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Evolutionary biology

The role of 'soaking' in spiteful toxin production in *Pseudomonas aeruginosa*

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The ubiquitous production of antibacterial toxins, such as bacteriocins, is an ecologically significant class of interbacterial interactions that have primarily evolved through their indirect fitness benefits to the producer. Bacteria release bacteriocins into the environment at a cost to individual cell, but individual bacteriocin-producing cells are unlikely to gain any direct benefit from their own toxin; indeed, cell lysis is required in many species. There is a growing body of research describing the ecological conditions that can favour the evolution of bacteriocin production. However, an important aspect of many bacteriocins has yet to be investigated: the ability of bacteriocin-producing cells to neutralize toxin ('soaking') produced by other clonemates. By competing *Pseudomonas aeruginosa* bacteriocin-producing wild-type and 'non-soaking' strains against a bacteriocin-susceptible strain, we find that soaking markedly reduces the fitness of a bacteriocin-producing strain at both high and low frequencies.

1. Introduction

Social interactions in microbes are widespread and have far-reaching implications [1]. Recently, there has been a proliferation of interest in social interactions that can be considered spiteful [2–7], where an actor pays a net cost to harm a recipient [8–10]. The production of antibacterial toxins, such as bacteriocins, which is common among bacteria [11], can be considered spiteful where the costs of toxin production (which are maximal in the many cases where cell lysis is required) outweigh the direct benefits obtained by the actor through the killing of local competitors. Recent theory and experiments suggest that spiteful bacteriocin production can be favoured when the spiteful lineage occurs at intermediate frequencies compared with bacteriocin-sensitive bacteria, within the interacting population [2,6,12]. This is because when spiteful individuals are at a low frequency in the population, the benefits created by the spiteful behaviour, such as reducing competition and 'freeing-up' resources will be shared by the sensitive, non-spiteful individuals as much as those exhibiting the spiteful trait. Similarly at high frequencies of the spiteful lineage, there will be a reduction in the benefit of the spiteful trait, as there are few competitors to harm and, therefore, fewer resources to gain. Only at intermediate frequencies is spite beneficial as it results in harm to competitors and allows exploitation of the resulting unspent resources by the spiteful lineage.

Despite qualitative consistency between the theoretical and the empirical studies where bacteriocin producers are competed against susceptible strains, a potentially important feature of bacteriocins may alter the frequency-dependent fitness of spiteful lineages: bacteriocin-producing cells have receptors that translocate their own bacteriocin which is then subsequently neutralized, as they possess an immunity protein encoded in gene clusters with the production gene or genes [11,13]. The overall effect of the bacteriocins will, therefore, be reduced as the bacteriocinogenic strain increases in both density and total proportion of the population. This 'self-soaking' of

toxins might alter the frequency-dependent fitness of bacteriocin producers and be important in explaining the persistence of sensitive strains even when they are rare in the population; especially if only a small percentage of the bacteriocinogenic strain are responsible for toxin production as it will then be more likely that the toxin will be neutralized by coexisting cells. To explore the role of 'self-soaking' in the evolution of bacteriocin production, we determine how the absence of the bacteriocin receptor affects the frequency-dependent fitness of bacteriocin-producing *Pseudomonas aeruginosa*.

2. Material and methods

(a) Bacterial strains

Pseudomonas aeruginosa strain PAO1 (bacteriocin-producing, 'soaker') and PW5036 (bacteriocin-producing, 'non-soaker') were competed with the bacteriocin-sensitive strain serotype O:9. PW5036 is isogenic to the wild-type PAO1 except for having its FpvA receptor rendered non-functional through transposon mutagenesis in the *fvpA* gene [14]. The stability of the transposon insertion was subsequently determined by PCR (see the electronic supplementary material). FpvA is the primary receptor for type I pyoverdine and the pyocin S2 produced by PAO1 [15]. In order to determine that no differences existed in bacteriocin production between PAO1 and PW5036, the sensitive strain (O:9) was cultured for 18 h in 1-day-old and 4-day-old PAO1 and PW5036 supernatants, respectively, and densities were determined through plating and counting colony-forming units (CFUs). Soft agar overlays of the sensitive strain (O:9) were performed on PAO1 and PW5036 to confirm this result (electronic supplementary material, figure S1; [16]).

