as the ratio of the number of doublings of each competing strain over time, as previously described [45]. Results from the competition experiments were analysed with GLM models using two distinct analyses for mixed and fixed effects with SAS software. The mixed model included the experiment and the marker orientation as random effects. This mixed effect analysis is conservative, requiring consistent results across experiments. We also use a fixed effect model as a less conservative test of significance. All data are presented for the mixed model, unless otherwise stated. This analysis allowed us to remove variance associated with the marker and differences between experimental runs, and thereby isolate the effect of the plasmid(s) by comparing least-square means of the different competitions. Results from both the mixed and fixed model analyses are summarized in the electronic supplementary material, table S3.

(e) *In vivo* competition between pAt+ and pAt− genotypes

Helianthus annuus seeds were surface sterilized and planted in sterile soil. Planting was staggered with approximately one week between each of six blocks. Each block contained three pots, two of which were inoculated with bacteria and one that was uninoculated as the sterile control. After two weeks of growth, seedlings were prepared for inoculation. Each plant was wounded by generating a 0.5–1 cm cut at the joint of the root and shoot using a sterile razor blade, and transferred to a double deep pot. *A. tumefaciens* C58 pTi+pAt+ and pTi+pAt− cultures (both marked and unmarked with an *aadA* antibiotic resistance gene, as described above) were grown in standard ATGN media to mid-exponential phase. Several hours prior to inoculation, all cultures were normalized to an OD_{600} of 0.25, and the appropriate strains were mixed at a 1 : 1 ratio. The starting ratio of pTi+pAt+ and pTi+pAt− cells was determined by selective plating of the mixed inoculum onto ATGN (± antibiotics), just prior to inoculation. Inoculated plants received 10 ml of culture, which was squirted directly onto the wound site using a sterile syringe. Subsequently, 10 additional ml of inoculum were mixed with 25 ml of sterile mqH_2O and flooded over the soil surface of the pot. Sterile pots received 20 ml of sterile ATGN medium and 25 ml of sterile mqH_2O.

At 11 weeks post-inoculation, four weeks after the primary leaves of the block 1 plants senesced, all pots were harvested. To harvest, 1–3 g of soil was collected from each pot from the same location, approximately 1 inch below the soil surface, and weighed. The soil from each sample was mixed with sterile water (9× the sample's wet weight) and slurried by vigorous shaking. A dilution series of this mixture was subsequently plated onto medium 1A [46] selective for *A. tumefaciens*. One hundred colonies from each sample were then patched onto ATGN plates (± antibiotics) to determine the final relative frequency of each genotype.

(f) Measurement of virulence gene induction

Strains ERM89 (pAt+pTi+) and ERM66 (pTi+) were transformed with the reporter plasmid, pSW209Ω, which carries a P_{virB}–*lacZ* fusion. Cells were grown for approximately 24 h in induction broth (pH 5.6, 50 µM phosphate and 200 µM acetosyringone) [47]. When cultures reached the mid-log phase of growth, they were assayed for β-galactosidase activity as previously described [43]. Activity is presented in terms of Miller units, a quantitative measure of specific activity that accounts for gene-expression mediated β-galactosidase activity, normalized to growth (see the electronic supplementary material).

3. Results

To determine the relative carriage costs of the At and Ti plasmids, pairwise competitions were carried out between isogenic derivatives of *A. tumefaciens* C58 (p−, pAt+, pTi+ and pAt+ pTi+) in carbon-limited media. Relative fitness was determined by calculating the ratio of the number of doublings of each strain over the course of competition (approx. 46 generations).

(a) Costs of At and Ti plasmids

The outcome competition revealed a strikingly high cost associated with the At plasmid (figure 1*a*) indicated by a significantly lower relative fitness of the pAt+ genotype compared with the plasmidless derivative ($W_{pAt+} = 0.871$, $p = 0.006$). The Ti plasmid, on the other hand, incurred a small cost that was not significant (figure 1*b*; $W_{pTi+} = 0.994$, $p = 0.323$). See the electronic supplementary material, table S3 for a summary of all mixed and fixed effect model analyses.

