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Report

Kin Recognition Protects Cooperators against Cheaters

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Summary

The evolution of sociality and altruism is enigmatic because cooperators are constantly threatened by cheaters who benefit from cooperation without incurring its full cost [1, 2]. Kin recognition is the ability to recognize and cooperate with genetically close relatives. It has also been proposed as a potential mechanism that limits cheating [3, 4], but there has been no direct experimental support for that possibility. Here we show that kin recognition protects cooperators against cheaters. The social amoebae *Dictyostelium discoideum* cooperate by forming multicellular aggregates that develop into fruiting bodies of viable spores and dead stalk cells. Cheaters preferentially differentiate into spores while their victims die as stalk cells in chimeric aggregates. We engineered syngeneic cheaters and victims that differed only in their kin-recognition genes, *tgrB1* and *tgrC1*, and in a single cheater allele and found that the victims escaped exploitation by different types of nonkin cheaters. This protection depends on kin-recognition-mediated segregation because it is compromised when we disrupt strain segregation. These findings provide direct evidence for the role of kin recognition in cheater control and suggest a mechanism for the maintenance of stable cooperative systems.

Results

Social cheaters are individuals that reap the benefits of cooperation without fully paying the associated costs [1, 2, 5]. Cheaters arise constantly among cooperators [1, 6, 7] and may overtake a population and collapse a social system if not controlled [3, 8, 9]. Multiple mechanisms may limit cheating, including pleiotropy, high relatedness, and the evolution of cheater resistance [10–12]. Another possible mechanism is kin recognition, by which altruists preferentially cooperate with genetically related individuals [3, 4]. Figure 1 illustrates how kin recognition may limit the spread of cheaters. A cheater mutation arises in a population of cooperators that recognize one another as kin (Figures 1A and 1B). The cheater propagates (Figure 1C), but new mutations that alter kin recognition arise as well (Figure 1D). Individuals that carry a new kin-recognition signal cooperate among themselves while being protected from the cheaters that carry the old kin-recognition signal.

Many studies have documented the emergence of cheaters among cooperators and the presence of kin recognition in the same social systems [13–15]. However, there has been no direct demonstration that kin recognition can protect against exploitation by cheaters. We used the social amoeba *D. discoideum* to address this problem.

D. discoideum are solitary soil amoebae that exhibit social behavior upon starvation [16]. Genetically distinct cells coaggregate and develop into multicellular structures of approximately 50,000 cells, in which they differentiate into spores and stalk cells. Only the spores survive, whereas 20% of the cells die altruistically as stalks, which are thought to facilitate spore dispersal and therefore increase inclusive fitness [16, 17]. Cheaters in *D. discoideum* are strains that make more spores than their fair share, where fair share is the ratio between the strains at the onset of development [18]. Wild isolates of *D. discoideum* can exploit one another [17, 19, 20], and the genetic potential for cheating is considerable, as a single mutation in any one of a large number of genes can lead to cheating [21].

Wild isolates of *D. discoideum* segregate from genetically distant strains after initial coaggregation and develop into mostly clonal fruiting bodies. We have described a kin-recognition mechanism in *D. discoideum*, in which cells cooperate with genetically related kin during multicellular development [22]. This mechanism is mediated by two transmembrane proteins, TgrB1 and TgrC1 [23, 24]. The *tgrB1* and *tgrC1* genes are highly polymorphic in natural populations [23], and a matching pair of TgrB1 and TgrC1 is not only necessary and sufficient for cooperation between strains, but is also the only component determining kin recognition in *D. discoideum* [24]. Syngeneic strains that express different pairs of matching *tgrB1* and *tgrC1* alleles initially coaggregate upon starvation. After 6–8 hr of development, these mixed strains segregate into distinct subaggregates and continue to develop into fruiting bodies that are mostly clonal [24]. The existence of cheating behavior and kin recognition in *D. discoideum* allows a critical examination of the role of kin recognition in stabilizing social systems.

