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Parallel emergence of negative epistasis across experimental lineages

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Abstract

Epistatic interactions can greatly impact evolutionary phenomena, particularly the process of adaptation. Here, we leverage four parallel experimentally evolved lineages to study the emergence and trajectories of epistatic interactions in the social bacterium *Myxococcus xanthus*. A social gene (*pilA*) necessary for effective group swarming on soft agar had been deleted from the common ancestor of these lineages. During selection for competitiveness at the leading edge of growing colonies, two lineages evolved qualitatively novel mechanisms for greatly increased swarming on soft agar, whereas the other two lineages evolved relatively small increases in swarming. By reintroducing *pilA* into different genetic backgrounds along the four lineages, we tested whether parallel lineages showed similar patterns of epistasis. In particular, we tested whether a pattern of negative epistasis between accumulating mutations and *pilA* previously found in the fastest lineage would be found only in the two evolved lineages with the fastest and most striking swarming phenotypes, or rather was due to common epistatic structure across all lineages arising from the generic fixation of adaptive mutations. Our analysis reveals the emergence of negative epistasis across all four independent lineages. Further, we present results showing that the observed negative epistasis is not due exclusively to evolving populations approaching a maximum phenotypic value that inherently limits positive effects of *pilA* reintroduction, but rather involves direct antagonistic interactions between accumulating mutations and the reintroduced social gene.

Keywords

epistasis; parallel evolution; experimental evolution; *Myxococcus xanthus*

Introduction

The potential impacts of epistatic interactions on evolutionary processes are manifold (Wolf et al. 2000). For this reason, there has been considerable effort to categorize and predict the structure and emergence of epistatic interactions (Szathmary 1993; Elena and Lenski 1997;

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Weinreich et al. 2005). In particular, the role of epistatic interactions in the process of adaptation has received attention because multiple mutations may interact with each other to either facilitate or constrain adaptation (Weinreich et al. 2006; Kryazhimskiy et al. 2009; Draghi et al. 2010; Khan et al. 2011; Chou et al. 2011).

Parallel evolutionary lineages offer independent samples in which to study the emergence, structure, and dynamics of epistatic interactions during adaptation. While different mutations may fix in each lineage, phenotypic responses to selection may be similar because alternative evolutionary genetic solutions may result in convergent evolution (Woods et al. 2006; Stern 2013). By quantifying the effects of epistatic interactions of accumulating mutations on a particular phenotype over evolutionary time, it is possible to assess whether patterns of epistatic interactions are driven by mutations specific to particular evolved phenotypes or whether these patterns arise from the fixation of adaptive mutations in general, irrespective of the particular phenotypes those mutation cause. Fundamental differences in the structure of epistasis between lineages that evolve qualitatively novel phenotypes and those that did not would distinguish between these scenarios. The presence or absence of common patterns among replicate lineages may help highlight generalities about the emergence of epistatic interactions.

Experimental evolution in the laboratory is an ideal approach to studying the consistency of epistasis in the context of parallel evolution. Many independent lineages with genetically identical ancestors can be propagated in parallel for many generations under identical selective conditions; ancestral and intermediate states can also be preserved by freezing, and subsequently reanimated (Elena and Lenski 2003). We studied epistatic interactions in four experimentally evolved lineages of the social bacterium *Myxococcus xanthus*.

Selecting cells at the leading edge of expanding *M. xanthus* swarms, Velicer and Yu (2003) evolved *M. xanthus* with increased surface motility from ancestors that were nearly non-motile on soft agar due to deletion of *pilA*, a gene necessary for the production of type IV pili. Two of eight replicate lineages in that study evolved qualitatively novel ‘tentacle’-like motility phenotypes, whereas six other replicate lineages did not, yet still exhibited significant increases in motility (Velicer and Yu 2003). By reintroducing *pilA*, Zee et al. (2014) studied epistatic interactions in one of the two ‘tentacle’ lineages (E8). They found that the changing genetic background of the lineage as it evolved over time first produced negative magnitude epistasis, in which the magnitude of positive effect of *pilA* on swarming decreased over evolutionary time, and later generated sign epistasis, in which the gene reintroduction actually had a negative effect on absolute swarming rate late in the evolutionary trajectory (Fig. 1).