(b) Interactive effects of the At and Ti plasmids

Based on independent plasmid costs, predictions can be made with respect to the other competitive interactions (figure 1). However, very few of these interactive effects can be described by the individual plasmid costs alone. One of the most striking of these interactions is that the pTi+ strain experiences a

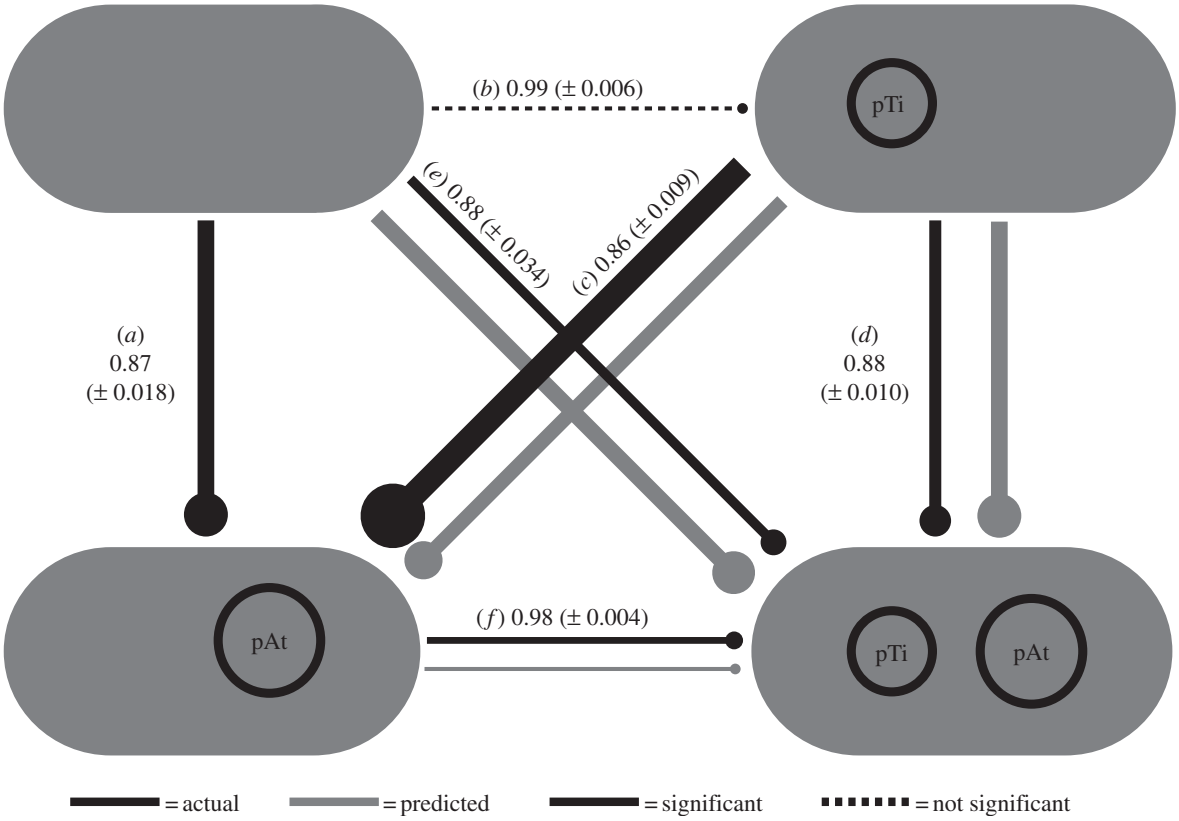

Figure 1. Relative fitness after pairwise competitions between plasmidless, pAt+, pTi+ and pAt+pTi+ cells normalized to a 50/50 starting ratio. Isogenic strains were competed in carbon-limiting media for 46 generations, with seven passages. Each bar represents a single competition pair, where the club points to the strain that declined in frequency. The thickness of the black bars is proportional to the strength of the interaction between the two strains, and relative fitness values of the less fit strain are presented adjacent to them. In all of these pairs, the strain with higher relative fitness is set to 1.00. The thickness of the grey bars represents the predicted strength of the interaction pair based on the individual plasmid costs when competed against the plasmidless derivative. These data summarize the results of four blocks of the experiment with four replicates per block of each competition pair. Values in parentheses represent s.e.m. values for each pair.