Cheating and kin recognition have been documented between different wild isolates in *D. discoideum*, but a recent study found that the genomes of these strains differ by about 40,000 single-nucleotide variations (E. Ostrowski, personal communication). It would therefore be difficult to examine the relationship between kin recognition and cheating using wild isolates because the results might be affected by uncharacterized genetic variations. We therefore took a reductionist approach to testing the interplay between kin recognition and cheating in a genetically defined system. *D. discoideum* genes can be precisely mutated or replaced at their natural chromosomal loci [16]. We used that property to generate strains that differed only in one cheating gene and/or in the *tgrB1/tgrC1* kin-recognition locus. These strains recognize each other as nonkin if they carry different sets of *tgrB1/tgrC1* alleles and cooperate as kin if they share the same sets of *tgrB1/tgrC1* alleles. These otherwise isogenic strains enabled us to test whether kin recognition protects against cheating without the potential confounding effects of other genetic differences. We also designed a cheater-protection assay to measure the cost imposed by cheaters on kin versus nonkin. We mixed cheater mutants with kin and nonkin victims in the test cases and replaced the cheaters with wild-type cells in the controls (Figure 2A). Victim strains are genetically

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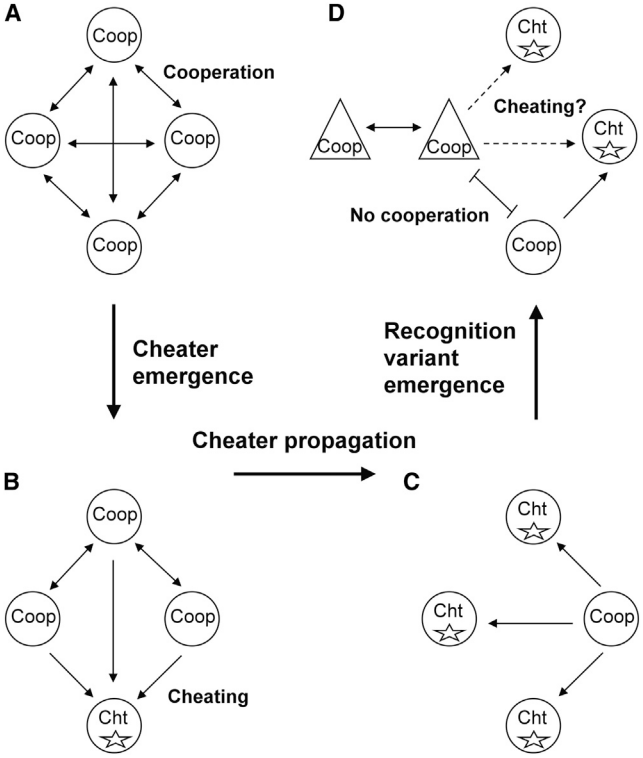

Figure 1. Evolutionary Relationships between Cheating and Kin Recognition

(A) Cooperation in a social group is manifested as reciprocal benefits (bidirectional arrows) between cooperating group members (Coop) that share a common kin-recognition signal (circles).

(B) Mutations (star symbols) can lead to the emergence of cheaters (Cht) within the population. The cheaters carry the common kin-recognition signal and they benefit from the cooperators without paying their fair share of the cost (unidirectional arrows).

(C) The cheaters propagate in the population and begin to outnumber the other cooperators because of the benefit they receive at a reduced cost.

(D) Other mutations can lead to the evolution of kin-recognition variants (triangles) among the cooperators. These new variants can cooperate among themselves (bidirectional arrows), but not with the original cooperators (barred line). We propose to test whether the new cooperators would be exploited by the old cheater (dashed arrows, null hypothesis) or whether they would be protected.

identical to each other and to the wild-type except for the designed differences in *tgrB1/tgrC1*, so each strain should sporulate according to its input proportions in the control, where no cheater is present. On the other hand, victim strains would exhibit reduced spore production in the experiments in which cheaters are present. We used the difference in spore production between the experiment and the control to evaluate the cost to the victims. Higher cost means the victim produced fewer spores when codeveloped with a cheater instead of the wild-type. The hypothesis that kin recognition protects from cheating would be refuted if the costs were indistinguishable between kin and nonkin and supported if the victims incurred a lower cost when they recognized the cheaters as nonkin.

