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Cooperative production of siderophores by *Pseudomonas aeruginosa*

Freya Harrison[1,2], Angus Buckling[1]

[1]Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS UK, [2]Current address: Department of Biology and Biochemistry, University of Bath, Bath BA2 7AY UK

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1. ABSTRACT

The production of iron-scavenging siderophores by the opportunistic animal pathogen *Pseudomonas aeruginosa* is a textbook example of public goods cooperation. This trait provides an excellent model system with which to study cooperation. Further, the links between siderophore production and *P. aeruginosa* virulence allow us to investigate how pathogen ecology, social behaviour and pathology might be connected. We present here the results of basic research on the evolution and ecology of siderophore cooperation in this species. In particular, we explore the effects of population and community structure, iron regime and genomic mutation rate on the relative success of siderophore cooperators and cheats. We also present preliminary data on the links between siderophore production and another clinically-relevant social trait, biofilm formation. It is our hope that more realistic laboratory studies of siderophore cooperation in *P. aeruginosa* will eventually cast light on the roles played by social traits in long-term microbial infections.

2. INTRODUCTION: SIDEROPHORES ARE A 'PUBLIC GOOD.'

Iron is a major limiting factor for bacterial growth (1). Under aerobic conditions, iron exists in the largely insoluble ferric form, and within animal hosts is generally sequestered by high-affinity iron-binding proteins (1, 2). Iron is therefore not easily accessible, especially to pathogenic bacteria. In order to deal with this problem, bacteria have evolved numerous mechanisms to scavenge iron. One common solution is the production of iron-scavenging molecules which are released into the environment in response to iron deficiency (3).

One important and well-characterised group of such molecules is the siderophores (4). These chelate iron from host protein complexes, re-enter the bacterium via specific receptors and release their cargo of iron to cellular metabolism. Their production is facultatively regulated in response to iron availability (1): low iron availability increases siderophore production. As siderophores can be

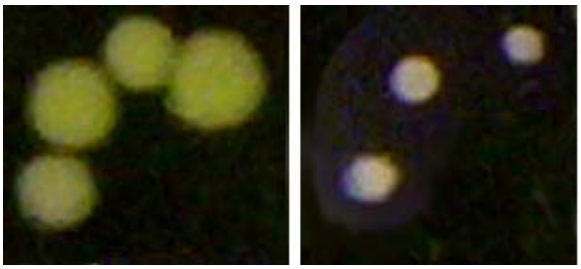

Figure 1. Colonies of pyoverdine non-producers (white) are easily distinguished from colonies of pyoverdine producers (yellow-green) on iron-limited agar. (Colonies shown were grown overnight on casamino acids (CAA) agar: 5g casamino acids, 1.18g $K_2HPO_4.3H_2O$, 0.25g $MgSO_4.7H_2O$ per litre, supplemented with 12g/L agar powder).

taken up by any cell with the cognate receptor, regardless of its own levels of siderophore production, siderophores represent a form of public goods cooperation: while individually metabolically costly to produce, siderophores confer a group-level benefit (5). This is predicted to make populations of siderophore producers open to invasion by non-producers (see (5-8)). Simple experiments using the opportunistic bacterial pathogen *Pseudomonas aeruginosa* (9) have confirmed the metabolic cost and growth advantage of siderophore production. First, in iron-limited growth media, monocultures of producers reach higher densities than do monocultures of non-producers. Second, when mixed together, non-producing clones can outcompete producing clones; and non-producing clones reach higher densities in the presence of siderophore producers than they do in monoculture (9). This ability of non-producers to exploit producers allows us to recognise them as social defectors or "cheats" (10, 11).

Production of siderophores by fluorescent Pseudomonads in fact represents a remarkably tractable model system for studying the evolution and ecology of cooperation. The primary siderophore of *Pseudomonas* spp. is the yellow-green pigment pyoverdine (1, 3, 4, 12). Cells which do not produce pyoverdine form white colonies on iron-limited agar, providing an easy visual score for cooperator *versus* cheat clones (Figure 1)(9). Further, a simple and cheap colorimetric assay for total siderophore content of culture supernatants exists (13). We have shown that the results of visual scoring and this assay are consistent with one another (8).

The relationship between siderophore production and bacterial growth rates led to the suggestion that siderophore production might enhance bacterial virulence. Consistent with this, mutants deficient in siderophore production have been shown to exhibit reduced virulence (12, 14, 15). Therefore, understanding the evolution and ecology of siderophore production should not only serve as an excellent basis for advancing our understanding of cooperation in general, but also provides us with an opportunity to study direct links between cooperation and virulence. In fact, efficient host exploitation by many microbial pathogens often relies on the cooperative production of siderophores (14-16), other nutrient-scavenging molecules (17), toxins (18), products that interfere with the host immune response (19, 20) or enzymes that degrade antibiotics (21). (See (22) for a review of social behaviours in microbes). That links between pyoverdine production and other virulence-related traits have been demonstrated (23, 24) makes this molecule even more interesting as a model public good.

Understanding how *P. aeruginosa* virulence is determined is of particular interest as this species is an important opportunistic human pathogen. Acute infections of burn wounds and nosocomial colonisation via medical artefacts (e.g. catheters) are risk factors for hospital patients (25). More importantly, this species is one of the most common and dangerous pathogens for cystic fibrosis (CF) sufferers. Almost all CF patients become chronically colonised by this species and colonisation severely worsens prognosis, promoting more frequent acute exacerbations, a general decline in lung function and significant increases in patient morbidity (26-28). *P. aeruginosa* can also interfere with the host immune system and chronic colonisation may increase the probability of further pathogenesis (19, 29, 30). Most people with CF die as a result of respiratory failure resulting from chronic microbial infection (31).

We and our co-workers have carried out a number of experiments using *P. aeruginosa* to elucidate the importance of population and community structure and evolution in determining levels of siderophore cooperation. We have also researched how cooperation mediates the outcome of mixed infections, and have begun to explore the links between siderophore production and another cooperative trait linked with pathogenicity, biofilm formation. In this review, we will present the results of these investigations and make suggestions for future work based on our observations. The path diagram in Figure 2 illustrates the hypothesised connections between the factors investigated.