significantly greater advantage when in competition with the pAt+ strain than would be predicted based on single pTi+ and pAt+ costs (figure 1*c*; $W_{pAt+} = 0.862$, $p = 0.003$). Despite the low pTi cost, the dramatic advantage observed against the pAt+ only strain is largely abolished when the pTi is also present in its competitor (figure 1*d*; $W_{pAt+Ti+} = 0.879$, $p = 0.001$). This difference in the competitive advantage of the pTi+ strain against the pAt+ strain is significantly different from its competitive advantage against the pAt+pTi+ genotype ($p < 0.0001$). Thus, the presence of the Ti plasmid in the pAt+ competitor diminishes this strong advantage of the pTi+ only strain.

(c) Co-inhabitant plasmid costs

Cells harbouring both the At and Ti plasmids also exhibited high cost when in competition with the plasmidless derivative (figure 1*e*; $W_{pAt+pTi+} = 0.879$, $p = 0.006$). However, the relative fitness of the pAt+pTi+ strain was significantly higher than predicted by the individual pAt and pTi costs ($p = 0.012$) demonstrating that the combined cost of co-inhabitant At and Ti plasmids was non-additive. This non-additivity was also observed in competitions with the pTi+ only strain, such that the relative fitness of the pAt+pTi+ strain is 0.017 higher than the pAt+ only strain ($p = 0.0001$; figure 1*d*). Surprisingly, this non-additive effect of pAt and pTi was not maintained in competition with the pAt+ only genotype (figure 1*f*). Here, the pAt+pTi+ strain exhibited a lower relative fitness compared with its pAt+ only competitor ($W_{pAt+pTi+} = 0.98$, $p = 0.017$).

(d) The At plasmid negatively affects expression of Ti plasmid virulence genes

Previous reports have demonstrated that the presence of the At plasmid has a positive influence on tumour size in some strains of *A. tumefaciens* [48]. We were thus interested in this pAt and pTi interaction because of its potential to have significant effects on the ecology of the species. Using a reporter plasmid carrying a P_{virB}–*lacZ* fusion (a representative virulence gene that is activated in response to plant host signals), we were able to quantify virulence gene expression in pAt− and pAt+ cells (figure 2). We found that the presence of the At plasmid exhibits a clear repressive effect on *vir* expression with a significant twofold increase in the absence of pAt ($p < 0.0001$). The effects of this decrease on the ability to cause disease are not yet clear, but given the high cost associated with expression of the virulence genes [16], the presence of the At plasmid might decrease the burden of the Ti plasmid under *vir*-inducing conditions (low pH, low phosphate, sugars and plant phenolics).

(e) *In vivo* competition between pAt+ and pAt− genotypes

To test for the predicted At plasmid-conferred rhizosphere-specific benefits, pAt+ and pAt− genotypes were competed within the rhizospheres of *A. tumefaciens* infected *H. annuus* plants. To determine the effect of a productive rhizosphere, the relative frequency change of each genotype was determined

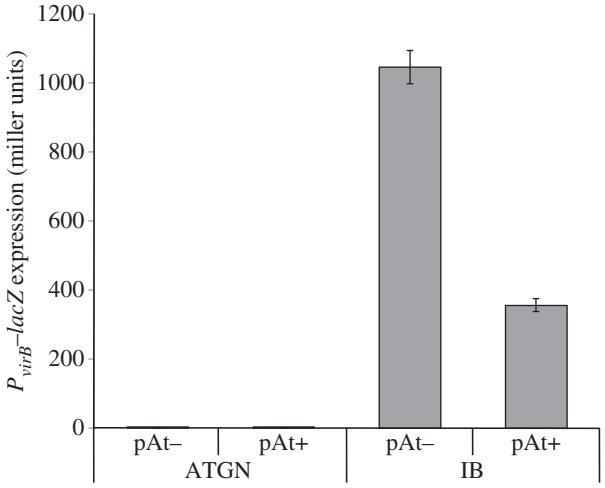

Figure 2. Expression of P_{virB}–*lacZ* in *A. tumefaciens* C58 pAt− and pAt+ strains. Cells were grown in liquid for 24 h in either ATGN minimal media (pH 7.0, 50 mM phosphate) or IB (virulence-induction broth) and assayed for β-galactosidase activity. Error bars represent standard deviation, $p < 0.0001$, unpaired Student *t*-test.