Kin Recognition Protects against the Obligatory Cheater *fbxA*$^-$

We first tested the hypothesis with the *fbxA*$^-$ mutation, which confers one of the strongest cheater phenotypes [6]. We labeled the victims with green or red fluorescent proteins (GFP and RFP, respectively) to facilitate spore quantification. We mixed the *fbxA*$^-$ strain, which was made in the AX4 background, with the compatible strain $tgrB1^{AX4}tgrC1^{AX4}$-GFP and an incompatible strain, $tgrB1^{QS31}tgrC1^{QS31}$-RFP or $tgrB1^{QS38}tgrC1^{QS38}$-RFP. We expected *fbxA*$^-$ to cheat on the compatible strain (AX4), as published [6], so this experiment tested whether the strain with the switched recognition cues was protected from *fbxA*$^-$. The results in Figure 2B show that both incompatible strains incurred lower costs than the compatible $tgrB1^{AX4}tgrC1^{AX4}$-GFP, indicating that the incompatible alleles protected the would-be victims from cheating by *fbxA*$^-$.

To test for potential confounding interactions between the *fbxA*$^-$ mutation and the $tgrB1^{AX4}tgrC1^{AX4}$ alleles, we generated the *fbxA*$^-$ mutation in the $tgrB1^{QS31}tgrC1^{QS31}$ background and repeated the experiment. Figure 2C shows that the incompatible strains $tgrB1^{AX4}tgrC1^{AX4}$ and $tgrB1^{QS38}tgrC1^{QS38}$ incurred a lower cost than the compatible strain $tgrB1^{QS31}tgrC1^{QS31}$. These results further support the finding that kin recognition can protect cooperators from cheating. They also indicate that the *fbxA*$^-$ mutation confers cheating in the $tgrB1^{QS31}tgrC1^{QS31}$ background and that the $tgrB1^{QS31}tgrC1^{QS31}$ strain is susceptible to cheating if the cheater carries compatible *tgrB1-tgrC1* alleles. We conclude that the *tgrB1-tgrC1* alleles, which mediate kin recognition, confer resistance to cheating by *fbxA*$^-$.

We also examined the protective effect with different cheater frequencies in the populations. We found that the incompatible victims were protected at cheater-victim ratios of either 8:1:1 or 2:9:9 (Figure S1 available online), suggesting that kin recognition protects cooperators against cheaters and the protection is independent of the genetic background and the cheater frequencies.

A potential complication is that the cheater-compatible victim might somehow distract the cheater from interacting with the incompatible victim. To test this possibility, we mixed the cheaters with two incompatible victims, excluding the compatible victim from the assay system. Figure S2 shows that both incompatible strains were protected from the cheater, suggesting that the protection is cell autonomous and independent of the other mixing partners.

A Merodiploid Strain Bridges between Incompatible Cells

When cells with incompatible *tgrB1/tgrC1* allele pairs develop in chimerae, they segregate from one another during the aggregation stage and eventually form clonal fruiting bodies [24]. To test whether this segregation is required for protection from cheaters, we used a condition that prevents segregation of incompatible strains. *tgrB1/tgrC1* merodipoids are strains that carry two pairs of *tgrB1/tgrC1* alleles. These strains cooperate with haploid strains that have either one of the matching *tgrB1/tgrC1* pairs [24]. Figure 3A shows that merodiploid cells can bridge between incompatible haploid strains and thus prevent segregation during aggregation. When the incompatible haploid strains $tgrB1^{AX4}tgrC1^{AX4}$ and $tgrB1^{QS31}tgrC1^{QS31}$ were mixed with the merodiploid strain $tgrB1^{+/QS31}tgrC1^{+/QS31}$ at equal proportions, the incompatible strains cooperated with one another such that the three strains were equally distributed throughout the aggregate (Figure 3A, left). We propose that the merodiploid cells bridge between the incompatible haploid cells, because decrease of the proportion of the merodiploids in the mix resulted in partial segregation of the haploid cells (Figure 3A, middle). Bridging is also

