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Report

Character Displacement Promotes Cooperation in Bacterial Biofilms

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Summary

Resource competition within a group of cooperators is expected to decrease selection for cooperative behavior [1–6] but can also result in diversifying selection for the use of different resources, which in turn could retard the breakdown of cooperation. Diverse groups are likely to be less susceptible to invasion by noncooperating social cheats: First, competition repression [7] resulting from character displacement [8, 9] may provide less of a selective advantage to cheating; second, cheats may trade off the ability to exploit cooperators that specialize in one type of resource against cooperators that specialize in another [10]; third, diverse communities of any kind may have higher invasion resistance because there are fewer resources available for an invader to use [11, 12]. Furthermore, diverse groups are likely to be more productive than clonal groups if a wider range of total resources are being used [13–15]. We addressed these issues by using the cooperative trait of biofilm formation in *Pseudomonas fluorescens* [3, 16]. Character displacement through resource competition evolved within biofilms; productivity increased with increasing character displacement, and diverse biofilms were less susceptible to invasion by cheats. These results demonstrate that diversification into different ecological niches can minimize selection against cooperation in the face of local resource competition.

Results and Discussion

When propagated in spatially heterogeneous environments (a static glass microcosm containing nutrient-rich medium [16]), populations of the ancestral smooth (SM) *P. fluorescens* genotype rapidly diversify, generating by mutation a range of niche-specialist genotypes that are maintained by negative frequency-dependent selection [16]. The wrinkly-spreader (WS) morph is ecologically dominant [17] and forms a biofilm at the air-broth interface through constitutive overproduction of cellulosaic polymer [18]. Although overexpression of cellulosaic polymer is individually costly (as demonstrated by the reduced exponential growth rate of WS relative to SM [3, 19]), its production provides a group benefit to WS because colonization of the air-broth-interface niche allows improved access to oxygen, a limiting resource [3]. Clonal WS biofilms have been found to be susceptible to invasion by SM genotypes that arise by mutation from WS [3]. In this context SM are cheats, gaining the benefit of inhabiting the air-broth interface while making no contribution to the integrity of the biofilm, which is significantly weaker in the presence of cheating SM genotypes [3]. Crucially, multiple WS morphs have been observed to evolve and coexist within single populations [16]. However, no empirical studies have addressed whether this divergence is adaptive, nor have they addressed the effect of this divergence on cooperative biofilm formation. We founded nine replicate populations with equal numbers of *P. fluorescens* SBW25 and an isogenic marked strain (*P. fluorescens* SBW25 ΔpanB) in static microcosms. These populations were allowed to diversify for 6 days, after which time a single WS colony of each marker type was isolated at random from each population.

If diversifying selection for using different resources had taken place as a result of local competition in the biofilm, coevolved pairs of WS would be expected to display reciprocal negative frequency dependence (a fitness advantage of a genotype when that genotype is rare). By contrast, reciprocal negative frequency dependence would be less common when random pairs of WS were competed because it is more likely that genotypes would be competing for the same resources. To test this, we performed reciprocal invasion-from-rare (1:100) competition experiments for both coevolved and random pairs of WS. The results support the hypothesis that coevolved WS pairs had partitioned the biofilm niche, with coevolved pairs consistently showing negative frequency dependence, whereas this was not the case for random pairs (Figures 1A and 1B; 9/9 coevolved pairs versus 5/9 random pairs displayed reciprocal negative frequency dependence; 1-tailed Fisher's exact test. $p = 0.04$). This result demonstrates that character displacement resulting in the use of different resources occurs between coevolved WS genotypes and that there is variation in the magnitude of this character displacement.

Next we addressed whether divergence within the biofilm reduced the susceptibility of a cooperating group to invasion by cheats. If this were the case, then the frequency of cheats (i.e., SM genotypes that arose through mutation and persisted) in mixtures would be lower than expected from the frequency of cheats seen in the

