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The control of cheating is important for understanding major transitions in evolution, from the simplest genes to the most complex societies. Cooperative systems can be ruined if cheaters that lower group productivity are able to spread. Kin-selection theory predicts that high genetic relatedness can limit cheating, because separation of cheaters and cooperators limits opportunities to cheat and promotes selection against low-fitness groups of cheaters. Here, we confirm this prediction for the social amoeba *Dictyostelium discoideum*; relatedness in natural wild groups is so high that socially destructive cheaters should not spread. We illustrate in the laboratory how high relatedness can control a mutant that would destroy cooperation at low relatedness. Finally, we demonstrate that, as predicted, mutant cheaters do not normally harm cooperation in a natural population. Our findings show how altruism is preserved from the disruptive effects of such mutant cheaters and how exceptionally high relatedness among cells is important in promoting the cooperation that underlies multicellular development.

altruism | cellular slime molds | conflict | *Dictyostelium discoideum* | kin selection

Cooperation is a hallmark of major transitions in biological complexity: from molecules to genes, from genes to chromosomes, from primitive cells to complex cells, from cells to multicellular organisms, and from multicellular organisms to societies (1–3). Cooperative groups are vulnerable, however, to exploitation by cheaters, individuals that have access to group benefits without contributing their fair share (1–3). Among cells and individuals, high relatedness is thought to aid in selection against cheaters (4–6). High relatedness means that cheaters and cooperators will tend to be in different groups, which both limits opportunities for cheaters to exploit cooperators and exposes any group-level defects of cheaters to selection. Curiously, although such control is central to selfish-gene theory, tests at the genetic level have been limited by the kinds of information available. In large organisms, relatedness is often estimated, but cheater genes are unknown. In microorganisms, cheater genes can be found (7–13), but little is known about relatedness in natural social groups.

The life cycle of social amoebae presents a challenge to the importance of relatedness in promoting selection against cheaters and an opportunity to test it. When the normally solitary amoebae are starved of their bacterial food source, they gather into a multicellular aggregate that forms a fruiting body. Here, ≈25% of cells altruistically die, forming a stalk that holds up the remaining cells, differentiated as spores, for dispersal (14–17). Thus, unlike more familiar organisms that develop from one cell, development begins by aggregation of many dispersed cells. Different clones can mix and cheat each other (18, 19), for example by avoiding contributing to the sterile stalk (7). Models (20–22), experiments (7, 23, 24), and a natural observation (24), suggest that cooperative fruiting body formation can be threatened by the spread of mutant cheaters that harm group productivity. It is not known whether such cheaters are controlled by either high relatedness or alternative forms of cheater control (12, 25).

The best known social amoeba, *Dictyostelium discoideum*, is a model organism that, unusually, allows both estimation of relatedness in the field and the study of cheater mutants. Relatedness of vegetative *D. discoideum* cells naturally cooccurring in very small soil samples (0.2 g) has been estimated as 0.52 (26), but relatedness in actual fruiting bodies has not been estimated. In this study, we measure relatedness of actual fruiting bodies from nature and make a general prediction of how cheaters that incur a large group cost (i.e., socially disruptive) should be controlled. To explicitly demonstrate control by relatedness, we then examine one cheater mutant in the laboratory, showing that it devastates cooperation at low relatedness but does not spread at high relatedness. Finally, we test the prediction that such mutants should not be successful at disrupting cooperation in a natural population.