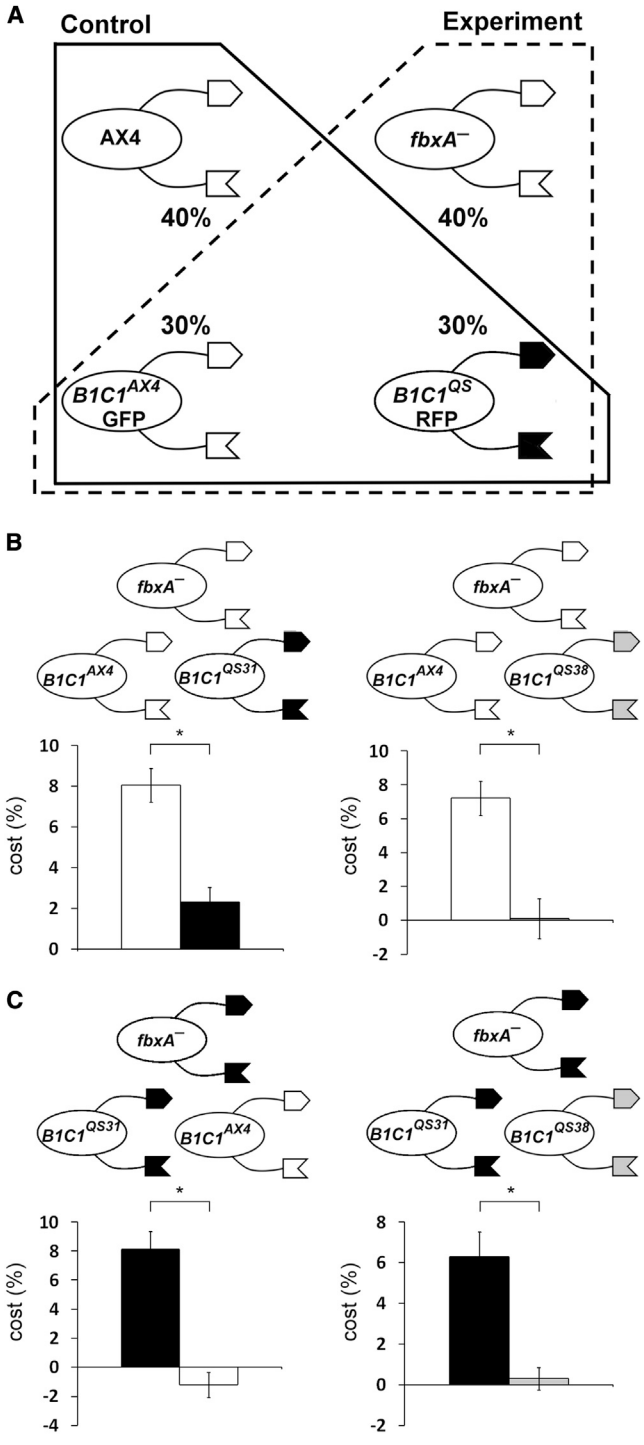

Figure 2. Kin-Recognition Protects against *fbxA*⁻ Cheating

Cells were codeveloped and spores counted to determine cost.

(A) Illustration of the assay system. Cells are drawn as ellipses with relevant genotypes within: AX4, laboratory wild-type strain; *fbxA*⁻, cheater strain; $B1C1^{genotype}$, *tgrB1/tgrC1* double-gene replacement strain where "*genotype*" indicates the allele origin; QS, wild-type isolate; GFP and RFP, green and red fluorescent protein labels, respectively. Extracellular arms indicate TgrB1-C1 proteins; the shading represents different alleles. Proportions (%) indicate the initial mixing ratios. Solid frame, control; dashed frame, experiment.

(B) Assays using *fbxA*⁻ in the AX4 background. Bars indicate the cost to the compatible strain $tgrB1^{AX4}tgrC1^{AX4}$-GFP (white) and the incompatible strains $tgrB1^{QS31}tgrC1^{QS31}$-RFP (black, left panel) and $tgrB1^{QS38}tgrC1^{QS38}$-RFP (gray, right panel).