3. SIDEROPHORE COOPERATION AND VIRULENCE

Many models of parasite virulence and evolution predict that mixed (multi-genotype or low relatedness) infections should be more virulent than single-genotype infections. Historically, this prediction gained wide acceptance, and it was attributed to increased resource competition in low-relatedness infections favouring more voracious host exploitation (32-36). However, empirical support for this hypothesis was, and remains, lacking. One likely explanation for this lack of support is that models often assume that simple resource competition represents the only possible interaction between coinfecting strains, whereas in reality other types of interaction exist: in particular, cooperation via public goods production. If some necessary virulence factors are public goods (14-16, 18-22), then this has serious implications for models of relatedness and virulence: kin selection theory predicts that public goods production, and so virulence, will be decreased at low relatedness.

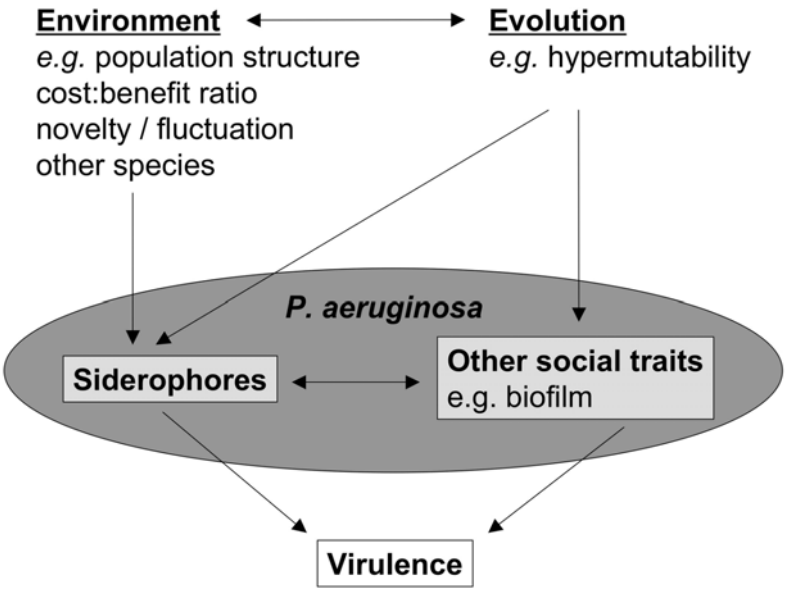

Figure 2. Our work has addressed how several aspects of the environment in which *P. aeruginosa* finds itself can affect siderophore cooperation. We are also interested in how evolutionary responses to this environment may affect siderophore production and in the possible links between siderophores and other social traits, most notably biofilm formation. (Arrows show direction of hypothesised influences).

Relatedness can be defined as the probability of two individuals sharing an allele at a particular locus, relative to the other members of the population (37). Low relatedness infections can thus comprise a mixture of public goods producers and non-producers. The total amount of public good produced, and hence the effect on the host, will be reduced in comparison with high-relatedness infections comprising only producers. Relatedness at loci governing cooperative behaviours is also a major determinant of the evolutionary success of cooperative traits. If the actor and beneficiary of a cooperative act share a cooperative allele, then cooperation can be maintained by natural selection due to its positive effect on inclusive fitness (10). Models of siderophore production and virulence have produced results consistent with the prediction that virulence can indeed be decreased at low relatedness (5).

We wanted to test empirically the hypothesis that virulence is decreased in mixed cooperator + cheat infections when public goods (siderophores) are important for bacterial growth. This investigation is published as (38). We inoculated waxmoth larvae (*Galleria mellonella*) with cells of a wild type, pyoverdine-producing strain of *P. aeruginosa*, a pyoverdine-minus mutant, or a 1:1 mixture of the two. Consistent with our prediction, we found that cooperator infections killed caterpillars on average two hours (15%) sooner than did cheat infections, and that mixed infections resulted in an intermediate time to death. These results are illustrated in Figure 3A. The growth rate of single- and mixed-clone infections showed the same pattern: cooperators grew faster than cheats, and mixed infections had an intermediate growth rate. These data demonstrate that the presence of siderophore cheats can reduce the growth rate of a bacterial population, and hence reduce virulence.

We next addressed whether cheats were more likely to be favoured in low *versus* high relatedness infections. We inoculated larvae with pure strains or a 1:1 mixture as above and incubated them for for eight hours before plating out homogenised tissue. We then calculated the number of cell doublings per gramme of fresh weight for each strain. The results are shown in Figure 3B: as predicted, cheat populations grow more rapidly in mixed, as opposed to single-clone, infections, while the opposite pattern is observed for cooperators. The selective advantage of cheating is therefore predicted to be higher in low relatedness infections. We then investigated the frequency-dependence of cheat fitness by inoculating larvae with mixtures containing 3-90% cheats and calculating cheat relative fitness after a period of growth. In these simple experiments, our 'cheats' never had a detectable selective advantage: at best, their fitness was the same as cooperators. This is in contrast with *in vitro* work, where the same strain was consistently able to to invade cooperating populations from initially low frequencies (9, 11, 38).

There are three likely explanations for this discrepancy. The first is the greater spatial heterogeneity within a caterpillar compared with a medium-filled tube, which is likely to increase the relatedness of immediate neighbours (37), and reduce the probability of direct cooperator-cheat interactions, bestowing a net benefit on cooperators. However, we have observed the *de novo* evolution of decreased siderophore production in *P. aeruginosa* populations growing in waxmoth larvae (D Racey, F Harrison & A Buckling, in preparation). The second possible explanation is the longer periods of time bacteria spend at high densities in tubes compared with larvae: tube assays reached densities of approximately 10^8