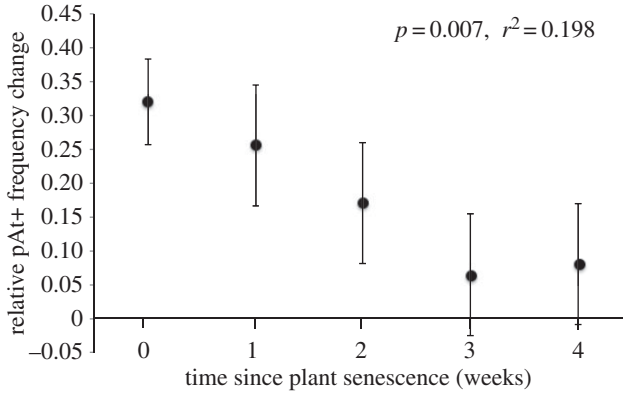

Figure 3. Relative frequency changes of pAt+pTi+ versus pTi+ only *A. tumefaciens* genotypes as a function of rhizosphere productivity. *Helianthus annuus* seedlings were wounded and inoculated with a 50/50 ratio of pAt+ and pAt− cells. At various stages post-senescence (0–4 weeks), the final frequencies of each genotype were determined. Points represent the least squared means of samples for each time of harvest. Error bars represent s.e.m. values for each time point.

across a gradient of plant senescence, with the prediction that the pAt+ genotype would increase in frequency when growing in an active rhizosphere, but decline after plant activity had ceased and plant-supplied resources were depleted.

Competitions were initiated by inoculating the wound sites of *H. annuus* seedlings with a 1 : 1 mixture of pAt+pTi+ and pTi+ only cells. Infection was induced, resulting in the development of visible galls. The relative frequency change of each genotype in the soils collected from the rhizospheres of both live plants and those at increasing times post-senescence, was determined (figure 3). Consistent with expectations based on rhizosphere-adaptive functions encoded on pAt, we observed a significant increase in the frequency of the pAt+ genotype in plants with an active rhizosphere, and a decline in pAt+ frequency correlating with the time following plant senescence ($p = 0.007$). We cannot exclude the possibility that conjugation of pAt occurred during the experiment as the strains were marked with antibiotic resistance genes integrated in the chromosome. However, if constitutive conjugation did occur it would have, if anything, resulted in an underestimation of the initial increase in pAt+ cells.

4. Model

(a) Resource competition model: environmental heterogeneity and selection for multiple plasmid genotypes

As a facultative pathogen, *A. tumefaciens* inhabits different resource environments depending on its plasmid genotype(s) and host availability. These environments can be classified based on the presence of a plant and the associated available resources. Based on competition studies measuring the costs of the At and Ti plasmids, and our understanding of their genetic composition and ecologies, if we assume that most competitive interactions are based on limiting resources, we can make predictions about their frequencies in these different environments. For the purpose of this model, we define three basic environments: the bulk soil, rhizosphere and tumour. We assume that general resources (G), usable to all genotypes, are present in all three environments. The two non-disease environments, the bulk soil and rhizosphere, are differentiated by the presence of plant-produced resources. These rhizosphere-specific resources (D) include plant-produced γ-butyrolactones (GBLs) and deoxy-fructosyl-glucosamine (DFG), both of which can only be catabolized by pAt+ cells [38]. The third environment, the tumour, is characterized by the additional presence of tumour-specific opines (O), which can only be used by pTi+ cells.

