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Evolutionary dynamics of rhizopine within spatially structured rhizobium populations

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Symbiosis between legumes and nitrogen-fixing bacteria is thought to bring mutual benefit to each participant. However, it is not known how rhizobia benefit from nodulating legume hosts because they fix nitrogen only after becoming bacteroids, which are terminally differentiated cells that cannot reproduce. Because undifferentiated rhizobia in and around the nodule can reproduce, evolution of symbiotic nitrogen fixation may depend upon kin selection. In some hosts, these kin may persist in the nodule as viable, undifferentiated bacteria. In other hosts, no viable rhizobia survive to reproduce after nodule senescence. Bacteroids in these hosts may benefit their free-living kin by enhancing production of plant root exudates. However, unrelated non-mutualists may also benefit from increased plant exudates. Rhizopines, compounds produced by bacteroids in nodules and catabolized only by related free-living rhizobia, may provide a mechanism by which bacteroids can preferentially benefit kin. Despite this apparent advantage, rhizopine genotypes are relatively rare. We constructed a mathematical model to examine how mixing within rhizobium populations influences the evolution of rhizopine genotypes. Our model predicts that the success of rhizopine genotypes is strongly dependent upon the spatial genetic structure of the bacterial population; rhizopine is more likely to dominate well-mixed populations. Further, for a given level of mixing, we find that rhizopine evolves under a positive frequency-dependent process in which stochastic accumulation of rhizopine alleles is necessary for rhizopine establishment. This process leads to increased spatial structure in rhizobium populations, and suggests that rhizopine may expand the conditions under which nitrogen fixation can evolve via kin selection.

Keywords: coevolution; mutualism; nitrogen fixation; population genetic structure; symbiosis; kin selection

1. INTRODUCTION

Symbiotic relationships between nitrogen-fixing bacteria and their eukaryotic hosts are thought to bring mutual benefit to each participant. However, little is known about how rhizobia benefit by nodulating legumes (Smith & Douglas 1987; Murphy *et al*. 1988). In fact, detailed natural history of the interaction points to a potentially severe cost to rhizobia of symbiotic nitrogen fixation.

Rhizobia entering the nodule commonly proliferate extracellularly in a structure called the infection thread which, in some host species, provides a refuge from competing rhizosphere bacteria, protozoan predation and phage attack (Gordon *et al*. 1996). However, rhizobia in the infection thread cannot fix nitrogen; if nodulation is arrested at this stage, infection is pathogenic to the host (Djordjevic *et al*. 1987). When nodulation proceeds normally, a subset of rhizobia are released from the infection thread into host meristem cells, surrounded by a host-derived peribacteroid membrane (Newcomb 1981), and differentiate into bacteroids, in which state they can use host carbon to fix atmospheric dinitrogen (Caetano-Anollés & Gresshoff 1991). However, differentiation appears to be a terminal developmental event and bacteroids lose the ability to reproduce (Zhou *et al*. 1985).

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In hosts with determinate nodules (Sprent *et al*. 1987), some bacteria remain undifferentiated within the nodule and can reproduce when released into the soil during nodule senescence (Gresshoff *et al*. 1977; Tsien *et al*. 1977; Gresshoff & Rolfe 1978; Sutton & Paterson 1979, 1980; van den Bos & Broughton 1981; Zhou *et al*. 1985). However, in other hosts, especially those with indeterminate nodules (Sprent *et al*. 1987), no viable undifferentiated bacteria survive nodule senescence (Almon 1933; Kijne 1975; Sutton *et al*. 1977; Sutton & Paterson 1979; Paau *et al*. 1980; van den Bos & Broughton 1981; Zhou *et al*. 1985). In either case, it appears likely that rhizobium cells that fix nitrogen are genetically dead. Nevertheless, nitrogen fixation might benefit their undifferentiated kin that remain reproductively viable in the rhizosphere (Jimenez & Casadesus 1989; Olivieri & Frank 1994) or infection thread.