Results

Natural dictyostelid fruiting bodies have been reported on dung (16, 23), and we found them primarily, but not exclusively, on dung of whitetail deer [see supporting information (SI) Fig. 5 and *SI Text*]. We used two methods to assess relatedness. First, we collected 88 fruiting bodies from 25 deer dung piles in October 2004 at Mountain Lake Biological Station (University of Virginia, Charlottesville, VA). For each, the entire fruiting body was genotyped at three highly polymorphic microsatellite loci (19, 26) that can distinguish >99% of clones. Sixty-eight fruiting bodies (77%) always showed single bands expected of clonal fruiting bodies, and 20 (23%) showed multiple bands (Fig. 1*A*). This yields a minimum relatedness of 0.86 (assuming clones in chimeras are equally represented; see *Methods*). To measure relatedness more directly, we clonally isolated 1,039 spores (13.85 ± SD 4.87 spores per fruiting body) from 75 additional fruiting bodies from various locations and times of year. Of these, 69 fruiting bodies (92%) showed only one clone, whereas 6 (8%) were chimeric for two or three clones (Fig. 1*B*). Relatedness within chimeric fruiting bodies was 0.684 ± SE 0.086 and within all fruiting bodies was 0.975 ± SE 0.012. Fig. 2 shows that this level of relatedness should be sufficient to control all costly cheaters that gain by avoiding the stalk. To test this prediction, we examined the success of a cheater mutant in low-relatedness and high-relatedness laboratory populations.

To demonstrate that high relatedness allows selection against cheating, we need to show not only that a cheater can threaten cooperation and invade at low relatedness, but that it cannot

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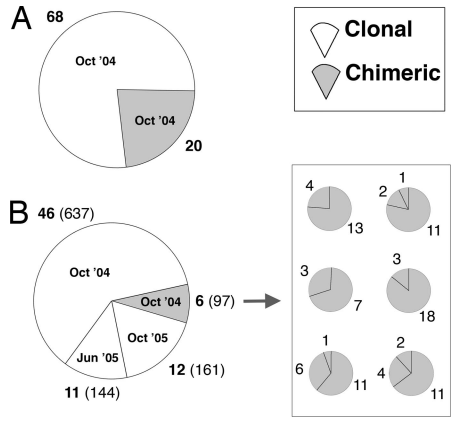

Fig. 1. Proportion of wild fruiting bodies that are chimeric (shaded slices). (*A*) Entire fruiting body genotyped at once. (*B*) Individual spores genotyped. Bolded numbers represent the number of fruiting bodies and nonbold numbers represent the number of spores. Smaller pie charts correspond to the six chimeras with individual spores genotyped; numbers represent number of spores for each different clone isolated from each fruiting body.

invade at high relatedness. We investigated the mutant *fbxA*$^-$(also known as *chtA*$^-$), a knockout of an F-box protein involved in degradation of a developmentally important phosphodiesterase (7, 27–29). *fbxA*$^-$ is ideal because it cheats in chimeras, but on its own it is developmentally deficient and produces few or no spores (7), so its spread through the population would be devastating.

First, we ask how damaging this cheater mutant would be at sufficiently low relatedness. A cheater cell's relatedness to groupmates is $r = (p_y - p)/(1 - p)$, where p_y is the frequency of the cheater allele in its group, and p is the population frequency (30). When $r = 0$, $p_y = p$: groups are thoroughly mixed and each group has the population frequency of the allele. *fbxA*$^-$ beats wild type at all mixture frequencies (Fig. 3*A*, and see ref. 7), which means that in a very low relatedness population, *fbxA*$^-$ will always win and will spread at least to the highest frequency tested (0.75). To confirm that *fbxA*$^-$ beats wild type because of social cheating rather than because of differences in growth during the vegetative stage, we compared growth rates of each strain on its own and found no significant difference in their growth (paired t test: $n = 10$, $t = -1.03$, $P = 0.330$).

In addition, we measured how damaging the increase would be; *fbxA*$^-$ results in fewer and more poorly developed fruiting bodies and, therefore, much lower spore production (Fig. 3*B*). How can the mutant spread despite this effect? At zero relatedness, all groups have the same genetic composition, so there is no opportunity for group differences in spore production to counter the within-group advantage of the cheater. Thus, at very low relatedness *fbxA*$^-$ is a severe threat; it will spread, and, as it does so, it will greatly reduce normal cooperative fruiting and spore production.