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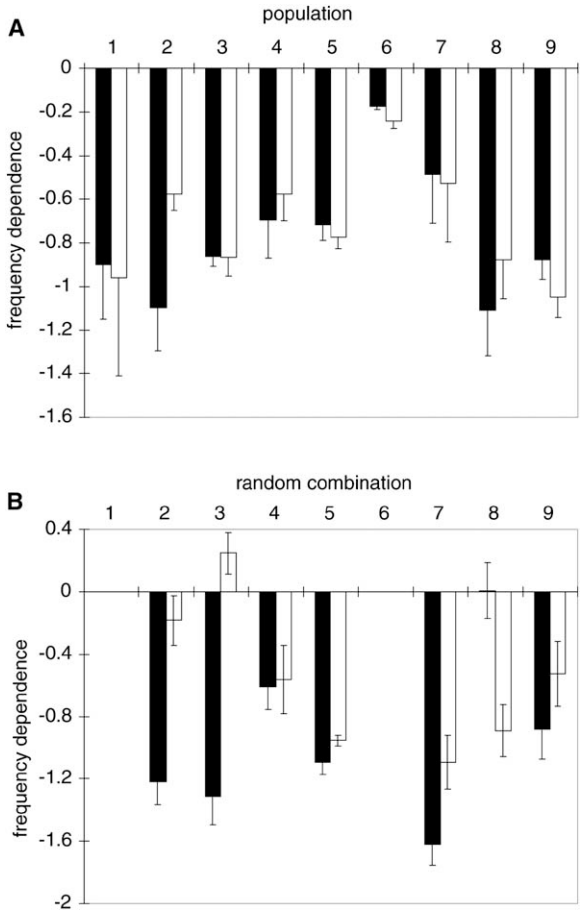

Figure 1. Character Displacement in Coevolved and Random Pairs of Cooperators

Frequency dependence of WS genotypes in coevolved (A) and random (B) pairings. Scale bars represent the mean frequency dependence for wild-type (black) and pan- (white) marked WS genotypes minus SEM. Missing bars represent instances where the rare type failed to invade above the measurable frequency.

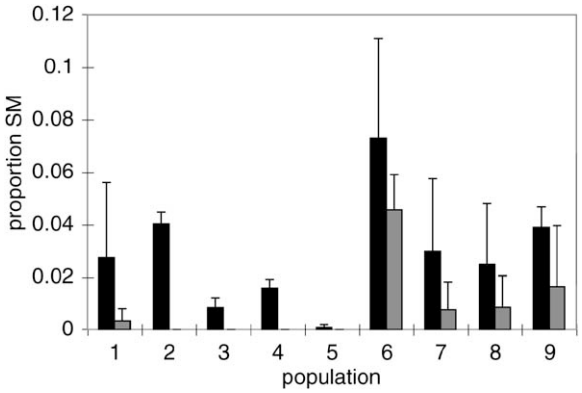

Figure 2. Observed and Expected Frequencies of Cheats

Expected and observed proportion of nonproducing cheaters after 6 days of static incubation. Black bars represent the mean expected proportion of SM colonies (calculated as a weighted mean of constituent monoculture values) + SEM; gray bars represent the mean observed proportion of SM colonies present in mixtures + SEM.

constituent monocultures. This could be determined by comparison of the cheat frequencies in the mixtures with an expected value that was an average of the two constituent monoculture values where each strain was weighted by its relative frequency (i.e., a weighted mean) in the mixed biofilm. To test this, we inoculated replicate microcosms with coevolved pairs of WS in mixtures (50:50) and monocultures and calculated the proportion of SM colonies after 6 days of static incubation. Note that growth in the broth phase was never observed in any population; hence, evolved SM were inhabiting the biofilm and not invading the broth phase. In support of our hypothesis, the proportion of SM cheats in mixtures was significantly lower than expected from monoculture values (Figure 2; paired t test, T = 5.23, df = 8, p = 0.0008), indicating that the observed adaptive character displacement prevented the establishment of the cheating SM phenotype.

Evolved diversity may increase the productivity of a group because a wider range of resources will be exploited [8]. The greater the divergence within the group, the wider the range of resources used and the higher the expected productivity. Typically, measures of productivity are partitioned into complementarity (change in productivity in mixture relative to monoculture as a result of resource partitioning) and dominance effects (change in productivity in mixtures relative to monocultures attributable to the disproportionate contribution of particular types) [20]. Our coevolved WS pairs displayed high between-population variance in the strength of reciprocal negative frequency dependence (Figure 1A; mean = −0.74, standard deviation = 0.26). Greater negative frequency dependence indicates that there is less competition for the invaders' niche and therefore a greater degree of character displacement. More divergent WS pairs (i.e., stronger negative frequency dependence) were expected to display greater niche complementarity, but no relationship was expected between frequency dependence and dominance. To test this, we inoculated replicate microcosms with coevolved pairs of WS in mixtures (50:50) and monocultures. After 6 days of static incubation, we measured productivity by counting the number of WS colonies on agar plates. As expected, there was a significant negative relationship between the strength of frequency dependence and complementarity (Figure 3; $F_{1,7}$ = 12.48, Rsq = 0.64, p = 0.009), but there was no significant relationship between frequency dependence and dominance (Figure 3; $F_{1,7}$ = 2.01, Rsq = 0.22, p = 0.2). Thus, productivity increased with increasing character displacement as a result of increasing niche complementarity.