Host-plant roots excrete into the rhizosphere many compounds that increase rhizobial growth (Hartwig *et al*. 1991), and in a nitrogen-limited soil, nodulation may increase the rate of excretion into the rhizosphere by improving host growth. Because bacteria largely reproduce asexually by fission, a proportion of cells in the infection thread, as well as cells outside the nodule, are likely to be genetically identical to bacteroids inside the nodule. Thus, although a differentiated bacterium may have sacrificed its own reproduction, this altruistic action

could be evolutionarily advantageous if, by enhancing the growth of its host, it offsets its own loss of reproductive capacity by increasing the fitness of its kin.

One problem with kin selection, however, is its vulnerability to cheaters. Enhancing host growth improves the nutritional environment for all microbes dependent on plant resources. There are two mechanisms by which kin selection may maintain the nitrogen-fixing mutualism. First, the population may be spatially structured, such that related bacteria are more likely to benefit from bacteroid sacrifices. For example, if each nodule represents a single infection, then in hosts with determinant nodules, all surviving undifferentiated bacteria in the nodule will be related to bacteroids, and any benefits from nitrogen fixation will accrue primarily to kin. However, in hosts that produce indeterminant nodules, the only viable kin occur in the rhizosphere, which is an intensely competitive environment (Lynch 1990). Bacterial populations in the rhizosphere may not be sufficiently structured for nitrogen fixation to evolve by spatially dependent kin selection. Instead, enhancing plant growth improves the rhizosphere environment for all microbes dependent upon plant resources, including unrelated non-mutualists, which does not guarantee an increase in the relative fitness of bacteroid kin. Even in this situation, however, kin selection could be an important process maintaining mutualism if the mutualistic system had a mechanism for accurately targeting resources to bacteroid kin. Either the host-plant or rhizobium could evolve the ability to produce a specialized nutritional resource exploitable only by reproductive kin of bacteroids. Some rhizobium strains produce a private nutritional source called rhizopine, which may broaden the conditions under which nitrogen fixation is advantageous to terminally differentiated bacteroids. In this paper, we develop a model to describe the evolutionary dynamics of the rhizopine trait under different degrees of spatial genetic structure.

2. RHIZOPINES

Inositol rhizopines are nitrogen- and carbon-containing compounds produced from plant precursors by bacteroids in nodules and catabolized by reproductive rhizobia (Tempé *et al*. 1982; Murphy *et al*. 1987, 1995; Saint *et al*. 1993; Rao *et al*. 1995). Rhizopine synthesis is directly regulated by the symbiotic nitrogen-fixation regulatory gene *nifA*, so that conditions in the nodule that initiate nitrogen fixation also initiate rhizopine synthesis (Murphy *et al*. 1988). Genes responsible for rhizopine catabolism are expressed in free-living rhizobia (Murphy *et al*. 1987). These two sets of genes are linked on the symbiotic plasmid, so that rhizopine produced by bacteroids in a nodule can be catabolized by reproductively capable cells of the same strain (Tempé & Petit 1983; Murphy *et al*. 1988; Saint *et al*. 1993). Non-rhizopine rhizobium and other soil bacteria apparently cannot catabolize rhizopine (Rossbach *et al*. 1995). Thus, rhizopine is a private nutritional source for rhizopine strains.

Given this unique advantage, it is difficult to understand why all rhizobia do not produce and catabolize rhizopine. Indeed several studies, including *in vitro*, pot and field experiments, indicate that rhizopine rhizobia have a competitive advantage and persist in the environment longer than non-rhizopine rhizobia (Jensen 1987; Evans & Howeison 1993; Murphy *et al*. 1995). Yet, rhizopine is not common in nature (Tempé *et al*. 1982; Eardly *et al*. 1990; Rossbach *et al*. 1995). In fact, in a survey of 332 strains, rhizopine synthesis and catabolism were found in only 13% of *Rhizobium meliloti* Division A (now classified as *Sinorhizobium meliloti* (De Lajudie *et al*. 1994)) and 12% of *R. leguminosarum* bv. *viciae* (Wexler *et al*. 1995).