Based on the principles of a basic Monod model for bacterial population growth, the change in population size for each competing genotype (plasmidless, pAt+, pTi+ and pAt+pTi+) was determined as a function of births (conversion of available environment-specific resources into cells) and deaths (a constant *per capita* rate that was assumed to be the same for all genotypes; see the electronic supplementary material, figure S1 for growth curves). Growth of each plasmid-bearing genotype was limited by the predicted plasmid-specific costs. The At plasmid cost was fixed, whereas the context-dependent pTi costs [16] have been incorporated such that they increase as a type II functional response to disease-associated opines. The empirically demonstrated non-additive pAt and pTi costs were incorporated into the model as a flexible modifier in order to assess its effect on the outcome of resource competition between all four plasmid genotypes. For a more complete description of the model and parameters, see the electronic supplementary material, table S4.

(b) Resource-dependent frequency distribution of multiple plasmid genotypes

Assuming that the costs of the At and Ti plasmids are strictly additive and all resources are available at equal concentrations and are mutually substitutable, the outcomes at equilibrium based on predicted consumer–resource interactions of all four genotypes (plasmidless, pAt+, pTi+ and pAt+pTi+) are depicted in figure 4. Figure 4*a* shows the zero-net growth isoclines (ZNGIs) of all genotypes for two sets of resources G (general) and D (DFG and GBLs), representing the intersection between the bulk soil and rhizosphere environments. Here, only pAt+ cells are able to use plant-produced resources of the rhizosphere, but these cells also incur the costs associated with carrying the plasmid. Thus, as long as the supply of