However, contrary to expectation, our regression analysis suggests that small amounts of character displacement resulted in a net reduction in levels of productivity (i.e., negative complementarity; Figure 3), and only relatively large amounts of character displacement were associated with a net increase in productivity (i.e., positive complimentarity; Figure 3). WS biofilm formation is a complex trait, relying on interactions among attachment factors, LPS, and cellulose fibers, and different WS have been shown to form different types of biofilm [21]. Interference at lower levels of character displacement could be explained by selection for complementary

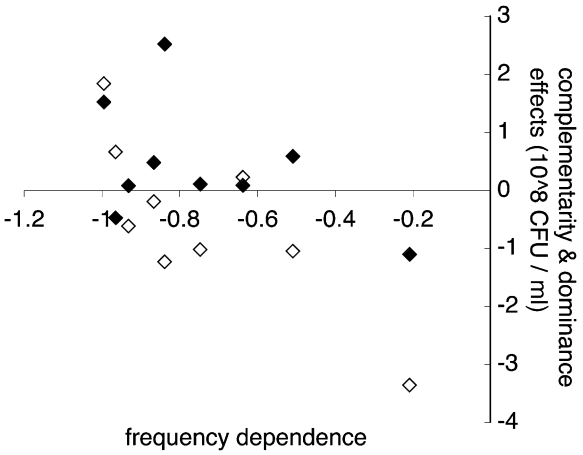

Figure 3. The Relationship between Frequency Dependence and Productivity

Black dots represent mean values of the dominance effect (change in productivity in mixtures relative to monocultures attributable to the disproportionate contribution of particular types), and white dots represent mean values of the complementarity effect (change in productivity in mixtures relative to monoculture as a result of resource partitioning) plotted against mean frequency dependence for each coevolved WS pairing.

resource use leading to pleiotropic changes in other aspects of biofilm formation that reduce the efficiency of cooperation. Similarly, strains of the cooperative bacterium *Myxococcus xanthus* that diverged in allopatry have been shown, in the majority of cases, to display reduced spore production when grown in mixtures as opposed to monoculture, presumably as a result of accumulated changes to the cooperative phenotype [22]. Crucially, our regression analysis suggests that for sufficiently high levels of divergence, the benefits of complementary resource use outweighed the costs of interference, leading to a net increase in productivity. Indeed, as further coevolution of diverged types would be predicted to lead to increasingly complementary resource use, productivity is generally likely to be higher in more diverged cooperative groups.

This study shows that adaptive divergence into different niche specialists driven by resource competition can have a net benefit to the cooperating group. Such diversity can increase resistance to invasion by cheats and increase net productivity—a situation that is likely to increase the severity of infections caused by pathogenic bacteria [23, 24]. Most theoretical and experimental studies suggest that competition between rather than within groups favors the maintenance of cooperation, under conditions where there is no direct benefit to cooperating [1, 25]. This study does not challenge this view but suggests that under certain conditions, where local competition results in diversification, the benefits of diversity may exceed the costs of cheating and favor the spread of cooperation.

Diversifying selection in cooperative systems can potentially arise from two modes of competitive interaction: resource competition, the focus of this study, and apparent competition (selection for enemy-free space [26]) mediated by social cheats, as recently hypothesized by Jansen and van Baalen [27]. These two forms of competition are not mutually exclusive, but they do give rise to different predictions regarding the mechanism of diversity's benefit and its effect on relatedness. We have discussed the benefits of diversity resulting from resource competition above: increased productivity, and increased invasion resistance potentially resulting from more efficient use of resources by resident cooperators, reduced competition, and cheats trading off their ability to cheat on cooperators that specialize on different resources. As such, resource-driven diversification need not directly reduce Hamiltonian relatedness (i.e., at the loci for social action); rather, divergence reduces relatedness at other genomic loci involved in inhabiting an ecological niche; however, as discussed, this could lead to indirect pleiotropic changes in cooperative phenotypes.

By contrast, diversification through apparent competition mediated by social cheats does reduce Hamiltonian relatedness by causing divergence at the cooperative-trait loci. Here, the benefit of divergence is that each divergent group cooperates differently, reducing the selective advantage of evolved cheats, which can only cheat on a portion of the population [27]. A possible example of such divergence may be provided by a recent study that reported evidence of diversifying selection acting on the pyoverdine locus (a gene coding for the production of iron-scavenging siderophore molecules that can benefit all bacteria in the neighborhood) in *Pseudomonas aeruginosa* [28]. However, further experiments would be required to confirm that such divergence is the result of apparent competition mediated by social cheats.