We now use the same data in a different way to ask what is the highest relatedness that would allow fbxA$^-$ to invade the population. At high levels of relatedness, cheaters will encounter themselves at high frequency within the group, and the within-group advantage of the cheater can be counteracted by the between-group cost. At invasion, when the cheater is rare, it must be more successful than wild type in pure wild-type groups. In Fig. 3*C*, we plot the fitness of *fbxA*$^-$ in the tested mixtures, relative to the fitness of wild-type fruiting alone, taking both the advantage of cheating (Fig. 3*A*) and the lowered productivity (Fig. 3*B*) into account. Finally, we note that, at invasion when the population frequency of the cheater p is near zero, $r = (p_y - p)/(1 - p) = p_y$. Thus, at invasion, relatedness equals the frequency of cheaters in the group. Fig. 3*C* shows that the mutant has lower fitness and cannot invade when it is in chimeric mixtures at >0.25 relatedness (Fig. 3*C*). High relatedness should prevent invasion of this potentially damaging cheater.

The size of the cheating advantage we found is consistent with avoidance of stalk, but an earlier study (7) found a stronger cheating advantage of *fbxA*$^-$. Could *fbxA*$^-$ spread with this larger advantage? Considering the group cost that we found, invasion would still be prevented at the observed level of relatedness (SI Fig. 6). This control depends largely on the complete fitness cost in clonal *fbxA*$^-$ fruiting bodies, which we confirmed also occurs on the natural substrate of dung (Fig. 4).

Fig. 2 predicts that no stalk cheater that has a high cost when alone should spread at the high relatedness in nature. Typical clonal isolations from soil do not necessarily address this prediction because they rely on the ability to distinguish dictyostelids from other soil microbes based on morphology (16). A clone with defective fruiting would normally be discarded because it does not resemble a dictyostelid (SI Fig. 7), and even if noticed, it may not propagate. We therefore collected wild fruiting bodies and plated out spores clonally to directly look for the *fbxA*$^-$ phenotype or other developmental defects (23, 24, 31). Of 3,316 spores germinated (34.9 ± SD 54.6 spores per fruiting body) from 95 wild fruiting bodies, however, all produced a robust wild-type pattern of development (SI Fig. 8). This suggests that *fbxA*$^-$ and other costly cheaters are not commonly cheating altruists in this population.

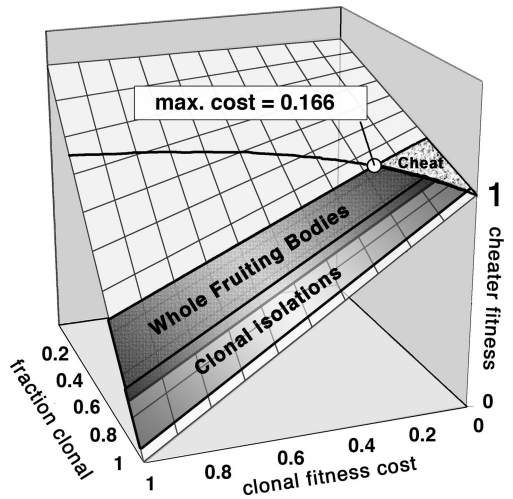

Fig. 2. Fitness at invasion of cheater that avoids stalk in fruiting bodies made of 25% stalk. The advantage of a rare cheater relative to wild type is $[c(1-k)0.75+(1c)]/0.75 > 1$. The 3/4 denominator is the fitness of cells in wild-type fruiting bodies, which have a 25% chance of dying in stalk. The numerator is cheater cell fitness averaged over a fraction c in clonal cheater fruiting bodies that lose k units of fitness, and $1 - c$ in chimeras, where they avoid the stalk and have fitness 1. The bands represent 95% confidence intervals (binomial distribution) for cheater fitness based on our two estimates of the percent of clonal fruiting bodies. The cheater will spread only at a relative fitness 1 (speckled gray region labeled "Cheat"), which means the clonal cost cannot exceed 0.166 (labeled point).

