Spite and virulence in the bacterium *Pseudomonas aeruginosa*

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Social interactions within populations of pathogenic microbes may play an important role in determining disease virulence. One such ubiquitous interaction is the production of anticompetitor toxins; an example of a spiteful behavior, because it results in direct fitness costs to both the actor and recipient. Following from predictions made by mathematical models, we carried out experiments using the bacterium *Pseudomonas aeruginosa* to test under what social conditions toxin (bacteriocin) production is favored and how this in turn affects virulence in the larvae of the wax moth *Galleria mellonella*. Consistent with theory, we found that the growth of bacteriocin producers relative to sensitive non-producers is maximized when toxin producers are at intermediate frequencies in the population. Furthermore, growth rate and virulence in caterpillars was minimized when bacteriocin producers have the greatest relative growth advantage. These results suggest that spiteful interactions may play an important role in the population dynamics and virulence of natural bacterial infections.

allelopathy | bacteriocins | disease | kin selection | microbial evolution

In recent years there has been a growing interest in understanding the evolution of social behaviors in microbes (1). The evolution of cooperation (behaviors that benefit the recipient) has received considerable theoretical and empirical attention, whereas the evolution of spite (behaviors that harm both the actor and recipient) has been relatively neglected. Conditions that favor the evolution of spite can be understood in terms of selection maximizing an individual's inclusive fitness (transmission of one's own genes and of one's own genes in other individuals). Spiteful behaviors can, therefore, theoretically evolve when they target individuals that are less likely to share the same genes as the actor than an average member of the competing population. That is, the relatedness between the recipient and the actor is negative (2–8).

Spiteful behaviors found in nature are surprisingly common, and one well-documented example is the production of bacteriocins. Bacteriocins are extracellular antimicrobial compounds produced by almost all bacteria (9). They can be considered spiteful, because they are costly to produce and because they kill susceptible cells via a range of mechanisms, including enzyme inhibition and the breakdown of DNA and cell membranes. The costs of production can be suicide (in *Escherichia coli*, for example, cell lysis is required to release the bacteriocins), but even where cell death is not required there will be an inevitable metabolic cost that is likely to be greater than the direct fitness benefits. Bacteriocins are highly diffusible; hence, the producing cell is unlikely to experience the benefit of killing a competitor (9, 10). Crucial for the evolutionary maintenance of bacteriocin production is that bacteriocins specifically target nonrelated individuals while doing no harm to the bacteriocinogenic cells, usually due to immunity factors that are genetically linked to the toxin (9). Note that relatedness in this context specifically refers to similarity at the bacteriocin loci between interacting individuals rather than average similarity across the whole genome. In this sense, bacteriocins can be viewed as spiteful green beards, whereby the same gene complex is capable of directing spite toward individuals that do not have the gene complex for the spiteful behavior (11).

A number of theoretical and empirical studies identify ecological conditions that favor the maintenance of spite (12–18). Assuming that individuals possess mechanisms to distinguish between related and unrelated individuals (2), spiteful behaviors are predicted to evolve to maximal levels when the frequency of individuals with the same spiteful trait makes up some intermediate frequency of the population (16). If the spiteful group is at a high frequency in the interacting population, spite will be less favored because the reduction in the competition resulting from the spiteful action will be small compared to the costs of being spiteful. Conversely, if the spiteful group is at a low frequency in the interacting population, the few individuals that are targeted will be on average no less related than the individuals that are not targeted. Hence, relatedness will be zero or weakly negative. This result leads to the prediction that spite will be most favored when the spiteful genotype is at an intermediate frequency in the interacting population. Note that in a previous paper (16) we refer to frequency of a particular genotype within the interacting population as "kinship." This term has a different meaning to relatedness, which refers to similarity between actor and recipient relative to the competing population as a whole.

An explicit test of the predicted unimodal relationship between spite and the frequency of spiteful genotypes has yet to be carried out. Existing empirical studies are, however, consistent with this prediction. Specifically, it has been shown in vitro that toxin producers can invade sensitive populations only when they are above a threshold starting frequency in both *E. coli* and the yeast *Saccharomyces cerevisiae* (12, 17).

