

Cheater-resistance is not futile

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Cooperative social systems are susceptible to cheating by individuals that reap the benefits of cooperation without incurring the costs¹. There are various theoretical mechanisms for the repression of cheating² and many have been tested experimentally. One possibility that has not been tested rigorously is the evolution of mutations that confer resistance to cheating. Here we show that the presence of a cheater in a population of randomly mutated social amoebae can select for cheater-resistance. Furthermore, we show that this cheater-resistance can be a noble strategy because the resister strain does not necessarily exploit other strains. Thus, the evolution of resisters may be instrumental in preserving cooperative behaviour in the face of cheating.

Dictyostelium cells propagate as unicellular amoebae in the soil. Upon starvation, they aggregate into multicellular structures and differentiate into viable spores and dead stalk cells³. Stalk-cell differentiation supports spore maturation and dispersal, but this altruistic behaviour can be exploited by cheaters that make more than their fair share of spores in chimaeric fruiting bodies⁴. The genetic potential for cheating is high⁵ and cheaters abound in nature⁴, but cheating behaviour can be restrained by various mechanisms, such as intrinsic lower fitness of the cheater⁶, pleiotropy of the cheater gene⁷, high genetic relatedness in natural populations⁸, and kin discrimination^{9,10}.

Evolution of cheating-resistance is another mechanism that could restrict the spread of a specific cheater allele in the population and it could be manifested in several ways. One way is the evolution of other cheaters, but such a mechanism could lead to an arms-race of cheating strategies that would contribute to the rapid demise of cooperation. We therefore tested whether selection for cheating-resistance in *Dictyostelium* could yield mutants that resist cheating while remaining cooperative. Our strategy was to mix a population of mutated cells derived from the wild-type AX4 with a cheater strain, allow them to grow and develop into chimaeric fruiting bodies, and select spores. Under these conditions, the cheater would exploit most of the cells in the mutant population, thus increasing the proportion of any cheater-resistant mutants. We chose a strong cheater mutant, LAS5 (ref. 5) (which we renamed cheater C, *chtC*) as the selector. This mutant strain has a plasmid insertion in the *DDB_G0290959* gene, which is predicted to encode a protein with a signal peptide anchor and a transmembrane domain at the amino terminus. We generated a population of 1,000 strains in the wild-type background, each containing one insertional mutation that also conferred resistance to the antibiotic blasticidin S (BSR). We mixed *chtC* (which is sensitive to blasticidin S) with the mutant population at a ratio of 4:1 and allowed the cells to develop into chimaeric fruiting bodies. We germinated the spores and allowed the amoebae to grow in the presence of blasticidin S, thus eliminating the *chtC* cells. We then mixed the enriched mutant population with fresh *chtC* cells and developed them again, to maintain similar levels of selection in six such cycles of selection (Fig. 1a). We predicted that most of the mutant strains in the population would be cheated upon by *chtC*, eventually leading to their disappearance,

and to the enrichment of any cheater-resistant strains that were present in the pool. We carried out this selection on several independent pools of mutants.

To test the population dynamics during the screen, we assessed population complexity of the mutant pool by Southern blot analysis of total genomic DNA, with a probe against the BSR marker. The initial population contained numerous strains, each giving a different banding pattern, which resulted in a smeared signal on the Southern blot (Fig. 1b). As the selection proceeded, the levels of various mutants fluctuated, as shown by the appearance and disappearance of bands on the blot. However, by the fourth cycle of selection, the population became enriched predominantly with a single mutant, suggesting that the presence of the cheater selected for a specific strain, which we predicted to be cheater-resistant. We isolated the enriched mutant and found an insertion in *DDB_G0271758*, a gene that has not been annotated in *Dictyostelium discoideum* and has no annotated homologues in other organisms. We then interrogated the above Southern blot with a probe against *DDB_G0271758* and found that the wild-type allele disappeared from the population, whereas the mutant allele became abundant as the selection progressed (Fig. 1c). We also used quantitative PCR (qPCR) to quantify the progressive enrichment of the mutant allele during the selective process and observed a 100-fold increase in the allele abundance by the sixth selection cycle (Fig. 1d).