Two kinds of rhizopine have been identified: *scyllo*-inosamine (*S*I or *s*Ia) (Saint *et al*. 1993) and L-3-*O*-methyl-*scyllo*-inosamine (3-*O*-M*S*I) (Tempé *et al*. 1982; Murphy *et al*. 1987). Both compounds consist of a six-carbon ring decorated with a nitrogen-containing amine group, and can be distinguished by the presence of a methyl group in the latter compound. However, only one strain has been identified that can synthesize *s*Ia (Wexler *et al*. 1995), and is probably a deletion mutant derived from a 3-*O*-M*S*I-producing strain (Rao *et al*. 1995). Further, all rhizopine strains so far identified can catabolize both 3-*O*-M*S*I and *s*Ia. Because production of *s*Ia appears to be a rare mutation that has not spread, we focus our model on the evolution of the rhizopine genotype that produces and catabolizes 3-*O*-M*S*I.

3. MODEL

Although the rhizopine phenotype comprises two traits, synthesis and catabolism, we treat it as a single-gene trait. This simplification is in part justified because the synthesis (*mos*) and catabolism (*moc*) genes are closely linked on the symbiotic plasmid (Murphy *et al*. 1988) and appear to be inherited together (Wexler *et al*. 1996), suggesting that synthesis and catabolism evolve as a single functional unit (Murphy *et al*. 1988; Saint *et al*. 1993). This simplification also assumes no independent mutation of the two loci.

Our model includes two genotypes: rhizopine, which possesses a $mos^{+}moc^{+}$ *Sym* plasmid, and non-rhizopine, which possesses a $mos^{-}moc^{-}$ *Sym* plasmid. We assume initially that each nodule harbours only one rhizobial genotype. Double infections occur in artificial inoculation experiments (Lindemann *et al*. 1974; Lucas *et al*. 1992), but the rate of double infection under natural conditions is unknown. However, given assumptions of symmetry, we find that inclusion of multiple infections does not change the results of the model (Appendix A).

The two genotypes, then, each occur in two states: as bacteroids inside the nodule, which do not reproduce, and as reproductive (undifferentiated) cells in the nodule or surrounding rhizosphere. As both rhizobial genotypes are assumed to be equivalent in other aspects of their life histories, the relative fitness of their reproductive cells depends upon the genotypic identity of bacteroids in the adjacent nodule. We assume that all rhizosphere cells have equal access to non-rhizopine plant root excretions and grow on them with equivalent efficiencies. Thus, when non-rhizopine bacteroids occupy a nodule, all adjacent reproductive cells experience equal fitness, which we set to one. We further assume that when rhizopine genotypes occupy a nodule, they divert a proportion, s, of root excretions to rhizopine and that the

Table 1. *Fitness and probabilities of occupancy of adjacent nodule, conditional on reproductive cell genotype*

		fitness when adjacent to nodules with bacteroids of genotype		probability of being adjacent to nodules with bacteroids of genotype	
		mos$^-$ *moc*$^-$	*mos*$^+$ *moc*$^+$	*mos*$^-$ *moc*$^-$	*mos*$^+$*moc*$^+$
reproductive	*mos*$^-$ *moc*$^-$	1	$(1-c)(1-s)$	$\Phi+p(1-\Phi)$	$q(1-\Phi)$
	mos$^+$ *moc*$^+$	1	$(1-c)$	$p(1-\Phi)$	$\Phi+q(1-\Phi)$

extra synthetic steps involved in producing rhizopine involve a carbon cost, c. When rhizopine genotypes occupy the nodule, the fitness of an adjacent reproductive cell of the non-rhizopine genotype is reduced both by having access to only the non-rhizopine component of the available carbon $(1-s)$ and also by the cost, c, of rhizopine production. Thus, the fitness of non-rhizopine genotypes adjacent to rhizopine nodules is the product $(1-s)(1-c)$. In contrast, reproductive cells of the rhizopine genotype can catabolize rhizopine as well as other root exudates. Thus, when adjacent to rhizopine nodules their fitness $(1-c)$ is reduced only by the cost of rhizopine production, as summarized in table 1.