Understanding how the genetic population structure of microbial pathogens affects production of bacteriocins has important applied implications, most notably in terms of the amount of harm infections cause their hosts (virulence). Attenuated virulence is predicted to coincide with maximal levels of spite (16), because under these conditions the growth rate of the infecting population will be lowest, as a result of increased killing and investment into the spiteful behaviors.

Here we use the opportunistic human pathogen *Pseudomonas aeruginosa* and a caterpillar model to explicitly test the predictions that (*i*) bacteriocin production is most favored when the spiteful genotype is at intermediate frequencies in the interacting population and (*ii*) that this results in minimal in vivo population growth rate and virulence. We also extend our previous evolutionary mathematical models to confirm that the qualitative predictions still hold in the ecological context of this experimental system.

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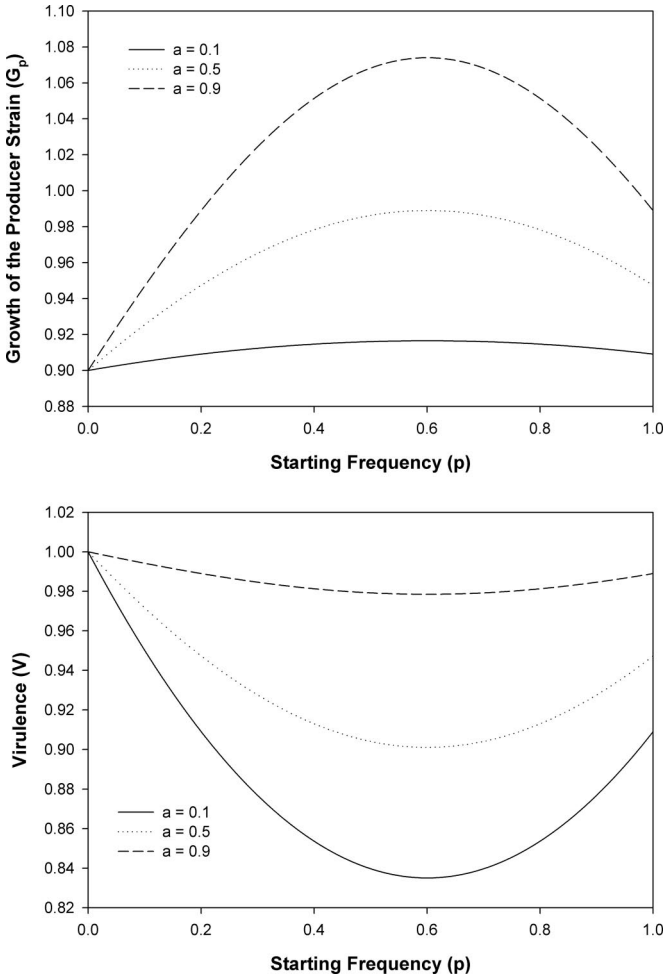

Fig. 1. Modeling the relative growth and virulence of a bacteriocin producer. Output from our mathematical models across a range of resource competition (*a*), when $c = 0.1$ and $k = 0.5$, showing that producers growth (*Upper*) is maximized at intermediate frequencies and at these frequencies virulence (*Lower*) is attenuated.

Results

A simple mathematical model was developed to describe the ecological conditions that favor bacteriocin production. In this model, a focal lineage of bacteriocin-producing bacteria compete with sensitive bacteria and remain insensitive to the bacteriocins of its sensitive competitors. Under these conditions (which are described in more detail in *Materials and Methods*), bacteriocin production provides the greatest advantage when producers are at intermediate frequency in the population and virulence is reduced at these frequencies. Examples of the growth rate of the producing strain at different frequencies and extent of local competition are shown in Fig. 1. Note that same qualitative relationships hold if the *y* axis displays the selection coefficient of the producing strain (assuming a minimum susceptible strain growth rate that is greater than zero, as observed in the experiment).