To verify that the insertion in *DDB_G0271758* caused the observed phenotype, we regenerated the insertion in fresh wild-type cells, and named the resulting mutant and the mutated gene *rccA* (resister of *chtC* A). Both growth and development of the *rccA* mutant were indistinguishable from the wild-type AX4, suggesting that the *rccA* mutation did not confer obvious fitness costs. We then carried out direct cheating assays. We mixed either wild-type or *rccA* mutant cells at a 1:1 ratio with *chtC* and determined the proportion of spores formed by each strain. When mixed with *chtC* cells, the *rccA* mutant formed almost 50% of the spores. This proportion was significantly higher than the number of spores formed by AX4 in a mix with *chtC* cells (Fig. 2a), but not significantly lower than the hypothesized value of 50% (one-sample one-tailed *t*-test, $P = 0.28$). This observation supports the hypothesis that the *rccA* mutant can resist cheating by *chtC*. We also tested whether *rccA* was a specific resister of *chtC* or a resister of other cheaters as well, by performing similar mixes with LAS1, another facultative cheater that was isolated from the screen that yielded *chtC*⁵. We found that *rccA* did not resist cheating by LAS1 (Fig. 2a), indicating that *rccA* is unable to resist all cheaters. Therefore, it is likely that the ability to resist cheating will depend on the cheating mechanism of the specific cheater.

Cheating-resistance might be due to counter-cheating, such that the presence of a specific cheater would select for equivalent or even stronger cheaters. An alternative would be that cheater-resisters would be neutral, or noble, so they would not cheat on strains victimized by the original cheater. To distinguish between these

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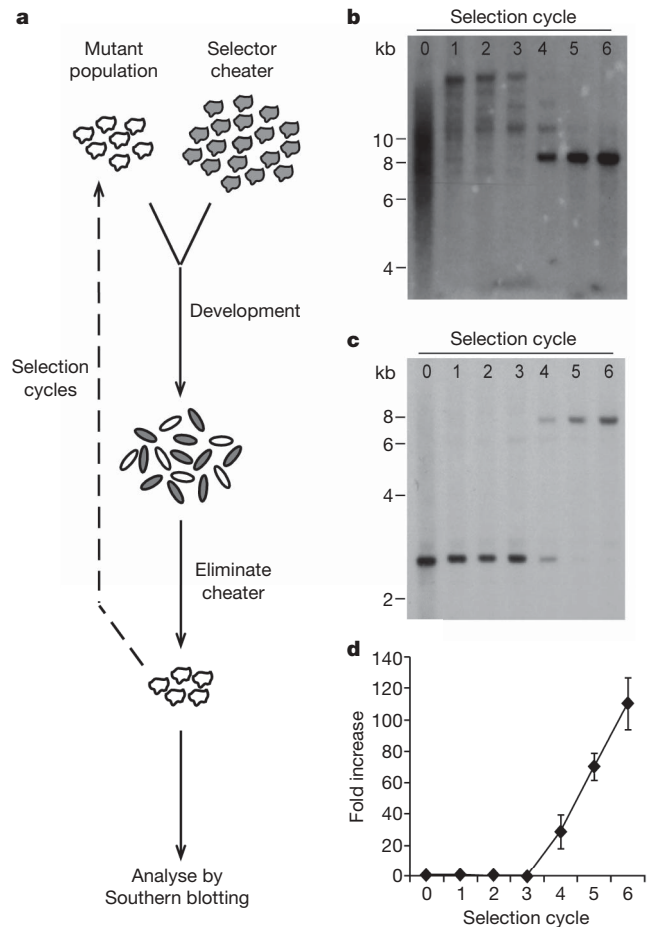


Figure 1 | The presence of a cheater selects for cheater-resistance.

a, Selection: we mixed *chtC* with mutant populations, grew and developed the mixtures, collected spores and then eliminated *chtC*, for six selection cycles. **b**, Southern blot analysis of DNA from the population at each cycle, probed against the insertional plasmid. Decreased population complexity is reflected by progressively simpler banding patterns. kb, kilobases. **c**, The same Southern blot as in **b** interrogated with a probe against *rccA*. The lower band (wild-type allele) disappeared during the selection, whereas the higher band (insertion allele) became enriched. **d**, qPCR on DNA from each selection cycle to determine the levels of the *rccA* insertion allele. Results (means \pm s.d. of three technical measurements) are depicted as the fold change relative to cycle 0.

possibilities, we developed either AX4 or *rccA* cells in 1:1 chimaerae with green fluorescent protein (GFP)-labelled AX4 (AX4-GFP) cells and tested the proportion of the green-fluorescent spores in the population. The results support the latter alternative—the *rccA* mutant formed a similar number of spores as the AX4 control in 1:1 chimaerae with AX4-GFP (Fig. 2a), indicating that *rccA* is not a cheater.