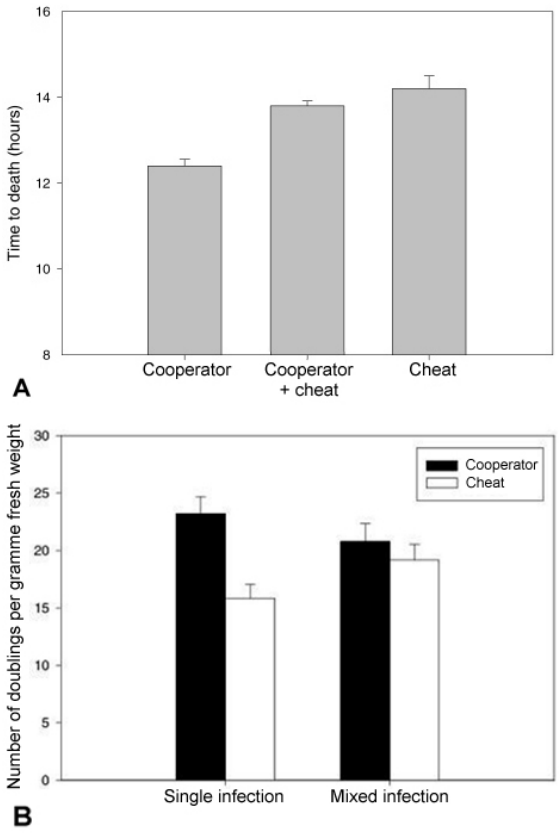

Figure 3. A Time to death (mean ± standard error) of waxmoth larvae inoculated with pure cooperator (PAO1), pure cheat (PAO9) or mixed infections of *P. aeruginos*a. Each treatment group comprised thirty larvae and mixed inocula contained cheats and cooperators in a 1:1 ratio. . Larvae were inoculated with c. 100 colony-forming units of bacteria, incubated at 37ºC and scored hourly for death. Cooperator infections resulted in a shorter time to death than did cheat infections and mixed infections resulted in an intermediate time to death (Kruskal-Wallis test H=42.76, $P < 0.0001$) (Reproduced with permission from (38)). B The number of doublings per gramme of fresh weight (mean ± standard error) of cooperator (PAO1) and cheat (PAO9) clones in single and mixed infections of waxmoth larvae. Each treatment group comprised twenty larvae and mixed inocula contained cheats and cooperators in a 1:1 ratio. There was a significant interaction between number of infection clones and cheat relative fitness (ANOVA on log-transformed data: ($F(1,75) = 4.97$, $P < 0.03$) (Reproduced with permission from (38)).

cells / ml (9), while insect assays reached densities of approximately 10^7 cells / ml. The higher the population density of bacteria, the greater the likelihood cheats will come into contact with cooperators (17, 37). Siderophore cheats have been observed at appreciable frequencies in chronic, clinical *P. aeruginosa* infections of the CF lung (7), where *P. aeruginosa* densities can reach 10^8 - 10^{10} CFU per ml of respiratory secretions, and pyoverdine production has been known to decrease over the course of chronic infection (39). As most CF patients are initially colonised by a single, environmental clone (40-42), any cheats present will most likely have arisen within the patient and invaded from an initially low frequency. These observations strongly suggest that cheats *can* enjoy a selective advantage in longer term, high density infections. The extent to which this apparent advantage is frequency dependent is not known. The evolutionary success of *de novo* cheating mutants in CF lungs and waxmoth larvae lead us to suggest that the apparent discrepancy in our results may reflect a peculiarity of the cheat strain used. This strain may, for instance, have a slightly reduced capacity for siderophore uptake or otherwise differ from naturally-evolved cheats as a result of extra mutations incurred during its manufacture by UV mutagenesis. Further work on the *de novo* evolution of siderophore cheats in larvae should clarify this issue.

4. TESTING HAMILTON'S RULE USING SIDEROPHORES: THE IMPORTANCE OF POPULATION STRUCTURE

Having established that siderophores are important virulence factors, understanding the ecological factors that influence their production takes on a practical significance. If siderophores are indeed a public good, we might expect the evolutionary dynamics of siderophore production to conform to the predictions of kin selection theory (10).

The *P. aeruginosa* siderophore model system was, in fact, successfully used to test two key predictions of kin selection theory. Hamilton (10) stated that altruistic cooperation is favoured when $rb > c$, where r is the genetic relatedness between actor and beneficiary, b is the benefit to the recipient and c is the net direct fitness cost to the actor. Hamilton also suggested that high population viscosity (limited dispersal) should favour cooperation as it will lead to increased r. This sparked a debate in the theoretical literature (see references in (9)), as high population viscosity is also expected to lead to increased competition between relatives: any increase in fitness accruing to the recipient of a cooperative act would therefore be achieved at the expense of relatives. This type of competition, where individuals compete with one another within a habitat patch, can be termed local competition. This contrasts with global competition, where groups inhabiting discrete patches within a metapopulation compete with one another. In this scenario, the probability of a given individual contributing to the next generation depends not only on its growth rate relative to its neighbours, but also on the total growth rate of its local patch relative to neighbouring patches. As the presence of cheats will have a negative impact on population density, subpopulations containing higher frequencies of cooperators are more likely to contribute to the next generation and so global competition is expected to favour cooperation (37, 43).

In the real world, populations are likely to experience a mixture of local and global competition (44, 45), so it is necessary to employ an extension of Hamilton's

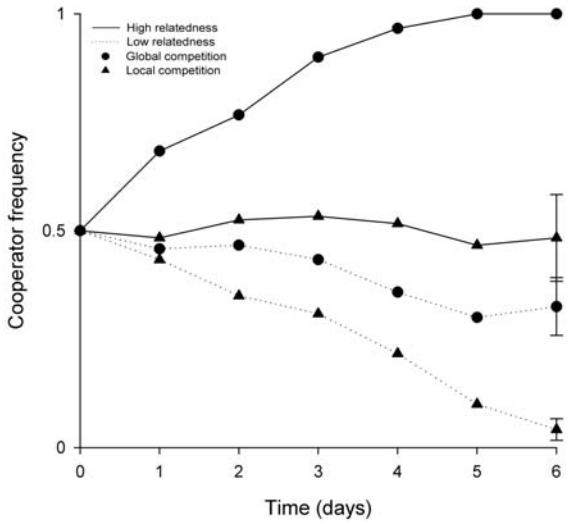

Figure 4. Relatedness and the scale of competition were independently manipulated in a two factorial ANOVA design. Replicates consisted of one population subdivided into twelve subpopulations (glass tubes containing casamino acids broth made iron limited by the addition of 100μg/ml human apotransferrin and 20 mM sodium bicarbonate). For high relatedness populations, six tubes were inoculated with cooperator (PAO1) cells and six with cheat (PAO6609) cells. After 24 hours' growth, populations were plated out and individual colonies transferred to fresh medium. Local competition was imposed by plating each tube individually and then choosing random colonies from random plates to inoculate fresh tubes. This meant that the productivity of a tube did not influence the likelihood of it contributing clones to the new metapopulation. Global competition was achieved by mixing tubes within a metapopulation together prior to plating, increasing the chance of clones from more productive cultures contributing to the new metapopulation. High relatedness was achieved by inoculating single colonies into tubes; low relatedness by inoculating two colonies into tubes (initially one cheat and one cooperator). Each treatment was replicated four times; graphed data points are the mean of these four replicates and standard error bars are shown for the final time points. ANOVA showed that both relatedness ($F(1,13) = 73.6$, $P < 0.0001$) and the scale of competition ($F(1,13) = 44.8$, $P < 0.001$) had significant effects on the final proportion of cooperators. There was also a significant interaction such that relatedness had a weaker effect on cooperator success when competition was local ($F(1,12) = 7.4$, $P < 0.02$) (Redrawn from (9)).