Discussion

It is increasingly recognized that many microorganisms are social and can cheat (7–13), but there have been no estimates of relatedness from natural populations at the scale relevant to natural selection against cheating. Here, we have shown that relatedness in cooperative groups of *D. discoideum* is very high, higher even than in most eusocial insect colonies (32). It is not clear what maintains high relatedness in *D. discoideum*. As in most eusocial insects, kin discrimination might be important, and

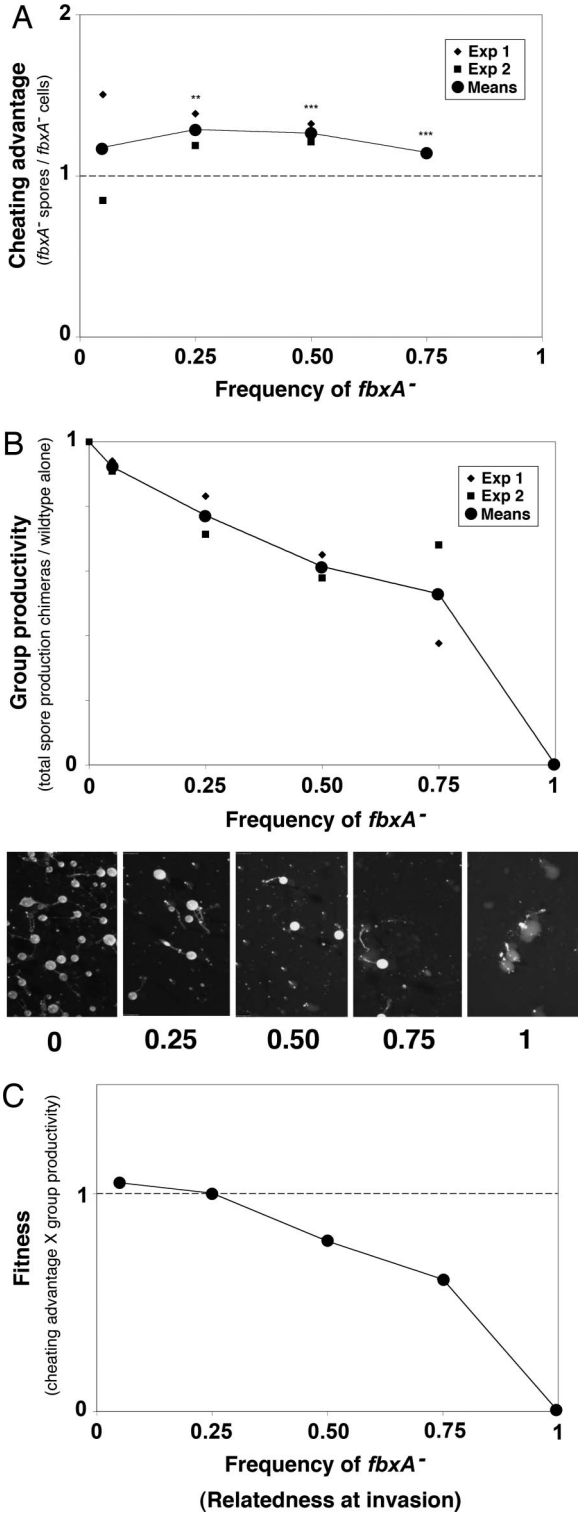

Fig. 3. Fitness of *fbxA*$^-$ knockout relative to wild type at different frequencies. (*A*) Cheating advantage of *fbxA*$^-$ measured as the ratio of the percentage of *fbxA*$^-$ in final spores to its initial percentage in the cell stage of development (Fisher's exact test versus no change, two replicates: **, $P < 0.005$; ***, $P < 0.001$ for each test; *N* at least 1,152 plaques for each test). (*B*) Group productivity (total spore production) declines as a function of the percentage of *fbxA*$^-$ in fruiting bodies (Spearman's rank correlation on mean values. $r_s = 1$, $n = 6$, $P < 0.01$). Photos show fruiting bodies from each mixture. (*C*) Estimate of *fbxA*$^-$ fitness as its cheating advantage times its group productivity. When fitness is <1, *fbxA*$^-$ will not gain an advantage. The line crosses at $R = 0.25$.