(C) Assays using *fbxA*⁻ in the $tgrB1^{QS31}tgrC1^{QS31}$ background. Bars indicate the cost to the compatible strain $tgrB1^{QS31}tgrC1^{QS31}$-GFP (black) and the incompatible strains $tgrB1^{AX4}tgrC1^{AX4}$-RFP (white, left panel) and $tgrB1^{QS38}tgrC1^{QS38}$-RFP (gray, right panel).

Data are means ± SEM, n = 4 per group, Student's t test. *p < 0.01.

allele-specific because inclusion of the merodiploid strain $tgrB1^{+/QS4}tgrC1^{+/QS4}$, which is compatible with only one of the haploid strains (AX4), resulted in segregation of the two haploid strains (Figure 3A, right).

Kin-Recognition-Based Protection Depends on Strain Segregation

We examined the effect of merodiploid-bridging on cheater protection. We mixed *fbxA*⁻ with the incompatible haploid $tgrB1^{QS31}tgrC1^{QS31}$ and the bridging merodiploid $tgrB1^{+/QS31}$ $tgrC1^{+/QS31}$, tested the cost to the two victim strains, and found that the costs were indistinguishable (Figure 3B, left). In the controls, where the merodiploid could not disrupt the segregation of the incompatible strains from the cheater, the cheater-compatible merodiploids $tgrB1^{+/AX4}tgrC1^{+/AX4}$ incurred a higher cost than the incompatible $tgrB1^{QS31}$ $tgrC1^{QS31}$ haploids (Figure 3B, middle), as did the compatible merodiploid $tgrB1^{+/QS31}tgrC1^{+/QS31}$ compared with the incompatible haploid $tgrB1^{QS38}tgrC1^{QS38}$ (Figure 3B, right). These results suggest that strain segregation is required for kin-recognition-mediated protection against cheaters.

Kin Recognition Protects against Different Facultative Cheaters

We tested whether kin recognition could protect against different types of cheaters. The *fbxA*⁻ cells are obligatory cheaters, which depend on the presence of a victim for sporulation [6]. We therefore tested facultative cheaters that can sporulate well in pure populations. Strains LAS43, LAS44, and LAS105 were generated in the AX4 background and are distinct in their cheater mutations and phenotypes [21]. We tested them in the cheater-protection assay with the incompatible strains $tgrB1^{QS31}tgrC1^{QS31}$ and $tgrB1^{QS38}tgrC1^{QS38}$. Figure 4 shows that the incompatible strains incurred a lower cost than the compatible $tgrB1^{AX4}tgrC1^{AX4}$ in all three cases, suggesting that kin recognition provides general protection against cheaters.

Discussion

Our results support the hypothesis that kin recognition protects against cheaters because victims that were incompatible with the cheaters incurred a lower cost compared with cheater-compatible victims. However, that protection was not absolute, as nonkin victims incurred some cost in many cases. Kin-recognition systems are inherently imperfect [3, 25], so imperfect protection from nonkin cheaters could be the result of incomplete segregation between the incompatible strains. Indeed, segregation in *D. discoideum* is also not completely exclusive even between the most incompatible wild isolates [22]. Moreover, the merodiploid-cheater mixing experiment (Figure 3B) shows that segregation is a key factor in protection, so nonkin victims probably incur costs from the cheaters because of imperfect segregation. In addition, facultative cheaters can exploit victims by either "self-promotion" or "coercion" [19]. Segregation-mediated protection may be less effective against cheating through the "self-promotion" strategy. Finally, some cheaters could inflict damage prior to