rule which takes the extent of local competition into account. Frank's (37) extension to Hamilton's rule states that cooperation is favoured when $r(b - a(b - c)) - c > 0$, where a represents the proportion of competition that is local. Models constructed by other authors confirmed the role of local competition in general terms (43, 46) and with relevance to siderophore production (5).

Griffin, West & Buckling (9) then used experimental populations of *P. aeruginosa* to examine the roles of r and a in the outcome of *in vitro* competition between pyoverdine-producing and pyoverdine-negative strains. Replica metapopulations consisting of twelve glass tubes containing iron-limited medium were subjected to high and low relatedness and local or global competition in a factorial design. Populations were maintained for six days (c. 50 bacterial generations), with daily transfer to fresh medium. The results are shown in Figure 4 and were entirely consistent with theory: high relatedness and global competition favoured cooperation. Global, unlike local, competition allowed the more productive cooperative tubes to contribute more to the global pool; and high relatedness prevented cheats from coming into direct contact with cooperators. Under low relatedness conditions on the other hand, cheats were able to exploit the pyoverdin produced by cooperators, and increased in frequency, explicitly demonstrating a cost to pyoverdin production.

5. THE INFLUENCE OF THE ABIOTIC AND BIOTIC ENVIRONMENT ON SIDEROPHORE COOPERATION

We then moved on from investigating population structure to address the role of the benefit:cost ratio (b/c) of siderophore production. b/c is a key determinant of the strength of kin selection (10, 37). The effect of environmental nutrient status on b/c and, by extension, on cheat success, has been demonstrated by computational simulation (43). We wished to explore the ecological factors that might influence b/c, and so the evolution of *P. aeruginosa* siderophore cheats. Our investigation was specifically designed to allow inferences about CF lung communities to be made.

The most influential aspect of the abiotic environment with regard to b/c is presumably iron availability, as evidenced by the iron-dependent regulation of siderophore gene expression (1, 3). The biotic community in which *P. aeruginosa* finds itself may also also influence b/c. In the CF airways, *P. aeruginosa* exists alongside numerous other pathogenic microbes (see (47) for a detailed review of lung microbial ecology). Its most notable coinfections partners are *Burkholderia cepacia* complex and *Staphylococcus aureus* (28, 29, 48-51). Interspecific interactions with these and other species may mediate b/c: for instance, *P. aeruginosa* is capable of lysing cells of *S. aureus* and utilising the iron released to support its own growth *in vitro* (52, 53). It is not known how significant this extra iron is in the context of the CF lung, or how this behaviour is related to interspecific competition for environmental iron. We wished to determine whether *P. aeruginosa* responds to *S. aureus* as an iron source. Further, we wished to determine whether any effect of *S. aureus* on *P. aeruginosa* siderophore production could be dependent on the levels of environmental free iron, as tissue damage due to chronic infection leads to elevated free iron levels in colonised airways (54-56).

This investigation is reported in (57). We founded forty-eight populations of *P. aeruginosa* (siderophore producers) in casamino acids broth microcosms. Twenty-four microcosms were supplemented