Zee et al. (2014) presented multiple null hypotheses for how swarming might be affected by reintroduction of the *pilA* gene. The first, and simplest, null hypothesis is that there would be no genetic interactions, and that the phenotypic effect of *pilA* reintroduction would be constant across all tested genetic backgrounds (called ‘independence null’ in Zee et al. (2014)). Another possibility is that there is some physiological limit that constrains swarming to a ‘maximum’ rate. In this case, the reintroduction of *pilA* would return swarming to this same maximum rate regardless of genetic background (assuming that the

wildtype swarming is near a maximum swarming value), resulting in a pattern of diminishing returns epistasis (the ‘limit null’ in Zee et al. (2014)). While Zee et al. (2014) demonstrated a qualitatively striking departure from these null models for one experimental lineage of *M. xanthus* (Fig. 1), here we expand this work to several replicate lineages to test for consistencies in the emergence of epistatic patterns.

We reintroduced *pilA* into clones sampled from four parallel lineages of *M. xanthus* and compared the swarming-rate trajectories of untransformed clones with *pilA*-transformed clones through evolutionary time. The qualitative phenotypic differences among lineages enable us to probe whether the observed epistatic trajectory in the two lineages exhibiting a novel ‘tentacle’ phenotype is unique to those lineages or rather whether qualitatively similar patterns are observed across all lineages, irrespective of swarming phenotype. We found a qualitatively similar pattern of negative epistasis in all lineages, although the temporal dynamics of how this common pattern was manifested differed greatly across lineages and over time within lineages. Further, we present data that is inconsistent with the ‘maximum’ swarming rate hypothesis for diminishing returns epistasis, and suggests that antagonistic interactions underlie the pattern of negative epistasis.

Materials and methods

Study species and selection regime

Myxococcus xanthus is a social, Gram-negative soil bacterium with a global distribution (Dawid 2000). While best known for its ability to form multicellular fruiting bodies in starvation conditions, *M. xanthus* also exhibits other social traits such as predation of other microbial species (Shimkets 1990; Velicer and Vos 2009) and social swarming motility across solid surfaces (Zusman et al. 2007). For this study, the important trait to understand is a form of *M. xanthus* motility that has often been called “S-motility” or “type IV pilus-dependent motility” (Skotnicka et al. 2016). S-motility involves coordinated movement of cells across solid surfaces and requires cell-cell proximity and the protraction and retraction of pili that attach to surrounding cells or surfaces (Zusman et al. 2007). S-motility requires a functional *pilA* gene (Wu and Kaiser 1995), which encodes for a subunit of type IV pili. Beyond the pili-driven S-motility, *M. xanthus* cells also move via A-motility (‘adventurous’; Hodgkin and Kaiser 1979); this form of motility allows individual cells to move across solid surfaces, and is not *pilA*-dependent (Shi and Zusman 1993). These two mechanisms together contribute to wildtype surface motility.

All four lineages examined here (E1, E2, E7, and E8) evolved in the lab from an ancestral strain lacking type IV pili (*pilA*) required for S-motility that is largely non-motile on soft agar. E1 and E7 were evolved from an unmarked *pilA* strain (GJV4, aka ‘A1’ in Velicer and Yu 2003), whereas E2 and E8 were propagated from a version of this strain marked with rifampicin resistance (GJV6, aka ‘A2’ in Velicer and Yu 2003). GJV4 and GJV6 were generated by deletion of *pilA* from the “wildtype” lab reference strains GJV1 and GJV2, respectively, with both possess fully function A-motility and S-motility (Velicer and Yu 2003; Zee et al. 2014; Rendueles et al. 2016). All lineages were evolved under positive selection for numerical dominance at the leading edge of a growing colony on soft (0.5%) agar media. Populations were allowed to vegetatively grow and move outward from the

inoculated center of a plate for a period of two weeks, after which an area of set size at the leading boundary of the swarm (~3 mm × 5 mm) was selected to found the next selection cycle. These lineages underwent 32 two-week selection cycles before analysis by Velicer and Yu (2003). Preliminary genome sequencing data from these four lineages after 40 selection cycles show 21, 18, 21 and 15 mutations for E1, E2, E7 and E8, respectively (O. Rendueles and G. Velicer, unpublished data), suggesting relatively constant rates of genome evolution across the lineages. A recently published study examined the lineages highlighted here (among others) after 40 cycles with respect to swarming rate and other fitness-related traits (Rendueles and Velicer 2016). Lines E1, E2, E7 and E8 in this study correspond to evolved populations P49, P50, P55 and P56 in Rendueles and Velicer (2016), respectively.