(b) Competition assays

Overnight cultures of each strain were grown shaking at 0.65g and 37°C for 18 h and then diluted to an OD_{600nm} of 1.8 to ensure similar numbers of bacteria per millilitre. These cultures were subsequently grown on agar plates to determine the number of bacteria present using CFUs as an approximate measure. Thirty millilitre glass universals containing 6 ml of Kings Media B broth were inoculated with a total of 10⁴ cells with different starting frequencies of the individual strains. PAO1/PW5036 and O:9 were competed against each other at a range of starting frequencies. Cultures were propagated in a shaking incubator at 0.65g and 37°C and sampled at 96 h. All frequencies were replicated six times.

At 96 h, we calculated the growth of PAO1 (wild-type) relative to O:9 (bacteriocin-sensitive) and PW5036 (bacteriocin 'non-soaker') relative to O:9 (bacteriocin-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. The strains were easily distinguishable from one another because of unique colony morphology and size. PAO1 and PW5036 form large, smooth, green colonies, whereas O:9 forms small, wrinkly, white colonies. 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 strains (O:9 is resistant to rifampicin 312.5 µg ml⁻¹, PW5036 is resistant to tetracycline 312.5 µg ml⁻¹, and PAO1 is resistant to rifampicin 60 µg ml⁻¹ and tetracycline 6 µg ml⁻¹). Selection coefficients (*S*) were used to estimate at what frequency bacteriocin production is favoured in PW5036 relative to the wild-type PAO1 using the common competitor O:9, where $S = (m_{\text{PAO1/PW5036}} -$

$m_{\text{O:9}})/m_{\text{O:9}}$, and (*m*) refers to ln(final density/starting density) [17]. As no cells of the bacteriocin-sensitive common competitor O:9 could be detected at when present at frequencies of 1 and 10 per cent, we used the minimum detection level (200 cells ml⁻¹) to calculate our selection coefficients for these frequencies.

To control for intrinsic growth rate differences between PW5036 and the wild-type PAO1, they were also competed against each other directly at a 1:1 ratio for 96 h and subsequently plated on KB agar and KB agar containing tetracycline to distinguish between both strains. Selection coefficients were calculated and PAO1 displayed an overall 2.7 per cent fitness advantage when compared with PW5036 (results not shown). When calculating final selection coefficients, this growth rate advantage of PAO1 was used to scale the growth of PW5036 (by multiplying its growth, represented by the term *m* in the equation for calculating selection coefficients, by 2.7%) to allow for direct comparison between the two strains, thereby taking into account only the difference in FpvA production. However, even after taking into account this growth rate difference, we acknowledge that, though unlikely, other, unknown effects of losing the FpvA receptor may affect our results. All statistical analyses were performed in R (v. 2.9.2).

3. Results

In our experiment, we compared the frequency-dependent fitness of a wild-type bacteriocin producer (PAO1) strain when competed with a bacteriocin-sensitive strain (*P. aeruginosa* serotype O:9), with that of a bacteriocin-producing 'non-soaker' (PW5036, deficient in its bacteriocin, S2, receptor, FpvA, but otherwise isogenic to wild-type PAO1) when competed with the same bacteriocin-sensitive strain (O:9) [14]. We confirmed that growth inhibition of the susceptible strain did not differ in the supernatant of wild-type and non-soaker strains ($F_{1,24} = 1.23$, $p > 0.29$) and was unaffected by the length of time strains were cultured before supernatant was extracted ($F_{1,24} = 3.89$, $p > 0.083$), with no significant interaction term ($F_{1,24} = 0.23$, $p > 0.64$). This result was also confirmed when performing soft agar overlays containing the sensitive strain on both bacteriocin-producing strains (see the electronic supplementary material, figure S1) [16]. This strongly suggests that bacteriocin production did not differ between the wild-type and non-soaker strains, hence differences in frequency-dependent fitness between the wild-type and non-soaker strain are presumably the result of soaking effects.