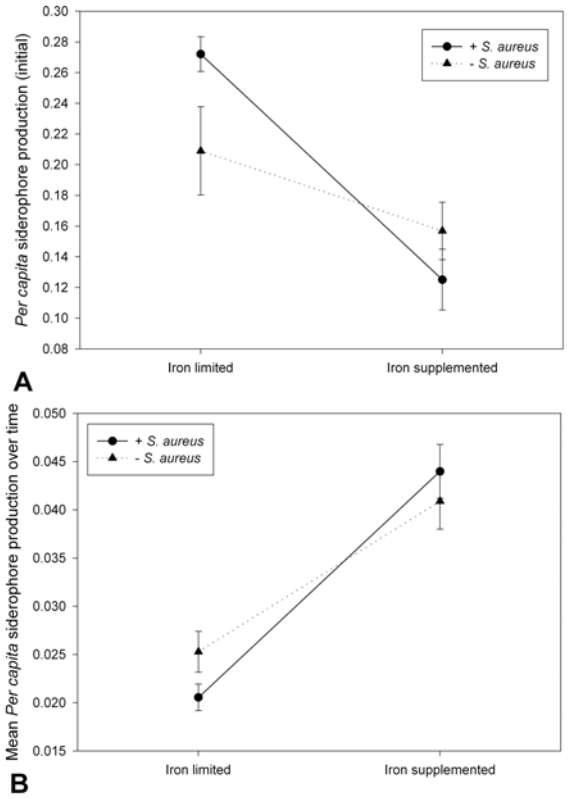

Figure 5. A. *Per capita* siderophore production by *P. aeruginosa* (mean ± standard error) after twenty-four hours growth at 37ºC. Cultures were grown in casamino acids broth in the presence of either 5 mM $Fe(III)Cl_3$ (+Fe) or 70 µg/ml human apotransferrin (-Fe) and in the presence or absence of c. 10^6 colony-forming units of *S. aureus* (±SA) in a fully factorial balanced design. Iron-limited cultures produced significantly more siderophores than did iron-supplemented cultures and this effect of iron was significant regardless of whether *S. aureus* was included in the model: ANOVA without *S. aureus* $F(1,45) = 31.33$, $P < 0.001$, with *S. aureus* included $F(1,43) = 34.34$, $P < 0.001$. *S. aureus* was not significant as a main effect ($F(1,43) = 1.09$, $P = 0.303$), but there was a significant interaction between iron and *S. aureus* ($F(1,43) = 5.21$, $P = 0.027$) such that siderophore production in iron-limited microcosms was higher when *S. aureus* was present. (Data originally reported in (57)). B. *Per capita* siderophore production by evolving *P. aeruginosa* populations (mean ± standard error), as measured in a common environment (iron-limited casamino acids broth; see main text) Fe = iron, SA = *S. aureus*. Mean *per capita* production of siderophores was increased under iron supplementation (ANOVA: $F(1,42) = 105.44$, $P < 0.005$). When iron was limiting, mean siderophore production was decreased in the presence of *S. aureus* (interaction $p < 0.05$). (Reproduced from (Harrison et al. 2007)). *S. aureus* was not significant as a main effect ($F(1,42) = 0.50$, $P = 0.483$), but there was a significant interaction between iron and *S. aureus* ($F(1,42) = 4.96$, $P < 0.005$) such that siderophore production in iron-limited microcosms was lower when *S. aureus* was present. (Data originally reported in (57)).

with iron and twenty-four made iron limited by adding an iron chelator (human apotransferrin). *S. aureus* cells were then added to half of the microcosms, yielding a fully factorial balanced design. We had previously ascertained that the *P. aeruginosa* strain used was capable of lysing the *S. aureus* strain. The populations were passaged daily into fresh growth medium for 20 days (c. 140 *P. aeruginosa* generations). Preliminary work had shown that the probability of carrying *S. aureus* over into the new tubes was minimal and fresh ancestral *S. aureus* was added at each transfer, removing any potential effect of *S. aureus* evolution.

We wished to separate the initial, physiological response of bacteria to the environmental benefit:cost ratio from any longer-term response to selection. The physiological response of *P. aeruginosa* was measured using data on siderophore production collected after the first twenty-four hours' growth in the treatment environments (chromeazurol sulphate assay as described by (13)). The evolutionary response was measured by storing samples of evolving populations at regular intervals and later growing these up in identical, iron-limited microcosms. Measurements of siderophore production *in situ* reflect a mixture of physiological downregulation and cheat evolution, whereas growth of samples in a common, iron-limited environment forces cells that had downregulated expression to begin producing siderophores once more, thus giving a reliable estimate of the extent of cheat evolution.

Our results showed that both iron regime and the presence of *S. aureus* affect the physiological regulation of siderophore gene expression and, by inference, b/c (Figure 5A). Consistent with iron-dependent regulation of siderophore expression (1, 3), iron-limited populations produced significantly more siderophores per CFU than did iron-supplemented cultures. Further, siderophore production by iron-limited populations was higher when *S. aureus* was present. This is not consistent with the hypothesis that *P. aeruginosa* was using *S. aureus* as an iron source. In this experiment, *S. aureus* instead seems to have an analogous effect to iron depletion, further increasing b/c when iron was already scarce.

As shown in Figure 5B, siderophore cheats evolved *de novo* much more readily under iron-limited conditions - i.e. when high values of b/c created a common pool of highly beneficial siderophore and hence a selective advantage to cheating. In iron-rich environments, siderophore production was physiologically downregulated due to a low b/c, resulting in little selection pressure for cheating. This is consistent with previous work describing short-term competition experiments between siderophore producers and a siderophore deficient mutant across a range of iron-limitation conditions (9). Figure 5B also shows that *P. aeruginosa* siderophore cheats were more common in iron-limited environments when *S. aureus* was present. This is consistent with the results shown in Figure 5A, where the presence of of *S. aureus* led to increased siderophore upregulation in iron-limited populations.

The simplest explanation for the observed effect of *S. aureus* is that it competes with *P. aeruginosa* for iron. However, we cannot rule out the possibility that other interactions between were responsible for siderophore upregulation in low-iron environments - cross-species quorum sensing, for example (58). In order to address this issue, we and our collaborators are carrying out experiments designed to determine whether the presence of *S. aureus* causes alterations in the expression of *P. aeruginosa* signal molecules (S. Diggle *pers. commun.*).

It would be interesting to see if *S. aureus* can ever serve as a significant iron source for *P. aeruginosa*, and whether this can ever outweigh the effect described above. The role of *S. aureus* evolution in long-term mixed populations is also a matter for consideration, as is any effect of environmental heterogeneity, and the net effect of the numerous suggested synergistic and antagonistic interactions between these species (31, 52, 53, 59-62). Experiments designed to explore questions such as these could add significantly to our understanding of the complexity of community interactions and the virulence of mixed infections.

6. HYPERMUTABILITY AND COOPERATION

The evolution of social traits should also be considered in the context of a population responding to various selection pressures. Evolutionary changes in non-social traits could influence mean trait values for cooperative behaviours - and vice versa - via pleiotropic effects or genetic linkage. We identified one commonly-selected trait that we thought could affect cooperator and cheat dynamics: mutation rate.

Bacterial populations readily evolve lineages with a 10-1000-fold increase in genomic mutation rate (63, 64). When adaptation is limited by the supply of beneficial mutations, as is the case in novel or changeable environments, such a 'mutator' genotype can hitch-hike with advantageous mutations to reach high frequencies (65-69). Mutators are common in bacterial infections, and Oliver *et al* (70) report that 37% of 128 CF patients they studied were colonised by a mutator genotype of *P. aeruginosa*. Hypermutability is believed to aid adaptation to changing host defences and chemotherapy (70-72). However, when a populations is well-adapted to its environment, the increased rate of deleterious mutations in mutator lineages means that hypermutability is selectively disadvantageous (65-67, 73, 74). Mutators may also be disadvantaged when population growth depends upon cooperation, as they are expected to generate cheating genotypes more readily and do decrease the relatedness of evolving populations. Mathematical models predict a negative correlation between mutation rate and cooperation when there is a sufficient element of local competition (34).