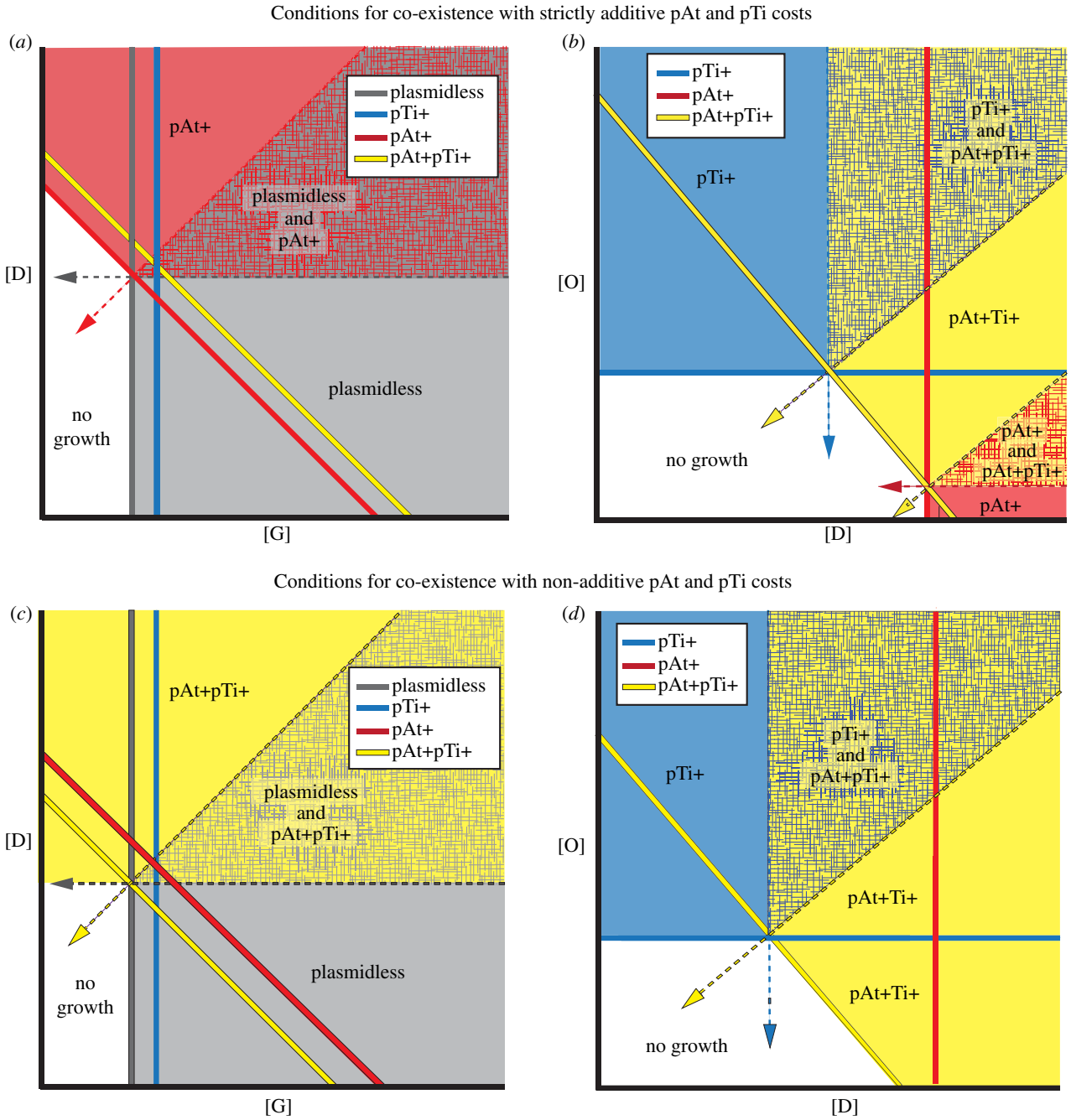

Figure 4. Model predictions for resource competition between plasmidless, pTi+, pAt+, and pAt+pTi+ genotypes along three resource supply axes: general resources (G), plant-produced resources (D) and tumour-produced opines (O). (*a*,*c*) Represent the intersection of the bulk soil and rhizosphere environments where G and D resources are available at some relative concentration, but D resources are only available to pAt+ cells. (*b*,*d*) Represent the intersection between the rhizosphere and tumour environments with D and O being specialized resources, available only to pAt+ and pTi+ cells, respectively. The grey, blue, red and yellow lines represent the minimum amount of resource required to support maintenance of the plasmidless, pTi+, pAt+ and pAt+pTi+ genotypes. Dashed arrows depict the consumption vectors for each relevant genotype. Each zone represents a range of resource concentrations, in which the persisting genotypes are listed. Zones are shaded according to the winning genotype(s). Note that the region in the lowest left sector (white) represents the range where resource concentrations are too low to support growth of any genotype. (*a*,*b*) Depict the model predictions for strictly additive pAt+ and pTi+ costs, whereas (*c*,*d*) have incorporated the empirically demonstrated non-additivity of plasmid costs.

plant-produced resources is sufficient that the benefits of catabolism outweigh the costs of the plasmid, pAt+ cells have an advantage over all other genotypes. This outcome is empirically supported by our greenhouse studies where pAt+ cells increase in frequency in the rhizosphere. The Ti plasmid provides no benefit under these conditions and thus, despite a minimal cost, does not persist in the population. Figure 4*b* shows the ZNGIs of all four genotypes for D and O resources, representing the intersection between the rhizosphere and tumour environments. Here, the dominance of each plasmid genotype shifts along a gradient based on the relative supply of pAt+- and pTi+-specific resources, D and O, respectively. Conditions for coexistence of each single- and double-plasmid genotype are met when there is sufficient supply of both resources. However, the range of conditions for coexistence between pAt+ and pAt+pTi+ cells is relatively narrow, because the costs of the Ti plasmid are directly tied to the presence of opines (disease), but the At plasmid costs remain constant.

(c) Non-additive costs eliminate conditions for maintenance of the single At plasmid genotype

When the empirically demonstrated non-additivity is incorporated into the model, the outcome of resource competition

shifts, so that cells harbouring both plasmids outcompete all other genotypes in both the rhizosphere and tumour environments (figure 4 and the electronic supplementary material, figure S2). Thus, as long as the difference between the strictly additive costs and the non-additive costs are higher than the costs of pTi alone, then the pAt+pTi+ genotype has the advantage. Because the cost of the Ti plasmid in the non-tumour environment is minimal, this difference does not need to be very large. Thus, in a community of multiple genotypes, non-additivity would favour the maintenance of pTi in the rhizosphere, when opines are not available (figure 4 and the electronic supplementary material, S2). Non-additivity also benefits the At plasmid, because the diminished associated costs expand the resource ranges in which pAt+ strains can persist.