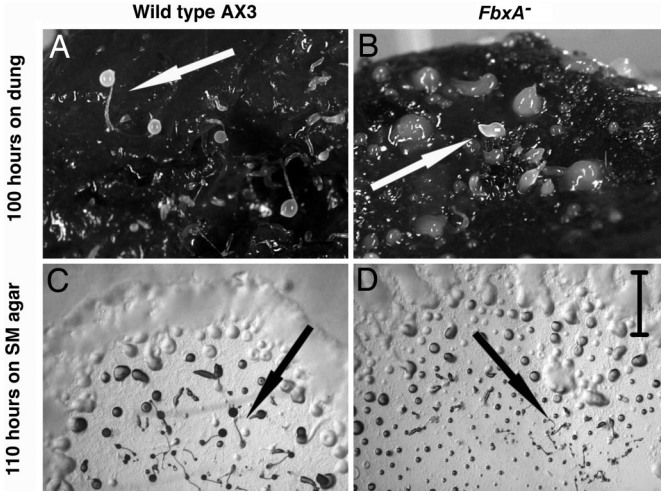

Fig. 4. The *fbxA*$^-$ mutant fails on both laboratory and natural substrates. Fruiting body phenotypes of wild-type AX3 (*A* and *C*) and *fbxA*$^-$ (*B* and *D*) on dung (*A* and *B*) and SM agar (*C* and *D*). Arrows point to mature fruiting bodies for AX3 and failed fruiting bodies for *fbxA*$^-$. Measure bar corresponds to 1.5 mm for agar photos and 3 mm for dung photos.

indeed, in the related *D. purpureum*, when different isolates are mixed together, they prefer to form fruiting bodies with kin (33). Nonetheless, previous work has shown for *D. discoideum* that genetically different clones mix in the laboratory (18). An alternative explanation for high relatedness is that *D. discoideum* may grow in isolated clonal patches in this natural population. Future work will need to test these hypotheses.

This high probability of fruiting alone in *D. discoideum* is predicted to allow selection against most cheaters that gain an advantage by refusing to become stalk cells (Fig. 2). The only exceptions are cheaters with little or no cost when fruiting alone, that is, those least likely to compromise the cooperative system. Cheaters that harm group productivity, those most likely to destroy cooperation if they spread, should be selected against.

Using the socially disruptive cheater *fbxA*$^-$, we confirm this prediction in a laboratory setting. Like cheating strains of *Myxococcus xanthus* (34), a prokaryote with a similar life cycle, *fbxA*$^-$ imposes a clear group cost. The spread of such cheaters can sometimes devastate cooperation, even to the point of causing extinction (35). Our data show that *fbxA*$^-$ is a severe threat to cooperation in low-relatedness populations and that it could invade and pose some threat to populations with relatedness up to 0.25 but that the high relatedness observed in the wild should keep it from invading, assuming *fbxA*$^-$ behaves similarly in the field. Indeed, the high relatedness should prevent the spread of any strongly socially destructive mutant. As expected, we did not find any such cheaters in fruiting bodies in the natural population, suggesting that they are absent or very rare. Strictly speaking, some could be present but unsuccessful at getting into fruiting bodies, but that would still mean that they are not successfully cheating or threatening cooperation.

Another *D. discoideum* mutant, *dimA*$^-$, shows both similarities and differences to *fbxA*$^-$. *dimA*$^-$ is a social defector that ignores the signal to become sterile stalk (33). *dimA*$^-$ is a net loser, regardless of relatedness, because of a pleiotropic effect that occurs late in development that disallows cheating (24). Cheating by *fbxA*$^-$ also carries a negative pleiotropic effect (lowered total spore production), but this effect is weak enough at low relatedness to allow cheating to succeed. High relatedness is what allows selection to operate strongly against *fbxA*$^-$. An alternative hypothesis is that negative pleiotropic effects in the vegetative

stage, which would be independent of relatedness, could contribute to selection against *fbxA*$^-$. However, vegetative pleiotropic effects are not important because our growth rate experiments show no significant differences in growth between the two strains. This is not surprising, because *fbxA*, like ≈25% of *D. discoideum* genes, is expressed primarily during development (36), with no transcript detected during the vegetative stage (7).