In our in vitro experiments we manipulated densities of both a bacteriocin-producing strain (PAO1, producer of pyocin S2) and a sensitive competitor (*P. aeruginosa* O:9) to create a range of different starting frequencies with respect to the producer (between 0 and 1) (19). As a control, we established the same range of starting frequencies for an isogenic mutant (PAO1150-2) of PAO1 that did not produce a bacteriocin that could affect the sensitive strain. Selection coefficients were used

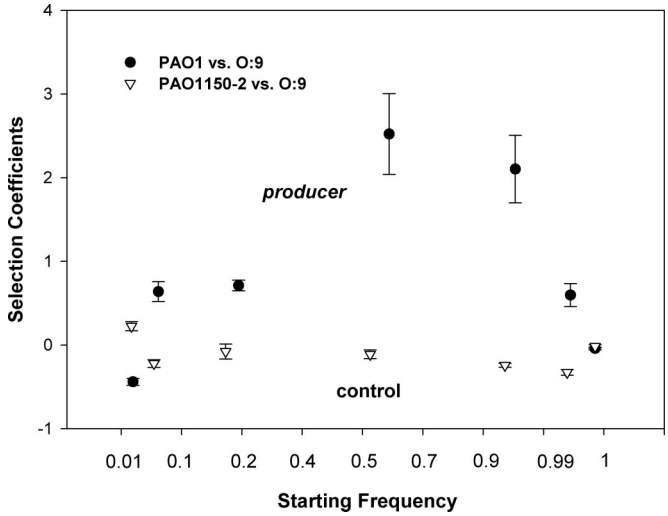

Fig. 2. Relative growth of bacteriocin producer. Relative growth rate of PAO1 vs. O:9. (producer, black circle) compared to relative growth rate of PAO1150-2 vs. O:9 (control, white triangle) along a range of different starting frequencies used to manipulate relatedness. PAO1 vs. O:9 shows a distinct peak in relative growth at intermediate frequencies (linear term, $F_{1,32} = 20.76$, $P < 0.001$; quadratic term, $F_{1,31} = 29.64$, $P < 0.001$).

to estimate the fitness of the producing and isogenic nonproducing strain relative to the sensitive strain. As predicted by the mathematical model, the fitness of the producing strains showed a unimodal relationship with starting frequency (Fig. 2), peaking at intermediate values (linear term, $F_{1,32} = 20.76$, $P < 0.001$; quadratic term, $F_{1,31} = 29.64$, $P < 0.001$). By contrast, the isogenic nonproducing strain showed a weakly negative relationship with starting frequency (Fig. 2), with a slope of -0.0975 (linear term, $F_{1,34} = 11.34$, $P < 0.002$; quadratic term, $F_{1,33} = 0.13$, $P > 0.721$). The ratio of the selection coefficients of the producing and isogenic nonproducing strain also displayed a significant unimodal relationship (linear term, $F_{1,32} = 23.44$, $P < 0.001$; quadratic term, $F_{1,31} = 25.85$, $P < 0.001$).

The effect of bacteriocin production on growth of the producer strain depends on the amount of time the bacteria spend competing. Specifically, only a weak relationship is observed between relative growth and starting frequencies before 96 h of growth, probably because of the time it takes for PAO1 to reach sufficient densities for pyocin to have an effect on O:9. Allowing bacteria to grow for longer periods of time does not qualitatively change the results but only makes detection of both PAO1 and O:9 harder at treatments with very high and low producer frequencies. As the overall numbers of bacteria decrease, there is an increasing build up of waste metabolites and depletion of resources. This dynamic is consistent with our model and is captured by the intensity of local competition ("*a*" parameter).