Assuming that both bacterial types are equally likely to nodulate and that bacteria mix freely within the soil, then the probability of a free-living reproductive cell being adjacent to a nodule of a given genotype depends solely on the genotype proportions within the population, which we denote as p and q $(q=1-p)$ for non-rhizopine and rhizopine genotypes, respectively. However, soil is a viscous matrix in which bacterial dispersal is limited (Lowther & Patrick 1993; Parco *et al*. 1994), leading to patchy distribution of soil organisms (Turkington & Harper 1979; Chanway *et al*. 1991; Bever *et al*. 1996). In such a structured system, there may be a greater probability than expected by chance that a reproductive cell is genetically identical by descent to bacteroids in the adjacent nodule. We define a coefficient of relationship, Φ, as the probability that bacteroids in a nodule are identical by descent to adjacent reproductive cells. If the rate of bacterial movement in the soil is high relative to the rate at which nodules are formed and senesce, then the spatial relationship between bacteroids and reproductive bacteria is random and $\Phi=0$. In contrast, $\Phi=1$ when there is no bacterial movement and a reproductive rhizobium is always associated with a nodule occupied by genetically identical bacteroids.

The probability of a reproductive bacterium being adjacent to a given bacteroid genotype is therefore determined by the genotype frequencies and the rate of mixing of bacteria in the soil. For example, the probability that a non-rhizopine reproductive cell is adjacent to a nodule occupied by non-rhizopine bacteroids is the sum of the probability of being identical by descent (Φ) and the probability of encountering that genotype at random, weighted by the probability of not being identical by descent $(p(1-\Phi))$. The probability that this same non-rhizopine reproductive cell is adjacent to a rhizopine nodule is the probability that the bacteroids are not identical by descent, weighted by the probability that they are rhizopine genotype $(q(1-\Phi))$. Similar reasoning produces the values for rhizopine reproductive cells in table 1.

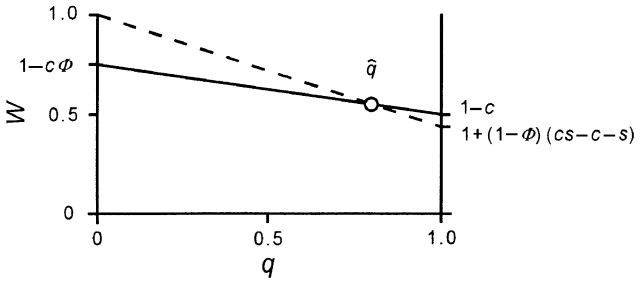

Figure 1. Fitness of rhizopine genotypes (dashed line = $w_{\text{non-rhizopine}}$, solid line = $w_{\text{rhizopine}}$) as a function of rhizopine genotype frequency under intermediate levels of cell mixing. The fitness functions cross at an internal equilibrium, $\hat{q}$.

We calculate the expected fitness of each bacterial genotype as the sum of its fitnesses in association with nodules of the two genotypes, weighted by the probability of encountering these types of nodules. Hence,

$$w_{\text{non-rhizopine}}=1+q(cs-c-s)(1-\Phi) \tag{1}$$

$$w_{\text{rhizopine}}=1-c(\Phi+q(1-\Phi)). \tag{2}$$

The fitness of both genotypes declines with rhizopine frequency, but non-rhizopine fitness declines more steeply. The two fitness functions may cross to produce an internal equilibrium, $\hat{q}$ (figure 1). The rhizopine genotype frequency in the next generation is given by

$$\Phi q'=\frac{qw_{\text{rhizopine}}}{\overline{w}}=\frac{q[1-c(\Phi+q(1-\Phi))]}{\overline{w}}, \tag{3}$$

where $\overline{w}$ is the average fitness of the population and

$$\overline{w}=1+q[p(sc-c-s)(1+\Phi)-c(\Phi+q(1-\Phi))]. \tag{4}$$

The change in frequency of the rhizopine genotype with each generation is then

$$\Delta q=q'-q=(pq/\overline{w})[qs(1-c)(1-\Phi)-c\Phi]. \tag{5}$$

Equilibrium is reached when $q=0$, $q=1$, and also when

$$\hat{q}=c\Phi/[s(1-c)(1-\Phi)]. \tag{6}$$

Analysis of local stability indicates that the equilibrium at $q=0$ will be stable and non-rhizopine will be fixed when