We tested this hypothesis in two experiments. The first experiment addressed the relative propensity of mutators to generate cheating mutants. We evolved wild-type and mutator populations of *P. aeruginosa* in iron-limited growth medium for c. 200 generations. The populations were subjected to entirely local competition to maximise the advantage of defection within a cooperating population. Siderophore cheats arose *de novo* in all populations, but cheats arose earlier, increased in frequency more rapidly and reached higher maximal frequencies in mutator populations. Furthermore, cheats were able to reach fixation in mutator populations but showed marked fluctuations in frequency in wild-type populations (Figure 6A). Despite the increased levels of cheating in mutator populations, the rate of decline in population density did not differ between wild-type and mutator populations (8). These results suggested that the mutator was able to generate fitter cheating genotypes than was the wild type. We then carried out competition assays to ascertain the ability of evolved cheat clones a) to invade cooperating populations from rare and b) to persist at high frequency in mixed populations.

We plated samples from frozen aliquots of our selection lines and isolated white colonies. These were then assayed for siderophore production in iron-limited medium and ability to invade cooperator populations from rare: clones which produced < 50% of the siderophores produced by their cooperative ancestor and which could invade from rare were taken to be true cheats and were competed with cooperator cells in iron-limited growth medium. Consistent with our suggestion, we found that while both wild-type-derived and mutator-derived cheats could all invade cooperators from a low starting frequency (c.5%), only the mutator produced cheats whose relative fitness exceeded one when mixed with cooperators at a starting frequency of c.50% (6/18 mutator-derived cheats had fitness >1 compared with 0/10 wild-type-derived cheats) (Figure 6B) (75).

Our second experiment (76) tested the hypothesis that mutators should be selected against under conditions that favour cooperation. Mutator and wild-type genotypes were competed in globally-competing metapopulations under conditions of high and low relatedness as described in section 4 and Figure 4. Consistent with predictions, mutators reached lower frequencies in the metapopulation under high relatedness than under low relatedness. Further, when relatedness was low, mutators were more likely to exceed 50% of the metapopulation. For each metapopulation, we then calculated the mean mutator frequency and mean pyoverdine cheat frequency over time. Crucially, the mean frequency of mutators within a metapopulation was positively correlated with the mean frequency of cheats (Figure 7; note the right-shift of the low-relatedness data relative to the high-relatedness data). In both of the mutator studies, data on visually-scored pyoverdine cheats was consistent with data on total siderophore production obtained using the chromeazurol sulphate colorimetric assay (13).

The effect of hypermutability on cooperation may help to explain observed distributions of *P. aeruginosa* mutators. Mutators are more common in clinical isolates than in conspecific environmental populations (77) and *P. aeruginosa* mutators are more commonly observed in long-

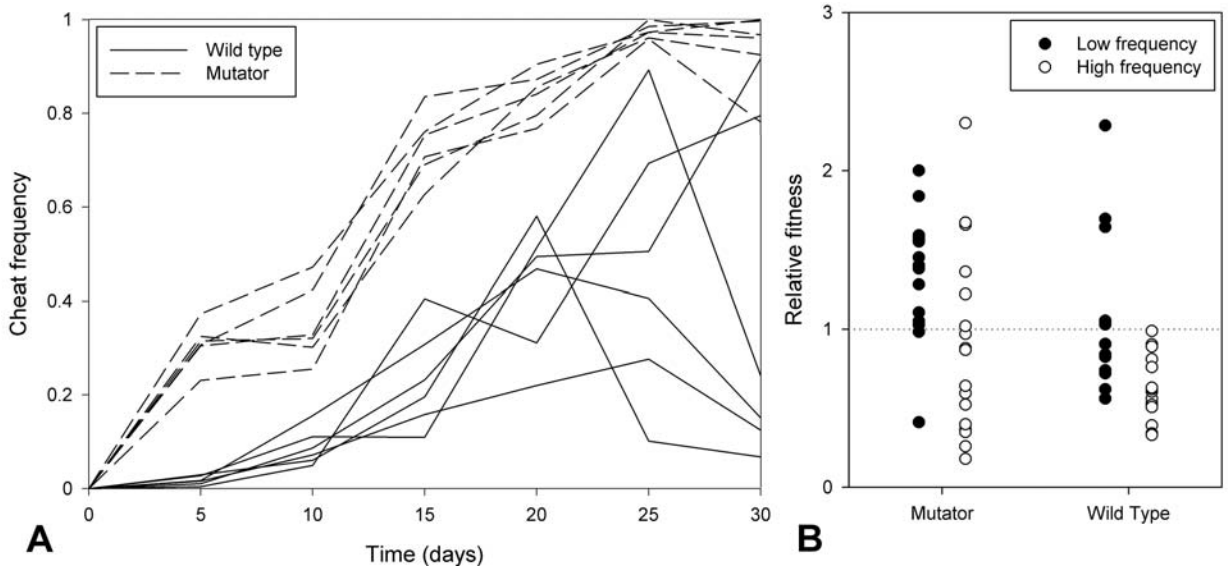

Figure 6. A. Cheat frequency over time in evolving populations of wild-type (solid lines) and mutator (dashed lines) bacteria. Six glass tubes containing casamino acids broth supplemented with 70 μg/ml human apotransferrin and 20 mM sodium bicarbonate were inoculated with c. 10^6 colony-forming units of wild type bacteria (PAO6049) and six with the same number of mutator bacteria (PAOΔ*mutS* (71)). Populations were allowed to evolve for 30 days (c. 200 generations), with daily transfer to fresh medium. In mutator populations, cheats arose earlier (T-test: $T(5) = 15.28$, $P < 0.005$), increased in frequency more rapidly ($T(5) = 6.72$, $P < 0.001$) and reached higher maximal frequencies ($T(9) = 3.11$, $P < 0.05$) than they did in wild-type populations. Mutator populations also showed less between-population variability in cheat frequency than did the wild-type populations (F-test on mean variance over all time points: $F(5,5) = 10.44$, $P < 0.05$). (Reproduced with permission from (8)). B. Fitnesses of a selection of evolved cheat clones in competition with cooperators. Cheats were isolated from the populations shown in Figure 6a as detailed in the text. Cheat clones were inoculated into casamino acids broth supplemented with 70μg/ml human apotransferrin and 20 mM sodium bicarbonate, along with an oppositely-marked cooperator clone. Cheats were competed at low (c. 5%) and high (c. 50%) frequency, relative to the cooperator. The fitness of each clone is expressed relative to the fitness of its cooperating ancestor in analogous competitions. In low-frequency competitions median fitness was not significantly different from one in the case of the wild-type-derived cheats (sign test: $P = 0.79$) and is greater than one in the case of the mutator-derived cheats ($P < 0.001$). In high-frequency competitions the median fitness of wild-type-derived cheats is less than one ($P < 0.001$) but the median fitness of mutator-derived cheats is not significantly different from one ($P = 0.24$). (Redrawn from (75)).