Reintroduction of the *pilA* gene

In order to characterize epistatic interactions between accumulated mutations and *pilA* that influence the swarming phenotype, we experimentally reintroduced the *pilA* gene carried on the plasmid pGVTu1-*pilA* into a temporal series of genetic backgrounds in each of the four experimental lineages by electrotransformation (Zee et al. 2014). Control transformants into which the plasmid pGVTu1 alone (without *pilA*) was transformed into GJV4 showed that the plasmid alone has minimal effect on motility (unpublished data). Upon electrotransformation, the plasmid is stably integrated into the *M. xanthus* chromosome at the Mx8 phage attachment site (Velicer and Yu 2003); it is maintained as an extra-chromosomal self-replicating genetic element. After integration, *pilA* is expressed from its own native promoter that is also included on pGVTu1-*pilA* (Zee et al. 2014). We generated multiple independent *pilA* transformants from each of several clones isolated from successive evolutionary time points (i.e., selection cycles) in each of the experimental lineages. Throughout the current work, we present previously published E8 lineage data as a comparison for the motility of the other lineages (Zee et al. 2014). For comparisons of *pilA*-transformed swarming to a hypothetical maximum swarming rate, here we define the ‘maximum’ as the mean swarming rate of the control, *pilA*-carrying parents of the *pilA* proximate ancestors GJV4 and GJV6, namely GJV1 and GJV2, which is the fastest swarming rate among any of the strains examined in this study. This swarming rate is among the fastest on soft agar found among either laboratory strains of *M. xanthus* strains or natural field isolates (Vos and Velicer 2008).

Swarming assays

Swarming was quantified by measuring colony size over time. *M. xanthus* cells were grown in shaken liquid culture of growth medium CTT (1% casitone, 8 mM MgSO₄, 10 mM Tris-HCl, 1 mM KH₂PO₄) (Hodgkin and Kaiser 1977), and resuspended to a density of ~5 × 10⁹ cells/μL. 10 μL of this cell resuspension was spotted into the center of a CTT soft (0.5%) agar plate, and then placed into a humidified incubator at 32°C (Zee et al. 2014). After both one and seven days, the boundaries of the swarms were outlined. As performed by Velicer and Yu 2003 and Zee et al. 2014, swarm sizes were quantified by as the mean of four radii of the swarm (or eight radii for highly asymmetrical swarms) between the one-day and seven-day swarm boundaries. All swarm sizes reported refer to the swarming over this six-day period. The delay of the start of the period over which swarming rates are measured until one day after spotting is intended to minimize effects of any slight variations in initial cell

density among inocula after resuspension. For any given evolutionary time-point, the difference in swarm size between the untransformed strains and the *pilA*-transformed strains measures the effect of the *pilA* gene on that genetic background. Changes over time in this difference for any given lineage represent changes in the epistatic relationship between *pilA* and the set of mutations accumulating in the lineage. There were no differences in the assay environment or procedure for untransformed and *pilA*-transformed strains.

Statistical analysis

Assays were conducted in three independent replicate blocks for each lineage, with each block containing three independently isolated, untransformed clones from each time-point and a set of two or three sister transformants for each of these unique clones. We performed linear regression analyses on data from replicate independent blocks. Swarming values from each set of sister transformants were averaged to a single independent value within each block. For comparisons across individual evolutionary time-steps, we compared motility rates using two-sided t-tests. Relationships between untransformed and *pilA*-transformed strains were tested with linear regression. All analyses were performed using the R Statistical Computing Environment (R Core Team 2014).