Our study suggests that the extreme altruism of *D. discoideum* persists in the face of cheating because high relatedness allows selection to remove all cheaters that would severely undermine group cooperation. We therefore confirm a principle that is thought to be widely important in other less tractable cooperative systems. High relatedness prevails both among the cells of multicellular plants and animals and among the individuals of social insect colonies, and the consequent ability to control cheater mutants may explain persistence and success of those cooperative entities.

Methods

Estimating Relatedness in Nature. We collected two deer scat pellets from each of 50 piles in both October 2004 and October 2005 samples, and each of 150 piles in June 2005. Each pellet was lifted with an entomological pin and placed carefully on a 2% agar/water plate and transported indoors, where it was incubated at room temperature. In the October samples, fruiting bodies typically appeared within 6 days, either fruiting directly on the dung or migrating onto the agar. This method yielded naturally constituted fruiting bodies before they could be dispersed. Sometimes arthropods hatched from the dung, died, and then fruiting bodies grew on the decaying animal (Fig. 1). This occurred most often in the June sample when dung arthropods were very abundant, delaying fruiting body formation. Individual fruiting bodies were collected with entomological pins or forceps and placed either directly into chelex (19), for genotyping whole fruiting bodies, or into water, for clonal isolation. We grew spores clonally by diluting fruiting bodies 0.1 M EDTA and plating at a density of 5–50 spores per plate on SM agar (37) with *Klebsiella aerogenes* as a bacterial food source. We examined all clearings for the mutant phenotype.

We extracted DNA directly from fruiting bodies (for whole fruiting body isolations) or from slugs and fruiting bodies (for clonal isolations), and three highly polymorphic microsatellite loci were amplified by PCR (19). Rare alleles could be missed in the whole fruiting body method (because of insufficient DNA), but this would only slightly underestimate relatedness because of their rarity. In the clonal isolations, however, there is no bias, because the probability of being detected is equal to the frequency. DNA was analyzed with an ABI 3100 Genetic Analyzer, Genescan, and Genotyper software (Applied Biosystems, Foster City, CA). We treated different isolates as being the same clone if they shared their alleles at all three loci and as different if they differed at one or more. Relatedness within fruiting bodies was estimated from spore genotypes by using Relatedness 5.08 (www.gsoftnet.us/GSoft.html), weighting fruiting bodies equally and jackknifing over fruiting bodies.

Allele sizes were binned in intervals of ≈3 bp by minimizing the variance from the centers of the bins. Based on allele frequencies (SI Table 1), the probability that two random clones would share alleles at all three loci independently was 0.005. Two assumptions allowed us to estimate a minimum relatedness from the genotyping of whole fruiting bodies (SI Table 2). First, because individual genotypes could not be inferred when a fruiting body showed multiple alleles at multiple loci, we assumed the maximum number of genotypes possible. Second, because we could not quantify the genotypes, we assumed that they were at equal frequencies. Both of these assumptions decrease the estimate of relatedness, so our estimate is conservative.

Searching for Cheater Mutants in Nature. The 88 fruiting bodies genotyped wholly were collected from 25 dung piles from October 2004, but 14 of these dung piles were sampled again for the clonal genotyping. This contributed 27 of the 75 fruiting bodies for this method. In all for the clonal genotyping, there were 46 deer dung piles sampled, one salamander dung pile, and three other locations in the leaf litter. For the June and October 2005 samples, all fruiting bodies were from separate locations.

For determining the presence or absence of mutants in the wild, we included additional spores from the 75 fruiting bodies isolated for genotyping, as well as an additional 12 fruiting bodies raised *in situ* on deer dung and 8 raised by adding concentrated bacteria to soil samples collected from Mountain Lake Biological Station in November 2003.