Selection coefficients were used to estimate the fitness of the bacteriocin-producing wild-type, non-soaker and sensitive strains [17]. Both the wild-type and non-soaking strain show a unimodal relationship between fitness and starting frequency, as previously described [6], with both the wild-type (linear $F_{1,31} = 20.76$, $p < 0.001$; quadratic $F_{1,30} = 29.64$, $p < 0.001$) and non-soaking strain (figure 1), peaking at intermediate values (linear $F_{1,31} = 10.7$, $p < 0.003$; quadratic $F_{1,30} = 8.98$, $p < 0.005$). However, when compared with the wild-type, the non-soaker peaks at higher starting frequencies and reaches overall higher levels of selection coefficients (intercept: $F_{1,61} = 35.01$, $p < 0.0001$; strain by frequency by frequency interaction: $F_{1,60} = 5.16$, $p < 0.027$). Pairwise comparisons between the two strains reveal greater fitness of the non-soaker at starting frequencies of 0.1 per cent ($p < 0.0001$), 1 per cent ($p < 0.003$), 10 per cent ($p < 0.01$), 90 per cent ($p < 0.0001$) and 99 per cent ($p < 0.002$) but no difference at 50 per cent ($p > 0.62$) after sequential Bonferroni correction for multiple tests [18].

Figure 1. Difference in growth between the wild-type strain (PAO1) with the ability to absorb and neutralize its own bacteriocins (i.e. 'self-soaking') and an isogenic strain lacking the FvpA receptor (PW5036) required for the absorption of the bacteriocin when competing against a sensitive strain (O:9). The receptor deficient strain shows an increased competitive advantage over the wild-type at higher and lower frequencies. Error bars show ± 1 s.e. of the mean. Filled circles, PW5036 (non-soaker) versus O:9; open circles, PAO1 (soaker) versus O:9.

4. Discussion

The costs of soaking are clearly most pronounced at both high and low frequencies. At high frequencies, this is entirely as expected because more wild-type cells will inevitably lead to more soaking. The low-frequency effect, however, is initially more surprising, as less soaking inevitably occurs with fewer bacteriocinogenic cells. However, theory suggests that the fitness reduction of competitors per bacteriocinogenic cell is critically important in determining the frequency at which a bacteriocinogenic lineage can invade a susceptible population, or is driven to extinction: the greater the reduction in competitor fitness, the lower the frequency at which bacteriocinogenic cells can invade [2,9]. This means that a reduction in competitor killing through soaking will have disproportionately greater effects at reducing fitness at lower frequencies of the bacteriocinogenic lineage. At intermediate frequencies, costs of self-soaking are likely to be minimal, because bacteriocins are produced in sufficient quantity to generate a large fitness advantage over susceptible bacteria.

As well as investigating the effect of soaking on frequency-dependent fitness, we were interested if soaking could contribute to the observed ability of sensitive cells to persist when at initially low starting frequencies [6]. Whereas approximately 10^5 cells ml^{-1} survived competition with the wild-type (when the sensitive strain (O:9) is at starting frequencies of 1 and 10%), none of the sensitive competitor strain (O:9) was detectable when competed with our non-soaking strain at these frequencies (with a detection threshold of approx. 200 cells ml^{-1}). Note that the resulting selection coefficients for these two treatments are a minimum and assume 200 cells ml^{-1} of the competitor. Soaking may, therefore, contribute to the maintenance of sensitive cells in both clinical and natural populations, and may contribute to the intransitive dynamics of producing, resistant and sensitive cells [19].

Under conditions where the producing strain is dominant in the population, one would also expect the loss of the bacteriocin receptor, as strains without it would be able to outcompete the wild-type as they no longer pay the costs of producing and expressing this receptor and are more effective at killing competitors. However, in this system, FvpA is also responsible for the uptake of pyoverdine type I [15], an important iron-scavenging molecule, so its loss would be unlikely to occur in iron-limited environments.

Soaking is likely to be important in the evolution of microbial toxin production in general. Many toxins target receptors that have important fitness consequences for competitors, which may often be shared by toxin-producing lineage. As such, selection to target common bacterial receptors may be constrained by the need to produce increased quantities of bacteriocins. Moreover, this soaking effect also suggests another important role of community context in driving evolutionary dynamics. The extent to which bacteriocins can bind to receptors for other species is unclear, but cases have been identified where interspecies inhibitions occur [11,13]. Finally, the increase in the short-term efficacy of receptor-less mutants, as well as their inevitable long-term cost through their inability to use siderophores, suggests a potential role in the biocontrol of clinical and agricultural infections.

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