Results

Phenotypic trajectories

All four lineages had significantly positive phenotypic responses to selection, displaying increased motility at the end of the evolution experiment (one-sided *t*-tests, $p < 0.01$ for all lineages) (Velicer and Yu 2003). The lineages, however, vary greatly in their phenotypic trajectories over shorter periods of evolutionary time (Fig. 2A). E1 has a distinctive trajectory in which swarming rate increases with each examined cycle until Cycle 15, but then greatly decreases between Cycles 15 and 18, after which there is no more significant change. The E2 lineage did not have the increase in motility during the first three selection cycles common to the other lineages, but did increase strikingly between Cycles 3 and 6 and more mildly thereafter over the rest of the trajectory. While three lineages exhibited at least one temporary decrease in swarming rate (E1, E2 and E7, Fig. 2A), the E7 lineage showed the most idiosyncratic phenotypic trajectory with the largest early increase in swarming followed by alternating periods of decrease (three) and increase (two) (Fig. 2A). E8 shows an early motility increase, a period of relative stasis, and then a late, large increase in swarming beginning after Cycle 18. Analysis of this lineage (both phenotypic and genomic) is reported in Zee et al. (2014). As in Velicer and Yu (2003), the swarming rates of the E7 and E8 lineages showed the greatest response to selection (Fig. 2A).

Here, we note a quantitative discrepancy between the swarming rate we measured for the Cycle 32 E7 population and the estimate of Velicer and Yu (2003). While the Cycle 32 swarming rates of E1 and E2 relative to that of E8 were very consistent across the two studies (~35% vs. 36% of the E8 swarming rate for E1 and ~36% vs. 41% for E2 here vs. the previous study, respectively), E7 was relatively slower in the experiments reported here (~56% of the E8 rate here vs. 87% in Velicer and Yu (2003)). This discrepancy is consistent with unpublished observations that the morphological and swarming-rate phenotypes of the

Cycle 32 E7 population are more variable across time and laboratory locations than are E8 phenotypes. (This study was performed in Bloomington, Indiana whereas the evolution experiment and original swarming rate assays were performed in Tübingen, Germany.) Nonetheless, despite this quantitative variation, in both studies E7 was substantially faster than E1 and E2 and significantly slower than E8.

Reintroductions of *pilA*

On the *pilA* ancestral genetic backgrounds of GJV4 and GJV6, reintroducing the *pilA* gene recovers the swarming of the original wildtype strains (GJV1 and GJV2, respectively) (Figure S1; one-sided t-tests, $p = 0.99$ (GJV4), $p = 0.37$ (GJV6)). However, in all four evolved lineages, *pilA*-transformed swarming decreases overall with evolutionary time (Fig. 2B), revealing that negative epistasis is a shared feature of all populations and is not unique to E7 and E8, the two lineages that evolved tentacle phenotypes (Velicer and Yu 2003). The general decrease in swarming rates of *pilA*-transformed clones over evolutionary time implies that not only does the positive phenotypic effect of *pilA* decrease (Fig. 2C), but also that it decreases specifically due to antagonistic interactions between accumulating mutations and *pilA*. This contrasts with the alternative hypothetical physiological maximum (limit null) scenario, in which the magnitude of positive *pilA* effects decrease not because of direct antagonism between accumulating mutations and *pilA*, but because evolving lines are approaching a maximum swarming value that cannot be surpassed. In this scenario, even upon *pilA* reintroduction, *pilA* effects are inherently reduced as untransformed lineages increase in swarming rate over time (Zee et al. 2014). Although we find a positive relationship between the distance of untransformed swarming rates from the phenotypic maximum and the phenotypic effect of *pilA* reintroduction (Fig. S2), the fact that *pilA* ceases to fully restore the swarming rates of evolved clones at later evolutionary time points (Fig. 2B) excludes the maximum limit null hypothesis as a primary explanation for why positive effects of *pilA* (Fig. 2C) decrease over time.

Despite the clear finding that *pilA*-transformed swarming decreases over time in all four lineages, it does so with very different temporal dynamics in the different lineages. In E1 and E8, there are large drops in *pilA*-transformed swarming already between the Ancestor and Cycle 3 and *pilA* never restores swarming to near ‘maximum’ (i.e. wild-type) levels in any evolved clone. In E2, the Cycle 3 samples are restored to wild-type levels before all subsequent time-point samples are restored only partially. In the E7 lineage, restoration to the maximum occurs intermittently until Cycle 12 before restoration become only partial thereafter.