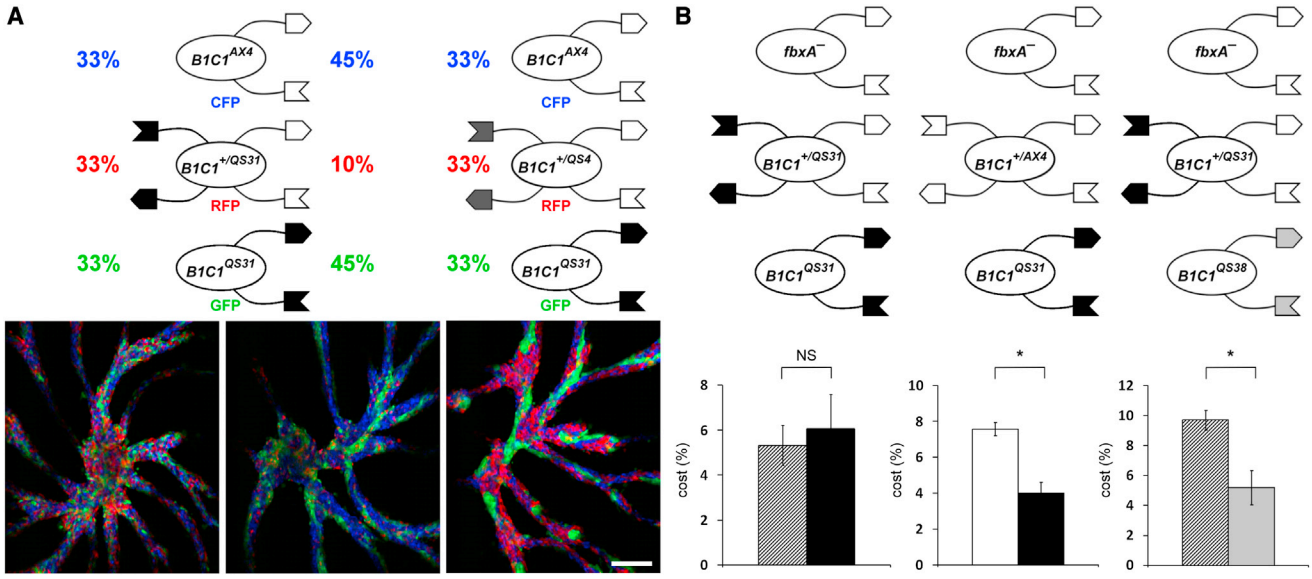

Figure 3. Protection from Cheaters Depends on Segregation

Cells were mixed at the indicated proportions (%) (A) or as described in Figure 2 (B) and allowed to develop.

(A) Fluorescence micrographs of mixed strains with different *tgrB1-C1* alleles during aggregation. Left and middle, incompatible haploids $tgrB1^{AX4}$ $tgrC1^{AX4}$-CFP (CFP, cyan fluorescent protein) and $tgrB1^{QS31}tgrC1^{QS31}$-GFP, and a merodiploid $tgrB1^{+/QS31}tgrC1^{+/QS31}$-RFP, which is compatible with both haploids; right, incompatible haploids $tgrB1^{AX4}tgrC1^{AX4}$-CFP and $tgrB1^{QS31}tgrC1^{QS31}$-GFP, and the merodiploid $tgrB1^{+/QS4}tgrC1^{+/QS4}$-RFP, which is compatible with the former haploid. The scale bar represents 125 μm.

(B) *fbxA*⁻ cheater-protection assays in the presence of merodiploid strains. Bars indicate the cost to the merodiploid strains $tgrB1^{+/QS31}tgrC1^{+/QS31}$-GFP (slashed) and $tgrB1^{+/AX4}tgrC1^{+/Ax4}$-GFP (white) and to the haploid strains $tgrB1^{QS31}tgrC1^{QS31}$-RFP (black) and $tgrB1^{QS38}tgrC1^{QS38}$-RFP (gray). Data are means ± SEM, n = 3–7 per group, Student's t test. *p < 0.03; NS, p = 0.57.

segregation [18], but we could not test them because relevant mutations are not known.

D. discoideum cheaters are abundant in nature and are found in close proximity with their victims [17, 20]. Our results suggest that kin recognition is a general defense mechanism that protects against various cheaters. This recognition system limits cheaters to their kin, whereas nonkin individuals are protected. However, kin victims are not completely defenseless from an evolutionary standpoint. The presence of cheaters in a population might select for new *tgrB1/tgrC1* allotypes (Figure 1), which could lead to an evolutionary "arms race" between cheating and kin recognition. This possibility is consistent with the observation that *tgrB1* and *tgrC1* are highly polymorphic in nature [23]. Other mechanisms also likely play a role in controlling *D. discoideum* cheaters that are limited to specific cheating mechanisms [10, 12].