We next determined the relationship between virulence (as measured by time to death of infected caterpillars) and frequency of the producing strain. We manipulated the infecting bacterial populations to give high (99%), intermediate (50%), and low (1%) frequencies of the producing strain relative to the susceptible strain. Consistent with the above theory (see also ref. 16), intermediate frequencies resulted in much longer time to death than the high and low frequencies (linear term, $F_{1,58} = 52.47$, $P < 0.001$; quadratic term, $F_{1,57} = 55.85$, $P < 0.001$) (Fig. 3*A*). The proposed mechanism behind this reduction in virulence at intermediate frequencies is reduced growth rate of the population as a whole, resulting from the high mortality rates of the susceptible strain. Consistent with this view, we found that bacterial density before the death of the insects showed an

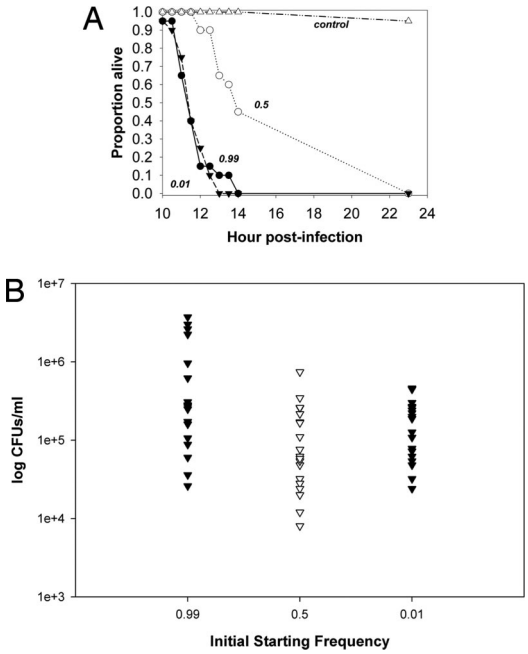

Fig. 3. Virulence and density affected by frequency of bacteriocin producer. (*A*) Time to death of caterpillars inoculated with PAO1/O:9 mixtures. Initial starting frequencies of PAO1 are indicated on the graph and correspond to the adjacent line. At the intermediate starting frequency death is significantly delayed (linear term, $F_{1,58} = 52.47$, $P < 0.001$; quadratic term, $F_{1,57} = 55.85$, $P < 0.001$). (*B*) The average total bacterial density of PAO1 and O:9 is indicated for the 3 different starting frequencies of the bacteriocin producer. A significant reduction in overall density occurs after 8 h of growth in the intermediate frequency treatment of PAO1 vs. O:9, where bacteriocin producers and sensitive non-producers are inoculated at initially near equal densities.

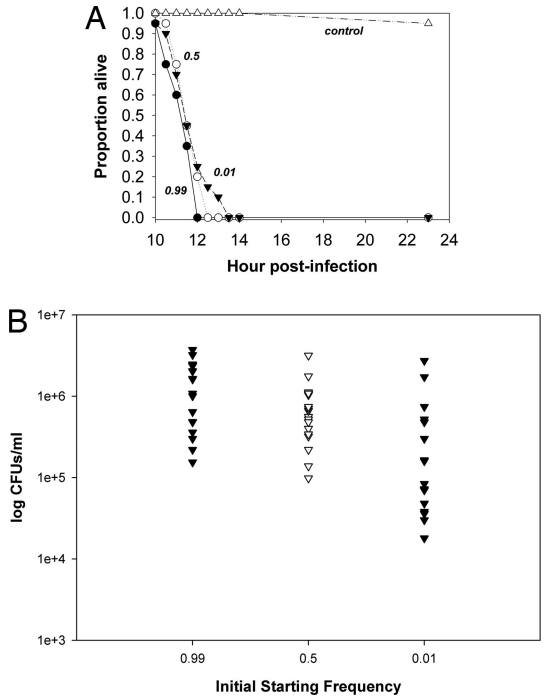

Fig. 4. Virulence and density are unaffected by frequency when bacteriocins are not produced. (*A*) Time to death of caterpillars inoculated with PAO1150-2/O:9 mixtures. Initial starting frequencies of PAO1150-2 are again indicated on the graph and correspond to the adjacent line, but in this case there is no significant difference delay in time to death (linear term, $F_{1,58} = 1.28$, $P > 0.263$; quadratic term, $F_{1,57} = 0.65$, $P < 0.422$) (*B*) The average total bacterial density PAO1150-2 and O:9 is indicated for the 3 different starting frequencies of the bacteriocin-negative mutant. There is no significant difference in overall density after 8 h of growth in the caterpillars between the different starting frequencies.