By looking at the difference between the untransformed and transformed swarming rates, we can isolate the phenotypic effect of *pilA* at each time point. This effectively measures the epistatic effect of each genetic background on *pilA* over evolutionary time. As mutations accumulate during the evolution experiment, we see consistent decreases in the positive effect of *pilA* effect across all lineages (Fig. 2C). Most of the largest decreases in *pilA* effect occur early during evolution. The largest decreases of *pilA* effect of lineages E1 and E7 occur between the Ancestor and Cycle 3, as does the second-largest decrease for E8. In E2, the largest decrease occurs over Cycles 3 – 6. As initially reported in Zee et al. (2014), the

E8 lineage shows a second massive decrease in *pilA* effect (and its largest) between Cycles 24 – 28, with a transition from positive effects through Cycle 24 to negative effects at the final two time-points. Such “sign epistasis” is not observed in any of the other three lineages (Fig. 2C).

Just as swarming-rate evolution over short time steps in the unmanipulated evolved populations did not always mirror the long-term swarming increases observed at the whole-lineage scale, analysis of single time-step changes also reveals that epistatic interactions between *pilA* and accumulating mutations can deviate from general, whole-lineage trends. Accumulating mutations negatively interact with *pilA* at the timescale of the entire evolutionary lineage for all lineages. However, similarly to the mean responses to selection, specific evolutionary changes during individual selection cycles have variably significant epistatic interactions with *pilA* (Table 1) and in one case a significantly positive interaction (E7, Cycles 9–12, Table 1).

The negative interactions between mutations fixing in the four lineages and the reintroduced *pilA* gene result in a negative overall relationship between the mean phenotypic response to selection (i.e., change in untransformed strains) during individual evolutionary time-steps and the mean change in swarming of *pilA*-transformed strains over the same time-steps when considering the pooled data from all lineages ($r^2 = 0.23$, $p = 0.002$, Fig. 3A). This relationship indicates that, on average across all populations, mutations that increased motility rates during the evolution experiment tend to interact antagonistically with *pilA* and reduce its positive effect on swarming upon reintroduction.

For all four lineages, there is a significant negative relationship between the untransformed and transformed swarming rates (Figure 3B; linear regression, E1: $p = 0.0008$; E2: $p = 0.003$; E7: $p = 0.04$; E8: $p = 0.02$). These patterns also point to antagonistic epistatic relationships. The physiological maximum model would generate a flat relationship between untransformed and transformed swarming rate for each lineage. (A positive relationship would reflect synergistic epistasis). It is also worth noting that the negative relationships in each lineage have different slopes and intercepts, reflecting the independent accumulation of mutation sets that differ quantitatively how they interact with *pilA*.

Discussion

Our results reveal how a reintroduced gene that underlies an ecologically important social behavior epistatically interacts with mutations fixed during evolution in four parallel lineages. We found that negative epistatic interactions emerged in all four lineages, with the largest antagonistic effects generally associated with mutations fixed early in the evolutionary trajectories. Thus, our results indicate that the emergence of negative epistasis is a generic property of all populations evolving in the same selective regime, irrespective of whether they evolved qualitatively novel tentacle phenotypes (E7 and E8, Velicer and Yu 2003, Fig. 2B) or (E1 and E2). Moreover, in E7 and E8, the largest decreases in swarming rates of *pilA*-transformed clones over successive evolutionary time-points (Cycles 6–9 and 12–15 in E7 and prior to Cycles 0–3 for E8) preceded the time-points at which the tentacle

phenotypes of these lineages most robustly emerged (Cycle 18 for E7 and Cycle 28 for E8, unpublished observations).

Our data shows departures from the expectations of both genetic independence and a physiological maximum swarming rate. While simply looking at the change in *pilA* effect over time (Fig. 2C) may be consistent with the maximum swarming rate hypothesis, the fact that the *pilA*-transformed strains are not at the maximum and decrease over evolutionary time indicates that there are antagonistic interactions between accumulating mutations and *pilA* (Figure 3B). The positive relationship in Fig. S2 can be interpreted as reflecting an inverse causal relationship between the number of accumulated adaptive mutations and the effect of the *pilA* gene.