To test whether *fbxA*$^-$ is detectable under normal field collection conditions, we grew it on hay infusion agar (16) at low density, as is the case in typical field collection, and it did not produce normal dictyostelid aggregation patterns at 64 h (SI Fig. 7), or fruiting structures with spores after 121 h.

To determine whether *fbxA*$^-$ could fruit on dung, we first grew *fbxA*$^-$ and wild-type AX3 in liquid medium (25). We put down aliquots of 100 ml (5×10^6 cells) on five autoclaved dung pellets for each. For *fbxA*$^-$, only one of the pellets produced visible fruiting structures (Fig. 4*B*). For AX3, four pellets produced fruiting structures containing spores. No spores had been produced by *fbxA*$^-$. Aliquots were taken from fruiting structures, examined under a microscope, frozen to kill any live cells, and plated out on SM agar at high and low dilution. We observed no spores, and no growth had occurred after 14 days.

Examining Cheating Advantage and Group Cost of *fbxA*$^-$. We assessed the fitness advantage that *fbxA*$^-$ gains relative to wild type at various frequencies. Six treatments were prepared: (*i*) 100% AX3, (*ii*) 95% AX3: 5% *fbxA*$^-$, (*iii*) 75% AX3: 25% *fbxA*$^-$, (*iv*) 50% AX3: 50% *fbxA*$^-$, (*v*) 25% AX3: 75% *fbxA*$^-$, and (*vi*) 100% *fbxA*$^-$. Strains were maintained in liquid medium (25). The complete mix experiment was performed twice at different times. We harvested cells of the two strains by centrifugation, washed them twice with water, and resuspended them in Pad Dilution Fluid (PDF) buffer (38). We added 1.25×10^7 cells of each treatment to a nitrocellulose filter in 125 ml of PDF (1×10^8 cells ml^{-1}), and total PDF on the filter pad and dish was made up to 2 ml. Two nitrocellulose filters were prepared for each frequency of *fbxA*$^-$. Filters were on top of damp paper filter pads, inside Petri dishes (60 × 15 mm) and placed in a plastic, humid box (35 cm × 14 cm × 13.3 cm) in the dark at 22°C for development.

To establish the frequency of *fbxA*$^-$ in mixtures, we plated out cells clonally and tested each clone for resistance to the toxin blasticidin (*fbxA*$^-$ was engineered to be blasticidin resistant) (7). These tests were done at three times: (*i*) before adding to filters (0 h), (*ii*) just before aggregation (6 h), and (*iii*) after development and fruiting (48 h). For the latter two times, we harvested cells and spores, respectively, from each nitrocellulose filter, by placing the filter in a 50-ml Falcon tube with 5 ml of KK2 (16.5 mM KH_2PO_4 and 3.8 mM K_2HPO_4) and then removing cells or spores by centrifugation. Cells or spores were counted by using a hemocytometer to estimate total spore production, and ≈50 cells or spores were added to each SM plate (16) with *Klebsiella aerogenes* as a food source (10 plates per treatment). For the 48-h time point, we added detergent (0.1% Nonidet P-40) to filters to lyse cells and leave only spores for clonal plating. Four days after clonal plating, we assessed blasticidin resistance by putting a few cells from individual plaques into HL5 with 5 mg ml^{-1} blasticidin (G418) in 96-well tissue culture plates (192 plaques per treat-

ment). These were examined after 1 week, and cells that grew were counted as *fbxA*$^-$. This allowed us to assess the cheating advantage. There were no significant differences in the frequency of *fbxA*$^-$ in cells at 0 and 6 h, so these data were combined (Fisher's exact test, $P > 0.1$ for all treatments).

To demonstrate that *fbxA*$^-$ beats wild type because of a cheating advantage during development rather than because of differences in growth during the vegetative stage, we compared growth rates of AX3 and *fbxA*$^-$ in liquid medium (25) over 10 different days. Each strain was maintained in its own flask at an initial density of 0.5×10^6 cells per ml, and after ≈24 h, we assessed the growth rate of both strains.

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