Although *rccA* resists cheating by *chtC* in a 1:1 mix, it is more likely to exist in mixtures that include both cheaters and victims. To test the behaviour of *rccA* under such conditions, we carried out a three-way mix between AX4-GFP, *chtC* and *rccA* at a 1:1:1 ratio, and determined the proportion of spores formed by each strain. The simplest prediction, on the basis of the respective pairwise mixes, was that *rccA* would remain unchanged in the population, whereas *chtC* would increase its proportion at the expense of the wild type. Surprisingly, we observed that both the wild type and *rccA* formed more spores than predicted (marginally significant) and *chtC* formed significantly fewer spores than predicted (Fig. 2b). This finding suggests that the presence of *rccA* may help to reduce the cheating of *chtC* on the wild type and might further contribute to restricting the spread of *chtC*.

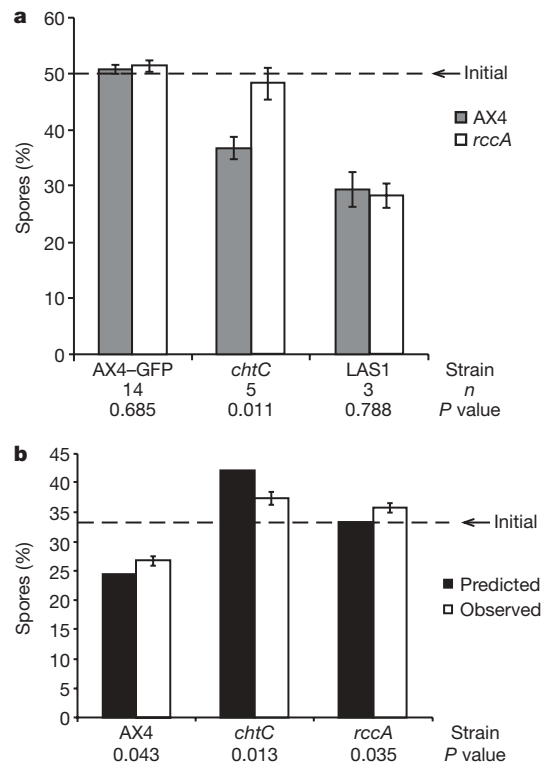


Figure 2 | *rccA* is a noble, specific cheater-resister. **a**, We determined the proportion of AX4 or *rccA* spores, when mixed individually with AX4-GFP, *chtC* or LAS1, as indicated. The results are means \pm s.e.m. The number of independent replications (n) and the P values for a Student's t -test on arcsine square-root transformed data are shown. *rccA* was significantly different from AX4 only when mixed with *chtC*. **b**, We mixed AX4-GFP, *chtC* and *rccA* and determined the proportion of spores formed by each strain. The predictions are on the basis of the respective pairwise mixes. The observations are means \pm s.e.m. of five independent experiments. All strains formed significantly different numbers of spores compared to the predictions. The P values for a one-sample two-sided t -test on arcsine square-root transformed data are shown.

Altogether, we carried out the selection for cheater-resistant strains (as described earlier and shown in Fig. 1a) separately on a total of seven different pools of mutants that were generated by independent mutagenesis experiments. In each of these pools, one or two mutants became enriched at different cycles during the selection (data not shown). From these pools, we obtained six mutants (other than *rccA*), which we named *rcc1*–*6*. All the mutants were resistant to *chtC* when mixed at a 1:1 ratio (Fig. 3), suggesting that selection for cheater-resistance is a common occurrence when a cheater is present in a population. Unlike *rccA*, one of the mutants (*rcc2*) significantly cheated on *chtC*, and two others (*rcc1* and *rcc4*) were close to significance. Notably, when we re-created these insertions in fresh wild-type cells, the new strains did not resist cheating by *chtC*. This finding indicates that the cheater-resistance of the original mutants was dependent on a genetic event other than the insertion, suggesting that spontaneous mutations that arose in the population contributed to cheating-resistance. These mutants retained their cheater-resistance phenotype in several experimental replications, suggesting that the underlying mutations were stable. We also tested the six original mutant strains against the wild-type AX4 and found that one was a cheater and five were noble (data not shown).