One possible explanation for the consistent negative epistasis we find is that the increased motility phenotypes are genetically co-opting remaining elements of the ancestral, *pilA*-driven S-motility (True and Carroll 2002; Prud'homme et al. 2007; Wagner and Lynch 2010). In both E7 and E8, the novel phenotypes are driven partly by increased exopolysaccharide (EPS) production that enhances cell-cell connectivity (Velicer and Yu 2003). New forms of EPS production and the PilA protein may functionally interfere with each other. Interactions between EPS and PilA have been demonstrated to contribute to *M. xanthus* social behavior (Black et al. 2006; Yang et al. 2010). Even in the absence of the evolution of the novel tentacle phenotype, it is possible that the mutations leading to the quantitative motility increases in E1 and E2 could show the negative interactive effects of genetic co-option. For example, if these lineages increase motility through modification of A-motility, these mutations could occur at loci that influence the ancestral S-motility as well. While A- and S-motility in *M. xanthus* are largely distinct genetically (Hodgkin and Kaiser 1979), several loci are known to contribute to both A- and S-motility simultaneously (Rodriguez and Spormann 1999; White and Hartzell 2000; Hartzell and Kaiser 2001). Further molecular genetic screens have identified further insertions that affect both motility systems, suggesting shared components between the two (Youderian et al. 2003; Youderian and Hartzell 2006). Molecular evolution at these loci could contribute to evolutionary co-option, and consequently, epistatic interference. If this type of genetic co-option is occurring, it could contribute to the generalized antagonistic epistasis that is manifest across lineages. Further experimental studies would be required to determine whether such co-option is truly underlying the patterns we find.

The negative relationship between changes in untransformed and transformed strains swarming (Fig. 3) suggests that mutations with positive effects on mean evolved motility often antagonize *pilA* driven motility. By contrast, many mutations that decrease mean motility in the lineages have a positive interaction effect with *pilA*, particularly in the E7 lineage (upper-left quadrant of Fig. 3B), although some others had negative interactions (lower-left quadrant). During the evolution experiment, cells were selected from the leading edge of the swarm to found the next selection cycle. Thus, in the context of the experiment, motility could be considered a proxy for fitness; however, this assumption could be flawed because the trait under selection is not necessarily increased swarming rate, but rather the ability of a genotype to be disproportionately represented at the edge of the swarm, irrespective of the behavioral mechanism by which it achieves this. It is therefore possible

that the cell population selected for founding the next selection cycles could contain ‘swarming cheaters’ that are carried to the edge of the swarm without themselves having mutations that increase motility. Such a mechanism could explain the decreases in motility in some lineages over individual evolutionary timesteps (e.g., E1, Cycles 15–18; Fig. 2A). Alternatively, populations may evolve mechanisms of interference competition that confer a competitive advantage against fast swimmers but trade off against intrinsic swarming rate. A recent study that examined both the lineages highlighted here (except after 40 cycles) and many others that underwent the same basic transfer scheme suggests that indeed some populations evolved mechanisms of interference competition without exhibiting significantly increased swarming relative to their ancestors (Rendueles and Velicer 2016).

Higher-order epistasis has recently drawn increased attention for its potential importance to evolutionary processes (Weinreich et al. 2013; Lali and Elena 2015). Over the course of the original evolution experiment, in some cases multiple mutations fixed between successive transfer cycles at which samples were stored frozen. For instance, in the E8 lineage, at least two mutations fixed (or at least reached high frequency) in each period between sample storage (Zee et al. 2014). It is important to note, then, that the epistatic interactions we observe between the genetic backgrounds and *pilA* could be a result of either pairwise interactions with specific mutations or higher-order epistatic interaction with a suite of mutations. Further, the changes in mean *M. xanthus* swarming between cycles could be the result of epistatic interactions between fixing mutations. At later time points when more mutations have accumulated, the opportunity for higher-order epistasis among fixed mutations, as well as with the reintroduced *pilA* gene, increases.

Our investigations with replicate experimental lineages allowed us to examine aspects of epistasis evolution that would be difficult to address with combinations of intentionally introduced mutations (e.g., Weinreich et al. 2006), particularly with respect to the emergence of spontaneously evolved novel phenotypes. Our differentiation of lineages relies on the *ad hoc* division into those that evolved ‘novel’ swarming phenotypes and those that did not. Novelty in evolution is difficult to rigorously determine (Pigliucci 2008) and is inherently biased towards conspicuous phenotypes (e.g., flowers (Frolich 2003), flight (Wheat and Wahlberg 2013), bioluminescence (Stansbury and Moczek 2014)), rather than changes at the molecular level that may significantly alter the phenotype. Though our division is based on the conspicuous ‘tentacle’ phenotypes of E7 and E8, we consider it justified because these phenotypes are associated with large quantitative increases in swarming rate (Velicer and Yu 2003). Our results with these experimental lineages allowed us to test among hypotheses regarding the potential relationships of epistatic interactions to qualitatively novel and spontaneously evolved social phenotypes versus to general processes of adaptive evolution irrespective of lineage-specific phenotypes. We find that negative epistasis between a reintroduced social gene and accumulating mutations in evolving lineages appears to be a generic feature of the process of adaptation in this experimental system rather than a feature unique to the emergence of more extreme phenotypes.