5. Discussion

Microbes experience constant shifts in selective pressures owing to spatial and temporal heterogeneity in the environment. As conditions of the local environment change, bacteria are forced to adapt. There is a great deal of evidence demonstrating that environmental heterogeneity selects for increased diversification and species richness of the associated microbial community [1,49,50]. Bacterial plasmids often confer traits that are beneficial within a specific environmental context, and the segregation of locally beneficial genes on plasmids may facilitate their proliferation in the environment [14]. Thus, in a population of bacteria with multiple plasmid genotypes, with different relative fitness in each environment, temporal and spatial environmental heterogeneity could favour the maintenance of all types. Our results demonstrate conditions under which this occurs in *A. tumefaciens*, and identify forces selecting for coevolution of co-occurring plasmids.

(a) Non-additive At and Ti plasmid costs

Coevolution of compatible plasmids experiencing shifts between intra- and inter-host competition can, as this work demonstrates, generate complex interactions between plasmids and plasmid genotypes. This is the first demonstration of non-additive costs between co-resident, naturally occurring plasmids. These results complement a recent study demonstrating rapid evolution of positive epistasis between laboratory-evolved co-resident plasmids [27]. In the case of the At and Ti plasmids, a reduced cost could have developed as a consequence of their overlapping ecologies. There are a number of studies demonstrating that coevolving bacterial chromosomes and plasmids undergo genetic changes resulting in higher fitness [28,51]. The same phenomenon of diminished costs could have occurred for coevolving At and Ti plasmids. The results of our resource competition model suggest that the empirically demonstrated non-additivity of the At and Ti plasmids could allow adaptive maintenance of both plasmids when faced with environmental heterogeneity. These findings thus show that in conditions where bacteria are experiencing selective pressures for the maintenance of multiple plasmids, such as those carrying genes for antibiotic resistance in a hospital setting [52], that a synergistic reduction in plasmid costs may evolve.

(b) Low cost of the Ti plasmid

The cost of the single Ti plasmid was determined to be not significant when competed against the plasmidless strain. This was expected, because many of the costly genes carried on the Ti plasmid are tightly regulated and were not expressed during these competitions. This regulation effectively minimizes the burden of the plasmid outside of the disease environment where it is not providing a benefit. The results of this study suggest that the energetic requirements associated with additional replication of DNA (Ti plasmid approx. 0.2 Mb) are relatively low. However, despite low costs, small differences in fitness can have dramatic effects over longer time scales. This is supported by evidence that pTi prevalence in natural isolates declines dramatically in the absence of suitable hosts [53], likely outcompeted by strains that lack the plasmid. This observation is also supported by our model, which incorporates both a small Ti plasmid carriage cost and a pAt-specific resource benefit for the rhizosphere and tumour environments, which predict the eventual loss of the pTi+ genotype in the rhizosphere (figure 4 and the electronic supplementary material, S2). Only with a significantly lower concentration of pAt+ catabolizable resources relative to opines, is there coexistence between pTi+ and pAt+pTi+ genotypes. Nonetheless, extended persistence of the Ti plasmid in the non-tumour environment likely depends on tight regulation of virulence and disease-associated genes.

(c) High cost of the At plasmid

The At plasmid, by comparison, demonstrated a strikingly large cost. This was a surprising result given that this plasmid was extremely difficult to cure, perhaps owing to the presence of multiple toxin–antitoxin (TA) systems on the plasmid (see the electronic supplementary material). These systems increase the likelihood that cells that lose the plasmid as a result of aberrant segregation during cell division, or displacement by incompatible plasmids, are effectively removed from the competing population. There are several putative TA systems that can be identified on the At plasmid, the toxin gene of one that was deleted to facilitate curing of the At plasmid. In addition to this, the conjugation system of the At plasmid is constitutively expressed under a wide range of laboratory conditions, suggesting that this would be also true in the soil [54]. When Ti plasmid virulence genes are induced, cells experience dramatic negative growth effects with doubling times nearly twice as long as those under non-inducing conditions [16]. A subset of the Ti plasmid *vir* genes are highly similar to the At plasmid conjugation (*avhB*) genes [23]. If the *avhB* genes are being constitutively expressed, then they might account for a portion of the At plasmid costs.