inverse unimodal relationship with frequency of the bacteriocin producer, such that density was lower for the 50% treatment (linear term, $F_{1,59} = 4.5$, $P < 0.038$; quadratic term, $F_{1,58} = 7.99$, $P < 0.007$) (Fig. 3*B*). Note that when the nonproducing strain was competed with the susceptible strain, there was no significant difference in virulence between the high, intermediate, and low starting frequency treatments ($P > 0.25$ for both linear and quadratic terms) (Fig. 4*A*), and only a linear relationship exists between density and starting frequency of the non-producer (linear term, $F_{1,58} = 16.24$, $P < 0.001$; quadratic term, $F_{1,57} = 2.66$, $P > 0.108$) (Fig. 4*B*).

Discussion

In this study, we show that a unimodal relationship exists between the growth of spiteful, toxin-producing bacteria when competing with susceptible strains and their starting frequency. Furthermore, we demonstrate that conditions that favor spiteful behaviors result in minimal virulence in caterpillar hosts, as a result of reduced population growth rate. Finally, we show theoretically that this unimodal relationship between the fitness of bacteriocin producers holds in both ecological and evolutionary contexts.

It is necessary to emphasize that in our experiments the producer differs from the sensitive strain in other ways than pyocin production and susceptibility, because the strains are not isogenic. This fact, however, does not alter our interpretation of the data, because the non-producer, which is isogenic to the producer, shows only a weak negative relationship between its growth and frequency, probably because of the slightly different resource uses of the different strains (20). Furthermore, virulence at intermediate frequencies is attenuated in our producer strain, whereas there is no difference in any treatment with the non-producer.

Our model suggests some very simple mechanisms to explain our results. When the producer is at low frequency, the benefits of reducing competition and "freeing-up" resources will be shared by the sensitive strain as much as producing strains; thus, there is little net benefit to the producing strain (relatedness is only weakly negative). Similarly, bacteriocin production has less benefit at high frequencies, because there are few competitors to kill, and hence there are fewer resources to be gained from costly bacteriocin production. Only at intermediate frequencies will bacteriocin production confer the greatest fitness advantage by killing competitors and thereby "freeing-up" resources.

The impact of spatial structure on the fitness of bacteriocin producers can also be understood in terms of the frequency of the producer in a competitive arena. In Chao and Levin's (12) experiments using *E. coli*, spatial structure was manipulated to give 2 scenarios: mass habitat and structured habitats. In mass habitats there was a frequency-dependent relationship to the success of bacteriocin production, where bacteriocin producers were only able to invade if relatively common. In structured habitats, however, bacteriocin producers are able to invade even when at low starting frequencies. Spatial structure makes individuals interact locally and through stochastic processes can result in higher local frequencies of the producer. These conditions lead to a situation in which the producer is at a high enough frequency and relatedness is sufficiently negative to allow bacteriocin producers to dominate. In our experiment we used homogeneously mixed environments similar to the "mass habitats" of Chao and Levin (12), but by considering a range of different relatedness structures we were able to show that frequency, facilitated by habitat structure, is driving this dynamic.

Consistent with theoretical results, virulence was greatly attenuated when mixing bacteriocin producers with sensitive bacteria. This result is consistent with recent studies showing that (*i*) a mixture of one bacteriocin-producing strain and sensitive strains of *Photorhabdus* and *Xenorhabdus* spp. resulted in lower virulence in caterpillars than the respective single strain infections (21) and (*ii*) mixing of *Xenorrhabdus nematophila* and its symbiotically associated nematode reduced virulence and increased susceptibility to bacteriocins (22). Here, we clarify these results by demonstrating that mixed infections show reduced virulence but only when the bacteriocin producers are at an intermediate frequency in the infecting population. Furthermore, we determine that attenuation of virulence at intermediate frequencies is almost certainly explained (as predicted) by a reduced growth rate of the infecting population as a whole, resulting from the high mortality rate of the susceptible strain. Density may not be the only important factor in determining virulence because intrinsic genetic differences between the various strains may also have a notable effect.