term, chronic infections than in acute infections (70). These observations have previously been attributed to the environmental fluctuations experienced during long-term *in vivo* growth (host immune responses and medical intervention: (66, 70-72, 78). We would suggest that there may also be weaker selection for cooperation, and hence weaker selection against mutators, in clinical *versus* environmental, and in chronic *versus* acute bacterial populations. In CF patients, *P. aeruginosa* infection generally results from colonisation by a single clone (40) and persists for several years and even decades. Patient-to-patient transmission is relatively rare. Taken together, these observations suggest that lung populations experience local competition. While little is known about the ecology of *P. aeruginosa* in soil and water, we would tentatively suggest that environmental populations could experience higher relatedness (due to shorter patch longevity) and more global competition (due to inter-patch dispersal) than would clinical populations.

The appearance of hypermutable variants of pathogenic bacteria is associated with increased antibiotic resistance (70, 71, 79) and as such mutators represent a significant risk factor for chronically colonised patients. Understanding the evolutionary ecology of mutators may thus benefit our understanding of chronic infection progression; indeed, understanding the evolution and ecology of pathogen communities may be vital in the development of new and more effective prophylaxis.

7. LINKS BETWEEN SIDEROPHORE PRODUCTION AND BIOFILM FORMATION

The sheer variety of social traits indulged in by any given species of microbe (22, 80) mean that these behaviours cannot be expressed or evolve in isolation from one another. A recent study (23) has shown a genetic link between siderophore production and a second virulence-linked cooperative trait: biofilm formation. Biofilms facilitate the colonisation of new niches, push daughter cells upwards to reach aerobic zones and increase resistance to antibiotics and ciliary clearance (81-83). The production of extracellular polymers necessary for biofilm formation is metabolically costly and so biofilms are open to exploitation by non-producing cheats (84, 85). Iron has been shown to be necessary for biofilm formation by *P.*

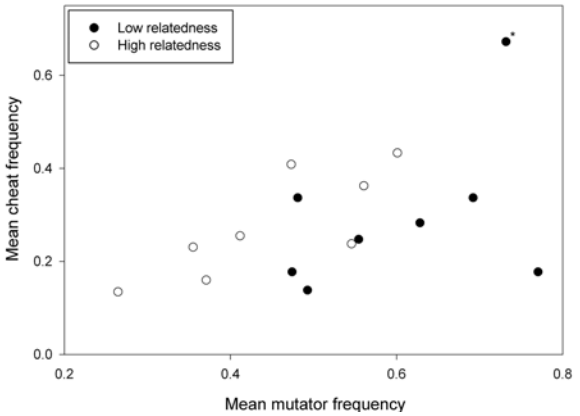

Figure 7. Mean mutator frequency over time and mean cheat frequency over time in sixteen experimental metapopulations of *P. aeruginosa.* Metapopulations were founded with wild type PAO985 and mutator PAOΔ*mutS* and subjected to either high or low relatedness under a global competition regime (as described in Figure 4 and in (9)). The mean mutator frequency was higher under low relatedness (ANOVA: $F(1,13) = 6.46$, $P < 0.05$). The mean equilibrium mutator frequency was not significantly different from 0.5 in high-relatedness metapopulations (T-test: $T(7) = 1.26$, $P = 0.25$) but exceeded 0.5 in low-relatedness metapopulations ($T(7) = 2.45$, $P < 0.05$). Across all metapopulations, mean mutator and mean cheat frequencies were positively correlated (ANOVA with relatedness treatment included as a factor: $F(1,11) = 5.30$, $P < 0.05$). This conclusion was not affected by excluding the outlier (*) from the analysis ($F(1,10) = 7.15$, $P < 0.05$). The strength of the relationship did not differ between relatedness treatments ($F(1,10)$ for interaction term = 1.59, P =0.24). (Reproduced with permission from (76)).

aeruginosa (86) and ferrisiderophores act as a signal to switch from planktonic to biofilm growth: as a result of this, siderophore-deficient mutants have reduced biofilm forming ability (23). We wished to expand upon these results and to determine their likely evolutionary consequences, using siderophore cheats that had evolved *de novo* in our lab. Specifically, we addressed the hypothesis that siderophore cheats could also act as biofilm cheats. The following work is detailed in (75) and will form the basis for a more detailed study of siderophores and biofilm.

Briefly, we grew biofilms of five of the evolved siderophore cheats described in section 6 and their siderophore-producing ancestor, PAO6049 (87) in iron-limited and iron-supplemeted growth medium, using the microtitre plate - peg lid system as described in (88, 89). Biofilm mass was assayed using crystal violet staining and the number of cells in the biofilm and planktonic subpopulations calculated from plate counts. The five cheats had lower total biofilm mass and allocated a smaller proportion of the total population to the biofilm than did their ancestor, both under iron limitation and under iron supplementation (T-tests: all P-values < 0.001 and remained significant after sequential Bonferroni correction). This was not a result of generally poor growth by the pyoverdine cheats, which in fact exhibited better planktonic growth than their ancestor in both treatment environments; this is probably due on the one hand to their previous period of adaptation to iron-limited broth and on the other to the costs of siderophore production when iron is supplied). Finally, all five evolved cheat clones had lower biofilm mass under iron-limited, as compared with iron-supplemented conditions (T-tests: all P-values < 0.006 and remained significant after sequential Bonferroni correction).

We also hypothesised that biofilms could represent an environment where siderophore-deficient cells would represent 'super cheats' (90) - not only do they not contribute to iron scavenging but they also contribute less to the structural integrity of the biofilm, exploiting two group-beneficial behaviours while paying the costs of neither. If this is the case, we would expect siderophore cheats to have a greater relative fitness advantage when competing with siderophore producers in biofilms than in planktonic populations. To test this hypothesis, we grew mixed biofilms consisting of the wild type PAO1 plus each of the evolved cheat clones under iron-limited conditions. The mixtures had significantly lower mean biofilm mass than did the pure wild type culture (T-tests: all P-values < 0.005 and remained significant after sequential Bonferroni correction), but none of the populations had significantly different biofilm *versus* planktonic cheat frequencies (Wilcoxon signed ranks test on paired data: all P-values ≥ 0.25). Interestingly, pyoverdine cheats evolved *de novo* in the PAO1 pure culture, but again there was no significant difference in cheat frequency between biofilm and planktonic subpopulations (Wilcoxon signed ranks test: P = 0.30).