Kin recognition has evolved independently in different organisms [26–31], and our results provide direct evidence that it may function in protection from cheaters. Polymorphism is a key element in recognition systems, and the prevalence of cheaters may be an evolutionary pressure that maintains such diversity, along with other selective pressures such as host-parasite interactions [32, 33]. Such evolutionary pressures have been demonstrated in numerous interspecific host-parasite and host-pathogen relationships [3], but not in intraspecific systems such as the one described here.

Experimental Procedures

Cell Growth and Development

Cells were cultured as described [23]. Media for *ura*⁻ strains were supplemented with 20 μg/ml uracil and drugs (10 μg/ml G418, 5 μg/ml Blasticidin S) were added as necessary but removed 48 hr before development. For development, cells were washed once with water, resuspended in PDF buffer (20.1 mM KCl, 1 mM $CaCl_2$, 2.5 mM $MgSO_4$, 9.2 mM K_2HPO_4, and 13.2 mM KH_2PO_4 [pH 6.4]), deposited on nitrocellulose filters at 1×10^6 cells/cm^2, and incubated in dark humid chambers for 48–72 hr.

Cheater-Protection Assay

Each set of experiments consisted of two three-way mixes. In the control, wild-type cells were mixed with GFP- and RFP-labeled strains at a 4:3:3 ratio. The fluorescently labeled cells differed in their *tgrB1-C1* alleles, whereas one of them was compatible with the wild-type. In the experiment, the wild-type was replaced with a cheater mutant that carried the wild-type *tgrB1-C1* alleles. Controls and experiments were performed side by side and developed under the same conditions. In each instance, we also developed pure populations of the fluorescent strains and measured the proportion of fluorescent spores produced by each strain. We only considered experiments in which the proportion of fluorescent spores was ≥90% and we used the particular proportion to scale the results of the experiments.

After development, we harvested the spores in 0.1% NP40 made in KK2 buffer. The total sporulation efficiencies of control and experiment were usually indistinguishable from each other. We measured the proportion of fluorescent spores by flow cytometry (LSRFortessa), counting at least 10,000 spores each time. Cost was calculated by subtraction of the percentage of green- or red-fluorescent spores in the experiment from the respective control:

$$\text{Cost}_{\text{GFP}} = \text{GSP}_c - \text{GSP}_e \text{ and}$$

$$\text{Cost}_{\text{RFP}} = \text{RSP}_c - \text{RSP}_e,$$

where GSP is the GFP spore percentage, RSP is the RFP spore percentage, c is the control mix, and e is the experiment mix.

For instance, if the GFP and RFP spore percentages in the control were 30% each, and in the experiment the GFP and RFP spore percentages were 23% each when mixed with a certain cheater, then the cost incurred by the GFP and RFP strains would be 30% – 23% = 7%. Each experiment was performed at least in triplicate, done on different days. We evaluated the significance of the differences between the costs to the two fluorescently labeled strains using a two-tailed Student's t test.