The specific shape (inverse unimodal) of the relationship between virulence and strain frequency of the infecting population is likely to depend entirely on the spiteful interactions (23). When other types of social interactions are more important than spite in determining the outcome of competition, different relationships are predicted. First, a monotonic negative relationship is predicted when bacteria are simply competing for resources because high diversity results in greater resource competition, leading to rapid host exploitation and increased virulence (24). Second, a positive relationship between virulence and diversity is predicted when bacteria need to cooperate to grow, because cooperation is most likely to be favored when diversity is low (25, 26). What remains to be investigated, both theoretically and empirically, is how the relationship between virulence and strain frequency is affected when multiple social interactions are important to the outcome of competition.

This study has provided experimental evidence of how strain frequency and, in turn, relatedness affects bacteriocin production. Note, however, that we have specifically measured local fitness and not global fitness under conditions that favor bacteriocins. We have also ignored any evolution of nonproducing resistance types (that would lead to a "rock–paper–scissors" interaction) (14, 15), because we are only competing producing versus sensitive or nonproducing versus sensitive strains. However, nonproducing resistance in competition with producing resistance represents a form of social cheating, and thus we can apply this kin selection framework to understand this problem in the future.

Here we have shown that spiteful behaviors, or more specifically, bacteriocin (pyocin) production is crucially affected by the frequency in the population of a given strain. We have also shown that pyocin production can have a major impact on the virulence of *P. aeruginosa* infections. The study may ultimately have practical applications in terms of manipulating the competitive arena such that toxin producers are favored and therefore reduce virulence. Pyocin production in *P. aeruginosa* is also likely to be important in a clinical setting, especially in diseases such as cystic fibrosis, where pyocin-producing strains are commonly found (27) and different strains are often outcompeted as the disease progresses.

Materials and Methods

Model. ***Bacteriocins.*** We consider 2 strains of bacteria growing under resource competition, with the focal strain making a relative investment c into bacteriocin production and the competitor strain making no such investment. We assume that the focal strain is immune to its bacteriocin, but a proportion pk of cells of the competitor strain is killed, where p is the proportion of the focal strain in the local medium.

The ''per capita'' growth of the focal (producing) strain (the growth scaled to that of a nonproducer strain in pure culture) is given by

$$G_{\mathrm{P}} = \frac{1-c}{1-a(pc+(1-p)pk)}, \qquad [1]$$

where a is the extent of local competition for resources (e.g., the degree of soft selection), and the growth of the competitor (nonproducing) strain is

$$G_{\mathrm{N}} = \frac{1-pk}{1-a(pc+(1-p)pk)}. \qquad [2]$$

The total growth is given by

$$G_{\mathrm{T}} = \frac{1-(pc+(1-p)pk)}{1-a(pc+(1-p)pk)}. \qquad [3]$$

Thus, in the extreme of complete local competition ($a = 1$), the total growth is fixed at $G_{\mathrm{T}} = 1$.

The growth of the focal (producing) strain is independent of its local frequency p in the absence of resource competition ($a = 0$), and is given by $G_{\mathrm{P}} = 1 - c$. Here, the bacteriocin producer always exhibits lower growth than a pure culture of the nonproducing strain (i.e., $1 - c < 1$). In the presence of local competition for resources ($a > 0$), the growth of the producing strain is dependent on its local frequency; the derivative

$$\frac{dG_{\mathrm{P}}}{dp} = \frac{a(1-c)(c+k-2pk)}{(1-a(pc+(1-p)pk))^2} \qquad [4]$$