$$c\Phi>0, \tag{7}$$

and the equilibrium at $q=1$ will be stable and rhizopine will be fixed when

$$\Phi<(sc-s)/(sc-c-s). \tag{8}$$

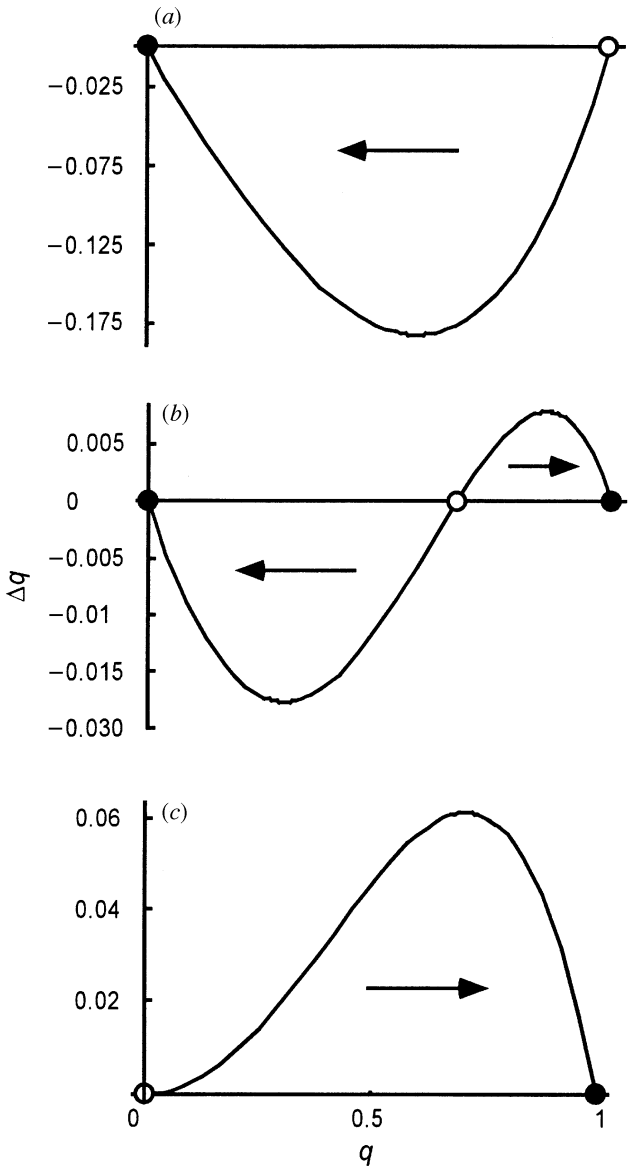

Figure 2. Predicted change in rhizopine gene frequency (Δq) as a function of rhizopine gene frequency, q, for three different values of Φ: (*a*) 1.0, (*b*) 0.25 and (*c*) 0. In these figures, both s and c are set to 0.5.

The internal equilibrium will exist and will be unstable when both inequalities (7) and (8) are true. Under these conditions the system exhibits positive frequency dependence with fixation of either rhizopine or non-rhizopine as potential stable outcomes (figure 2). By deterministic forces alone, initial establishment of the rhizopine genotype requires that rhizopine be produced without cost ($c=0$) or that the soil environment exhibits complete mixing ($\Phi=0$). Otherwise, stochastic forces must carry the rhizopine genotype frequency above $\hat{q}$.

The likelihood that rhizopine will establish via a stochastic process increases with decreasing values of $\hat{q}$. This event becomes more likely as the degree of mixing in the bacterial population increases (lower Φ), the proportion of exudate allocated to rhizopine increases (higher s), or the cost of rhizopine production decreases (lower c) (figure 3). A more efficient rhizopine genotype, as measured by high s and low c, is more likely to be established by stochastic forces. Moreover, as mixing in the population decreases (i.e. Φ increases), the likelihood of success for the rhizopine genotype becomes more dependent upon its efficiency of rhizopine production (figure 3).