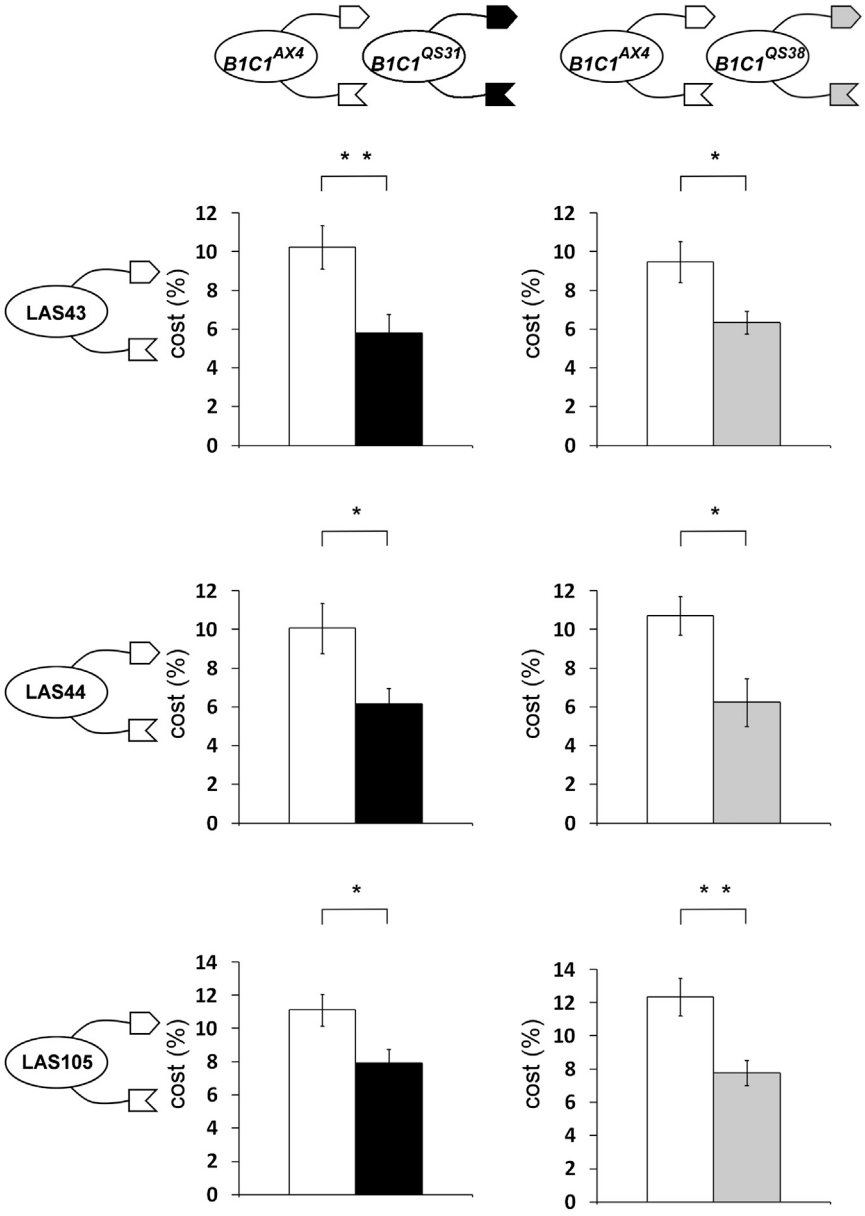

Figure 4. Kin-Recognition Protects against Various Cheaters

Cells were developed in chimera, and spores were counted to determine cost. The facultative cheaters LAS43, LAS44, and LAS105, made in the AX4 background, were used in independent assays as indicated on the left. Bars represent cost to the compatible strain *tgrB1*^{AX4} *tgrC1*^{AX4}-GFP (white) and the incompatible strains *tgrB1*^{QS31}*tgrC1*^{QS31}-RFP (black) and *tgrB1*^{QS38}*tgrC1*^{QS38}-RFP (gray). Data are means ± SEM, n = 4–5 per group, Student's t test. *p < 0.05; **p < 0.03.

To examine the potential effects of differential fluorescence labeling, we mixed *fbxA*$^{-}$ with a mix of compatible isogenic GFP- and RFP-labeled *tgrB1*^{AX4}*tgrC1*^{AX4} cells (Figure S3A). We also repeated the cheater-protection assay, which was described in Figure 2B, left panel, with strains in which we switched the GFP and RFP labels of the compatible and incompatible strains (Figure S3B). The fluorescent labels did not affect the results in either case.

Segregation Assay

We grew the fluorescently labeled cells in pure populations, washed the cells, mixed them at the indicated proportions at a density of 1×10^7 cells/ml in PDF buffer, deposited them in 40 μl drops on a 5 cm agar plate (2% Noble Agar in KK2 buffer), incubated them in a dark humid chamber, and photographed them at the streaming stage (8–12 hr) with fluorescence microscopy.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, three figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.06.049.

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