These preliminary results confirm the existence of a link between siderophore production and biofilm formation, highlighting the fact that adaptive change in social traits can be constrained by selective pressures on genetically linked traits. This is consistent with previous observations that cooperation can be stabilised by pleiotropic fitness costs of cheating (91). Our results are not, however, consistent with the hypothesis that siderophore cheats act as super cheats in a biofilm environment. We hope to extend this work in the future by investigating a wider range of cheat clones, testing biofilm resistance to antibacterial agents and assessing the ability of cheat clones to invade established biofilm communities. Both siderophore production (7, 39) and biofilm-forming ability (39, 92) have been observed to decrease over time in chronic *P. aeruginosa* infections of cystic fibrosis patients: are both changes the result of adaptation to the airways? Or might selection for siderophore cheating have the knock-on effect of reducing biofilm, even though biofilms aid persistence? This remains to be seen, but it is certainly an intriguing question.

8. CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK

We have successfully used a bacterial model system to explore the evolution and ecology of a clinically-

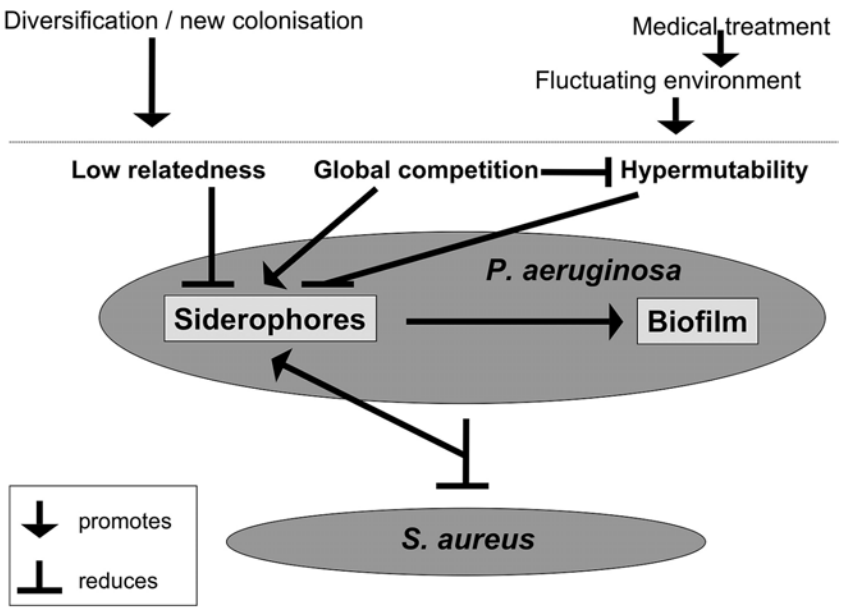

Figure 8. Path diagram showing how experimental work has begun to reveal an integrated picture of the links between siderophore cooperation, other traits, environmental factors and *P. aeruginosa* virulence. Factors specifically relevant to disease populations (e.g. in the CF lung) are shown above the dotted line.

relevant cooperative trait. From simple *in vitro* experiments, we have been able to confirm and extend theoretical models of cooperation and to highlight the importance of microbial sociality in mediating pathogen virulence and persistence. In addition to providing the first report of the *de novo* evolution of siderophore cheats in laboratory populations (8), we have carried out experiments with the following conclusions.

i. Kin selection theory (Hamilton's rule) is an appropriate theoretical framework within which to consider siderophore production by *P. aeruginosa*.
ii. The extent of local competition is as important a determinant of cooperator success, as are relatedness and b/c.
iii. Low pathogen relatedness leads to reduced virulence in acute infections when pathogenesis is promoted by cooperative behaviour.
iv. Interspecific interactions can affect b/c and thus selection pressure on social cheating.
v. Hypermutability accelerates the breakdown of cooperation under local competition and kin selection for cooperation can mediate mutator dynamics in bacterial metapopulations.
vi. Siderophore production affects investment in biofilm formation.

Figure 8 represents a synthesis of our results as they relate to the evolution, ecology and pathology of siderophore cooperation in *P. aeruginosa*. The work we have presented in this review suggests several avenues for further investigation, which we hope will not only extend social evolution theory but also help to improve our understanding of multi-genotype microbial infections. The main emphasis of our future work will be on increasing the realism, and by extension practical relevance, of our experiments. For instance, models generally assume that cooperative traits represent a binary choice (cooperate or cheat), but in fact many of these behaviours are continuous traits subject to physiological regulation based on environmental signals. Research on how reaction norms (93) of siderophore production evolve could inform much more realistic models of cooperation. In other words, rather than purely looking at how the *in situ* level or maximum possible level of siderophore production by cells changes over a period of evolution, it would be advisable to ascertain how siderophore production by specific cells changes in response to iron availability, and to determine whether the nature of this relationship is subject to selection. Investigating the evolution of suites of linked social behaviours, such as siderophore production and biofilm formation, is also an exciting avenue for future research. The ecological realism of laboratory experiments could also be improved: we have already looked at the effect of one inter-specific interaction on siderophore cooperation, but this work is only the initial step in a potentially much larger project to ascertain how community structure and evolution changes selection pressures on cooperation over time periods approximating to long-term, chronic infections of humans. The work we have begun has benefited from numerous collaborations; in the future, we hope that work on both this and comparable systems will be facilitated by the combined efforts of evolutionary biologists, microbiologists, clinicians and theoreticians

In conclusion, our work has begun to illustrate the importance of studying cooperative behaviours within the context of evolving communities inhabiting structured environments. Even among microbes, communities can be dynamic and complex.

9. ACKNOWLEDGEMENTS

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Abbreviations: CF: cystic fibrosis

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Send correspondence to: Freya Harrison, Department of Biology and Biochemistry, University of Bath, Bath BA2 7AY UK, Tel: 441225 385437, Fax: 441225 386779, E-mail: freya.harrison@zoo.ox.ac.uk

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