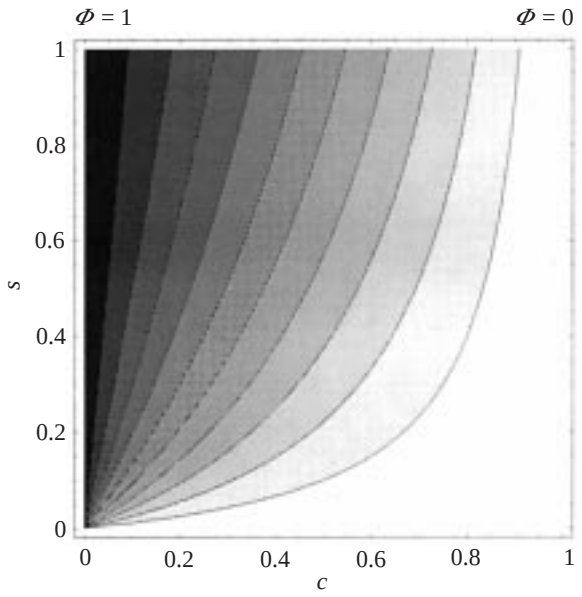

Figure 3. Isoclines of Φ, in the s and c space, for which $q=1$ (as in equation (8)). To the left of each line, s is sufficiently larger than c so that rhizopine can establish. To the right of the line, c becomes too large and non-rhizopine prevails.

4. DISCUSSION

Our model predicts that persistence of the rhizopine genotype depends upon some degree of bacterial mixing in the soil. Without bacterial mixing, the rhizopine genotype will be eliminated from the population if rhizopine synthesis entails any cost. This is true because, without mixing, reproductive cells occur only adjacent to nodules occupied by their kin. With no non-rhizopine cells to compete against, rhizopine reproductive cells realize no fitness advantage from exclusive access to rhizopine. Further, because of the cost of rhizopine synthesis, nodules occupied by rhizopine bacteroids produce less plant exudate than do those occupied by non-rhizopine bacteroids and, in the absence of mixing, this cost of rhizopine production is borne exclusively by rhizopine reproductive cells.

Mutualism may evolve via kin selection in spatially and genetically structured (unmixed) populations (Hamilton 1964; Wade 1979; Michod 1982). Because our model predicts that mixing enhances rhizopine persistence, it suggests that rhizopine could play a role in maintaining the nitrogen-fixing mutualism in less structured bacterial populations as well. This mechanism remains to be tested, but it clearly depends upon the evolutionary stability of the rhizopine trait.

Given intermediate levels of bacterial mixing in the soil, the spread of rhizopine depends upon a positive frequency-dependent process. Positive frequency-dependent selection for rhizopine production may generate strong spatial structuring of rhizobium populations. For a given set of environmental conditions and initial

genotypic frequencies, the population should fix at one or the other genotypic state. Mixed populations of rhizopine and non-rhizopine genotypes should be rare.

Positive frequency dependence also dictates that rhizopine can establish only if chance events allow accumulation of enough rhizopine genotypes to exceed $\hat{q}$. This requirement may help explain why surveys have revealed so few rhizopine genotypes; perhaps rhizopine can be successful, but its establishment is limited by stochastic processes. The location of $\hat{q}$, and thereby the probability of rhizopine establishment, depends upon the degree of mixing in the bacterial population, Φ, and the efficiency of rhizopine synthesis, which is indicated by the relationship between c and s (figure 3). An efficient rhizopine genotype can divert a large quantity of carbon to rhizopine (high s) at little cost (low c). More efficient rhizopine genotypes are more likely to succeed, and the degree of efficiency necessary for success increases with decreased mixing in the bacterial population (i.e. increased Φ).