In this study we identify and provide empirical evidence for a novel mechanism for the maintenance of cooperation in the face of local competition. However, unlike previous mechanisms for the repression of local competition within groups, adaptive divergence does not require complex policing mechanisms for cooperation to be maintained [7]. We therefore provide an additional mechanism [7, 27, 29] by which cooperation might be more resistant to the effects of individual selection than previously thought; hence, conditions favoring the maintenance of cooperation are much broader. In addition, this result may provide insights into how division of labor within multicellular conglomerations may initially have evolved in order to minimize functional redundancy and increase efficiency [30]. Finally, our results are relevant to determining the virulence of bacterial infections [23, 24]. Biofilm dwelling is likely to confer a selective advantage in vivo through increased resistance to phagocytosis, antibiotics, and harsh environmental conditions in general [31], resulting in more damaging and persistent infections. Under conditions where the production of resources for the common good is important for bacterial growth and survival (as is the case for the production of biofilm polymers), theory and data suggest that reducing the relatedness within an infection will reduce virulence because of selection for noncontributing cheats [23–25, 32, 33]. Our results suggest that reducing relatedness at loci that define the ecological niche (but not the cooperative trait) of cooperating individuals may increase the fitness of a cooperating group and thereby increase virulence. These results highlight that there is unlikely to be a general rule

determining the virulence of mixed versus clonal infections when social interactions are taken into account [23]. The interplay between relatedness at loci for social action and relatedness at other loci is likely to be particularly relevant to pathogenic microbes for which relatedness can vary greatly at different points of the genome through the action of mutation and recombination; however, this remains unconsidered by current theory.

Experimental Procedures

Culturing Techniques

Nine replicate microcosms (30 ml glass universal containing 6 ml of King's B nutrient media supplemented with 0.0024% pantothenic acid) were inoculated with equal volumes of *Pseudomonas fluorescens* SBW25 and an isogenic pantothenate auxotroph to a total of approximately 10^7 cells. These were statically incubated for 6 days at 28°C, after which time all populations were vortexed and an aliquot was diluted and plated onto vitamin-free KB agar supplemented with 4.8×10^{-6}% pantothenic acid. On this medium the pantothenate auxotroph strain is readily distinguished by its greatly reduced size. A single wrinkly-spreader colony of each marker type (wild-type and pantothenate auxotroph) was then isolated from each population for further study and stored at −80°C in 20% glycerol.

Reciprocal Invasion-from-Rare Competition Experiments

Competition experiments between coevolved and random pairs of reciprocally marked WS genotypes were carried out. Competitors were grown separately in KB microcosms supplemented with 0.0024% pantothenic acid (so that any fitness cost of the panB mutation was negated) for 24 hr at 28°C and shaken at 200 rpm so that they were in the same physiological state. A total of 10^7 cells were then inoculated into static KB microcosms supplemented with 0.0024% pantothenic acid at 1:100 and 100:1 ratios for each pairing and competed for 6 days. Each competition was replicated 3-fold. Relative fitness (W) was calculated from the ratio of the estimated Malthusian parameters (m) of the competitors, $m = \ln (N_f/N_0)$, where N_0 is the starting density and N_f the final density [34]. We determined densities by counting at least 2000 colonies grown on vitamin-free KB agar supplemented with 4.8×10^{-6}% pantothenic acid. On this medium the pantothenate-marked strain is readily distinguished by its greatly reduced size. Frequency dependence was calculated as the slope of the line of fitness against starting density for each genotype in each pairing.

Cheater Invasion and Productivity Assay

Coevolved WS genotypes were inoculated into KB microcosms supplemented with 0.0024% pantothenic acid, individually and in 50:50 mixtures to a total of 10^7 cells, and incubated for 6 days. All experiments were replicated 2-fold. Single-genotype populations were then plated onto KB agar plates supplemented with 0.0024% pantothenic acid; mixtures were plated onto both KB agar plates supplemented with 0.0024% pantothenic acid and vitamin-free KB agar supplemented with 4.8×10^{-6}% pantothenic acid. The frequency of WS and SM colonies was determined for each population from the KB agar plates; the proportion of each marker type in the mixtures was determined from vitamin-free agar plates. SM proportions were calculated from approximately equal quantities of colonies counted for monocultures and mixtures. Expected mixture values of SM proportion were calculated as means of monoculture values weighted by the proportion of each type in the mixture. Productivity data were partitioned into complementarity and dominance effects as described in reference [20].

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