(d) Rhizosphere-specific benefit of the At plasmid

Despite the high costs of the At plasmid, the results of within-plant competitions show that the benefits it confers, likely owing to catabolic functions, are sufficient to provide a competitive advantage to pAt+ cells. Until now, inference of the environment-specific benefits of the At plasmid was based entirely on specific gene functions, but had never been empirically demonstrated. Although environmental patterning studies are still needed to truly evaluate the ecological dynamics of this plasmid, these studies suggest a clear rhizosphere-specific benefit.

Outside of the rhizosphere, the high cost of the At plasmid could potentially be offset by conjugal transfer to new hosts. Constitutive conjugation incurs a significant burden on host cells, such that in the absence of potential recipients, or

compensatory environmental benefits, a plasmid investing in horizontal transmission would likely fail to be maintained in a population [55,56]. However, in the presence of suitable recipients, conjugation could compensate for higher costs and put a competing plasmid at a marked disadvantage. Established trade-offs between horizontal and vertical transmission [55] suggest that a plasmid that has evolved to use resources for conjugation might be compensating for costs incurred to the host, or as a consequence of within- or between-host competition. Perhaps resource competition with plasmidless or singly infected pTi+ strains is sufficient to have selected for this increased investment into horizontal transmission.

(e) Nonlinear interactions of the At and Ti plasmids

The individual costs of the At and Ti plasmids are not only quite distinct, but also exhibit context dependence, varying nonlinearly with the interacting partner. Competitions between pAt+ and pTi+ cells against their plasmidless derivatives do not predict their competitive interaction with one another, as pTi+ cells exhibit greater than expected competitive ability against pAt+ cells. One explanation for this is that cells carrying a Ti plasmid produce some factor(s) that increases the expression of a costly At plasmid function, or vice versa. The observation that this advantage is diminished when the pAt+ competitor also harbours pTi suggests that if this were the case, the production of this factor might depend on the presence of pTi− cells in the population, or that non-additive plasmid costs dampen this effect.

There are dozens of transcription factors on both the At and Ti plasmids, and these could be influencing the expression of genes on the other plasmid. A recent study by Lang *et al*. [57] demonstrates that At plasmid conjugation is affected by the expression of an opine-responsive transcriptional repressor encoded on the Ti plasmid. Another clear demonstration that the At and Ti plasmids interact in this way, which could result in diminished costs for cells harbouring both plasmids in the pre-disease environment, is pAt-dependent decreased expression of the costly Ti plasmid *vir* genes (figure 2). This could be an effect of direct regulation by the At plasmid, although we know of no shared regulatory elements for these genes. Alternatively, this could be an effect of competition for specific resources within the cell. As mentioned previously, a subset of the type IV secretion systems encoded by the Ti plasmid *vir* genes and the At plasmid *avhB* (conjugation) genes are highly homologous. If pAt conjugation genes are being constitutively expressed, specific resources such as nitrogen or carbon might be limited for the formation of these complexes, thereby limiting *vir* gene expression. This result is surprising in that it is contradictory to previous findings demonstrating an increase in tumour size for pAt+ cells, although a correlation between tumour size and relative expression of *vir* genes has never been established. Further studies with pAt mutants that are non-conjugal would help to determine the mechanism driving this effect.

6. Conclusion

The results of these studies show clear evidence for complex epistatic interactions between two commonly co-occurring megaplasmids that markedly affect the fitness of different plasmid genotypes, including the first demonstrated example of non-additive costs among multiple plasmids. We also show that this non-additivity is likely to have very strong effects on the outcome of microbial competition and plasmid population dynamics. Given that selective pressures (plasmid costs and benefits) and community dynamics are all environment-dependent, the variable lifestyle of *A. tumefaciens* as a facultative pathogen is likely to explain some of the complexity of the observed interactions. Although the precise selective forces that drive these interactions and the mechanisms that underlie them remain to be determined, this study demonstrates the importance of inter-plasmid dynamics on the outcome of species-level population dynamics.

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