With this dependence on mixing within the soil, our model predicts that across populations, rhizopine production will be correlated with bacterial traits or environmental factors that facilitate cell mixing. For example, numerous studies indicate that water facilitates bacterial movement through the soil (Carlile 1980; Breitenbeck *et al*. 1988; Issa *et al*. 1993). Thus, we predict that wetter soils are more likely to harbour rhizopine-producing rhizobia, whereas non-rhizopine rhizobia will occupy drier soils. Further, because cell motility is positively correlated with pore-neck diameter (Carlile 1980), and fine-textured soils have smaller pore-neck diameters for a given level of soil moisture, we also predict that rhizopine-producing rhizobia are more likely to occupy coarser soils. In addition, because physical mixing of soil also enhances bacterial movement, the rhizopine trait may be more likely to occur in soils mixed by ploughing or by the activities of fossorial mesofauna, such as earthworms, and larger fossorial animals, such as gophers. Finally, we also expect rhizopine production to be genetically correlated with the possession of a flagellum, as rhizobia vary in the occurrence and number of flagellae, and flagellae can improve cell motility (Soby & Bergman 1983; Issa *et al*. 1993).

Empirical surveys of rhizopine production among rhizobia are needed to test these predictions. As mentioned previously, a few studies have examined the distribution of rhizopine production among rhizobium taxa (e.g. Tempé *et al*. 1982; Eardly *et al*. 1990; Rossbach *et al*. 1995; Wexler *et al*. 1996). However, to our knowledge, none has evaluated the spatial clumping of rhizopine genotypes or attempted to correlate rhizopine production with other bacterial traits or environmental factors. Our model provides important clues about the requirements for the evolution of rhizopine and makes clear predictions to be evaluated by empirical surveys. It also suggests that rhizopine might expand the conditions under which kin selection can maintain the nitrogen-fixing mutualism. However, owing to positive frequency-dependent dynamics, the rhizopine trait is less common than originally expected because its establishment may be limited by stochastic processes. Further, relaxing the assumption that rhizopine synthesis and catabolism genes cannot mutate independently may further restrict the conditions under which rhizopine can evolve.

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APPENDIX A

We expanded the model to include double infection by denoting the frequency of doubly infected nodules as r. As above, we assume that the occurrence of particular combinations of genotypes in doubly infected nodules is proportional to the genotype frequencies and influenced by the degree of cell mixing. For a particular type of reproductive cell, the probability of a particular combination of bacteroid genotypes occurring in the adjacent nodule is presented in table A1. Further, if we assume that in a nodule that is doubly infected with both genotypes, half the total carbon is available to rhizopine bacteroids, then the relative fitnesses of reproductives are as denoted in table A1. Again, calculating bacterial fitness as the product of the relative frequency of each combination and the fitness of reproductives in that combination, we find that the relative fitnesses of the

Table A1. *Probabilities of occupancy of adjacent nodule (upper values), conditional on reproductive cell genotype, and relative fitnesses of reproductive cells (lower values), as a function of their genotype and that of bacteroids in the adjacent nodule, when nodules can be doubly infected*

bacteroid(s)	reproductive $mos^-\ moc^-$	$mos^+\ moc^+$
$mos^-\ moc^-$	$(1-r)[\Phi+p(1-\Phi)]$ 1	$(1-r)[p(1-\Phi)]$ 1
$mos^+\ moc^+$	$(1-r)[q(1-\Phi)]$ $(1-c)(1-s)$	$(1-r)[\Phi+q(1-\Phi)]$ $(1-c)$
$mos^-\ moc^-$, $mos^-\ moc^-$	$r[\Phi^2+2\Phi p(1-\Phi)+p^2(1-\Phi)^2]$ 1	$r[p^2(1-\Phi)^2]$ 1
$mos^+\ moc^+$, $mos^+\ moc^+$	$r[q^2(1-\Phi)^2]$ $(1-c)(1-s)$	$r[\Phi^2+2\Phi q(1-\Phi)+q^2(1-\Phi)^2]$ $(1-c)$
$mos^-\ moc^-$, $mos^+\ moc^+$	$2r[\Phi q(1-\Phi)+pq(1-\Phi)^2]$ $[1+(1-c)(1-s)]/2$	$2r[\Phi p(1-\Phi)+pq(1-\Phi)^2]$ $[1+(1-c)]/2$

three genotypes are identical to that presented in equations (1) and (2). Thus, double nodule occupancy has no effect on the outcome of our model.

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