In conclusion, by reintroducing a gene of known effect into multiple independent evolutionary lineages, we were able to study how epistatic interactions emerge and change over time under identical abiotic selection pressures. We found consistent negative epistasis

between the *pilA* gene and the temporally changing genomic environments across four lineages that evolved in the absence of this gene. This pattern is best explained by antagonistic interactions between the reintroduced *pilA* gene and the changing genomic backgrounds within each lineage. Our results complement previous studies that find a preponderance of negative epistasis (e.g., Chou et al. 2011; Khan et al. 2011), but our evidence for direct antagonistic interactions is unique.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Swarming colonies of an untransformed clone from lineage E8 isolated after Cycle 28 of the evolution experiment exhibiting the tentacle phenotype and fast swarming characteristic of this lineage at Cycles 28 and 32 (left, photo taken six days after inoculation) and a pGVtu1-*pilA* transformant (right) of the untransformed clone shown on the left. Reintroduction of *pilA* into this clone and other clones from E8 Cycles 28 and 32 eliminates the tentacle phenotype and decreases swarming rate.

Figure 2.

Phenotypic and epistatic trajectories. (A) Swarming rate increases overall in all lineages despite some transient decreases. (B) Swarming rates for *pilA*-transformed strains decrease over time, with largest effects at earlier time points. In all lineages, *pilA*-transformed strains are not at the 'maximum' swarming rate. Dashed line represent 'maximum'. (C) The positive effect of *pilA* reintroduction on swarming decreases with time at the whole lineage scale in all lineages except E1, in which the decrease is transient. Swarming rates are over six day experimental period. Dashed line represents no *pilA* reintroduction effect. Points show

the mean, and error bars represent standard errors. In all panels, E8 data from Zee et al. (2014). E1 = black triangles; E2 = red triangles; E7 = green circles; E8 = blue circles.

Figure 3.

(A) Negative relationship between mean changes in untransformed and *pilA*-transformed swarming rate. Each point represents a specific evolutionary period between frozen-sample storage for a specific lineage, with the x-axis showing mean change in untransformed swarming rate over that period and the y-axis showing the mean change in the *pilA*-transformed swarming. The solid black line shows the linear fit of data pooled from all lineages. (B) Negative relationships between swarming rates for untransformed and *pilA*-transformed swarming rates. Each set of points represents a separate lineage. Different colored points different lineages: E1 = black; E2 = red; E7 = green; E8 = blue.

Table 1

Stepwise t-tests for responses of swarming rate to selection and effects of *pilA* reintroduction.

Cycle interval	Response to selection				<i>pilA</i> effect			
	E1	E2	E7	E8	E1	E2	E7	E8
Ancestor-3	<0.001*	<0.001*	0.04*	0.23	0.00*	0.33	<0.001*	<0.001*
3-6	—	<0.001*	0.35	—	—	<0.001*	0.24	—
3-9	<0.001*	—	—	0.95	0.01*	—	—	0.42
6-9		0.07	0.03*			0.20	0.02*	
9-12	0.52	1.00	0.08	0.90	0.13	0.55	0.05*	0.22
12-15	0.49	0.01*	0.15	0.35	0.78	0.57	0.10	<0.001*
15-18	0.01*	0.63	<0.001*	0.99	0.13	0.72	0.01*	0.31
18-21	0.42	0.30	0.22	0.25	0.11	0.46	0.49	0.03*
21-24	0.43	0.96	0.14	0.09	0.75	0.14	0.27	0.27
24-28	0.74	0.67	0.95	<0.001*	0.68	0.50	0.36	<0.001*
28-32	0.37	0.72	0.55	0.22	0.25	0.69	0.28	0.15

All values shown are p-values. Asterisks represent p-values < 0.05. Note: E2 and E7 have data for Cycle 6, while E1 and E8 do not.