takes the same sign as $c + k - 2pk$, i.e., $dG_{\mathrm{P}}/dp > 0$ when $p < (c + k)/2k$ and $dG_{\mathrm{P}}/dp < 0$ when $p > (c + k)/2k$. Thus, the growth of the producing strain is a monotonically increasing function of its frequency if $c > k$, and a unimodal-shaped function of its frequency if $c < k$. In particular, the growth of the producing strain is $G_{\mathrm{P}} \to 1 - c$ as $p \to 0$, and $G_{\mathrm{P}} \to (1 - c)/(1 - ac)$ as $p \to 1$. Note that $(1 - c)/(1 - ac) < 1$ so, if $c > k$, the growth of the producing strain is always less than that achieved by a pure culture of the nonproducing strain. If $c < k$ then growth of the producing strain is maximized at the $p^* = (c + k)/2k$, and here it is given by $G_{\mathrm{P}} = 4(1 - c)k/(4k - a(c + k)^2)$, which exceeds the growth of the nonproducing strain in pure culture if $a > 4ck/(c + k)^2$. Note that c (the cost to the producer) must be $< k$ (the maximum cost experienced by the recipient) for pyocin production to be maintained by natural selection.

Assume that the above growth is occurring in a single subpopulation of a much larger structured population in which the producing strain is vanishingly rare and that the focal subpopulation is representative of all of the subpopulations in which the producing strain is located. Then the local frequency (p) of the producing strain is equivalent to the kin selection coefficient of relatedness (r) describing the genetic similarity of cells of the producing strain to the other cells growing in its locality. The producing strain is expected to invade from rarity if its growth is greater than the average in the whole metapopulation (nonproducing strain in pure culture), i.e., when $G_{\mathrm{P}} > 1$. This yields the condition

$$\frac{1-c}{1-a(pc+(1-p)pk)} > 1, \qquad [5]$$

which may be reexpressed as

$$\left(-\frac{ap}{1-ap}\right)(-(1-p)k) > c \qquad [6]$$

which is of the form $RB > C$, where $R = -ap/(1 - ap)$ is Queller's (28) form of relatedness (genetic similarity of social partners relative to competitors), and is equivalent to equation A2 in Gardner, West, and Buckling (16).

Virulence. Now consider that each subpopulation represents a single host individual carrying a bacterial infection. Assume that the virulence of the bacterial infection is proportional to its growth, i.e.,

$$V = bG_{\mathrm{T}}. \qquad [7]$$

Under the extreme of complete resource competition ($a = 1$), bacterial growth is $G_{\mathrm{T}} = 1$ and virulence is fixed at $V = b$. With less intense resource competition ($a < 1$), virulence is dependent on the frequency of the producing strain within the infection; the derivative

$$\frac{dV}{dp} = -b\,\frac{(1-a)(c+k-2pk)}{(1-a(pc+(1-p)pk))^2} \qquad [8]$$

has the opposite sign of $c + k - 2pk$, i.e., $dV/dp < 0$ when $p < (c + k)/2k$ and $dV/dp > 0$ when $p > (c + k)/2k$. The sign of dV/dp is always opposite of that of dG_P/dp, and so virulence is monotonically decreasing with the frequency of the producing strain when $c > k$ and is a U-shaped function of the frequency of the producing strain when $c < k$. In particular, virulence is maximized in the absence of bacteriocin production ($p = 0$), and is minimized when the producing strain is at the intermediate frequency $p^* = (c + k)/2k$.

Bacterial strains. *P. aeruginosa* strain PAO1 was used as the bacteriocin producer, and serotype O:9, as the bacteriocin-sensitive competitor PAO1, is a known producer of pyocin S2, whereas serotype O:9 is sensitive to S2 pyocins (19, 29). PAO1150-2, a transposon bacteriocin-knockout mutant of *psy2*, acted as a nonproducing, isogenic control strain (30). Bacteriocin production in *P. aeruginosa* can involve lysis, but it is not clear whether it is essential for the release of the soluble pyocins that are the focus of this study (10). Bacteriocin production, sensitivity, and insensitivity were confirmed by using a simple plate assay where the production of relevant bacteriocin is determined by overlaying bacteria mixed in semisolid agar on plates that have been spotted with bacteria of another strain, as described by Fyfe et al. (31). If the strain inoculated on the plate produces bacteriocin that kills the strain mixed with semisolid agar, a halo-shaped zone of clearing can be observed in the bacterial lawn after incubating at 37 °C for 18 h. The absence of a clear halo indicates that either the overlaid strain is insensitive to the bacteriocin producer or the inoculated strain does not produce any bacteriocin.

Competition Assays. Overnight cultures of each strain were grown with shaking at 0.65 × *g* at 37 °C for 18 h and then diluted to an OD_{600} of 1.8 to ensure similar numbers of bacteria per milliliter. These cultures were subsequently grown on agar plates to determine the number of bacteria present, with colony forming units (CFUs) as an approximate measure. Thirty-milliliter glass universals containing 6 ml of Kings Media B broth were inoculated with a total of 10^4 cells with different starting frequencies of the individual strains. PAO1 and O:9 where competed against each other at starting frequencies of 99%, 90%, 50%, 10%, 1%, and 0.1%. This exact design was replicated in the PAO 1150-2 and O:9 competition. Cultures were propagated in a shaking incubator at 0.65 × *g* at 37 °C and sampled at 48 and 96 h, allowing time for the effect of the bacteriocin to be observed.

At each time point (48 h and 96 h), we calculated the relative growth of the producer to sensitive and non-producer to sensitive at the different starting frequencies. This was done by plating the various treatments on KB agar plates and counting the number of CFUs for each strain. All strains were easily distinguishable from one another because of unique colony morphology and size. At the more extreme frequencies, antibiotic plates were required to give better resolution of colony counts, and this was possible due to the different antibiotic resistance profiles of the assorted strains (PAO1 resistant to 1,250 μg/ml streptomycin; O:9 resistant to 312.5 μg/ml rifampicin; and PAO 1150-2 resistant to 312.5 μg/ml tetracycline). Selection coefficients (*S*) were used to estimate at what frequency bacteriocin production is favored in PAO1 relative to 1150-2 using the common competitor O:9, where $s = (m_j - m_j)/m_j$, and *m* refers to ln(final density/starting density) of strain *j* (in this case either PAO1 or 1150-2) and strain *j* (O:9) (32). All frequencies were replicated 6 times, and statistical analyses were performed in Minitab 15. Selection coefficients were preferable to simply using growth rates (*m*) to control for between-tube variation.

In Vivo Virulence Bioassay. Virulence assays were performed as described by Harrison et al. (33). Briefly, overnight cultures of PAO1, O:9, and PAO1150-2 were diluted in minimal salt solution. Fifth-instar waxmoth (*Galleria mellonella*) larvae (Livefood UK) were randomly allocated to be inoculated with 10^4 CFUs of PAO1/O:9 and PAO 1150-2/O:9 mixtures. The starting frequencies of the bacterial combinations consisted of 99%, 50%, and 1% PAO1 and PAO1150-2 to O:9. Larvae were swabbed with 70% ethanol to prevent contamination of the injection site and were injected into the abdomen with Terumo 1-ml disposal syringes and BD Microlance 30-gauge 1/2 needles. The injection volume was 50 μl in all cases. Twenty larvae were assigned to each treatment, and a further 20 larvae were injected with 50 μl of minimal salt solution as negative controls. Larvae were then incubated at 37 °C and monitored for death at 30-min intervals between 10 and 14 h and again at 24 h after inoculation. Larvae were scored as dead if they failed to respond to mechanical stimulation of the head.

Overall density of the different bacterial strains within the caterpillar hosts was also measured. Caterpillars were inoculated as previously described and incubated for 8 h at 37 °C. Larvae were then weighed, dipped in 70% ethanol to kill surface contaminants, and homogenized with a plastic pestle in 500 μl of minimal salt solution. Homogenates were centrifuged at 455 × *g* for 3 min to pellet the solid, and aliquots of diluted homogenate were plated onto KB agar. Agar plates were supplemented with 15 μg/ml ampicillin to select against growth of native larval-gut bacteria (this concentration of ampicillin does not affect the growth of *P. aeruginosa*). Plates were incubated overnight at 37 °C and subsequently scored for CFUs.

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