Cheater mutants can be either obligate, such that they cannot cooperate among themselves, or facultative, such that they cooperate among themselves, but cheat other strains. Experiments in the bacterium *Myxococcus xanthus* have shown that mutations that restore cooperation in an obligate cheater genotype can be selected for, and these mutations might also confer cheater-resistance against the

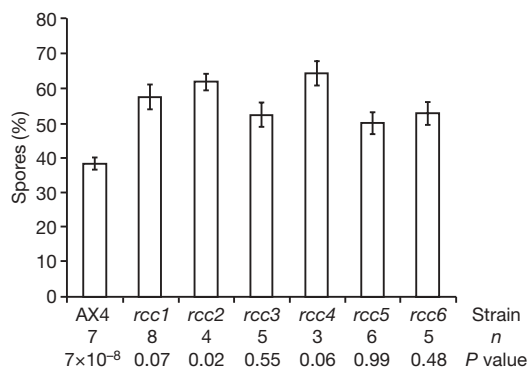


Figure 3 | Cheater-resistance is a general phenomenon. All of the independently derived mutants (*rcc1*–*rcc6*) formed more spores than AX4 when mixed at a 1:1 ratio with *chtC* (Student's *t*-test on arcsine square-root transformed data, $P < 0.005$). Results (percentage mutant spores recovered) are shown as means \pm s.e.m. The *rcc2* strain made more spores than *chtC*, and the other strains were not significantly different from the hypothesized value of 50%. The number of independent replications (*n*) and the *P* values for a one-sample two-sided *t*-test on arcsine square-root transformed data are shown.

parental cheater strain¹¹. Our experiments demonstrate that the introduction of a facultative cheater into a population can directly select for cheater-resistance behaviour in other strains. Thus, cheater-resistance may be an important mechanism of cheater control and might provoke molecular arms-races, similar to those seen in host–pathogen interactions¹². More importantly, the resistant strains can be noble strains that do not cheat, suggesting that such selection could yield strategies that allow cheater-resistance while preserving cooperation.

Cheater resistance in animals takes forms such as policing¹³, punishment¹⁴ and partner choice¹⁵, which are hard to study at the level of individual genes, but it has been largely neglected in microbial systems, where cheating is easily studied at the genetic level^{16–18}. This could be because cheating in many microbial social systems takes a passive form of declining to produce and secrete public goods, and it is difficult to resist such cheating without converting the cheaters back to being producers. The active cheating in social amoebae is more similar to animal sociality, and is therefore a good model system for exploring the complex evolutionary dynamics of genes affecting cooperation, cheating, and cheater-resistance.

METHODS SUMMARY

Strains, cell growth and transformation. We generated all of the mutations in the laboratory wild-type AX4 strain¹⁹. AX4–GFP⁷ was used for mixes. The *chtC* mutant has the same insertion site as LAS5 (ref. 5), with the pLPBLP plasmid²⁰ replacing pBSR1, and the BSR cassette removed by transformation with the pDEX-NLS-Cre plasmid²⁰. We grew cells and carried out plasmid transformation essentially as described¹⁹.

Mutagenesis, selection and identification of the mutated gene. We performed restriction enzyme-mediated integration (REMI) mutagenesis, and pooled 1,000 mutants²¹. We mixed spores from the pool and from *chtC* at a ratio of 1:4, grew and developed the mixture, collected genomic DNA and selected spores⁵. We then germinated the spores, and eliminated *chtC*. The selection cycle was repeated with the mutant pool and a fresh culture of *chtC*. This was done individually on seven independent pools of mutants. We carried out plasmid rescue as described²².

Nucleic acid analysis. We prepared genomic DNA as described²³ and performed Southern blot analysis by standard methods²⁴. We used linearized pBSR1 (ref. 21) as a probe for the BSR cassette, and a PCR fragment for the *rccA* gene²⁵. We performed quantitative PCR as described²⁶ using primers for the *rccA* mutant allele and the unrelated *DDB_G0276785* gene for normalization.

Mixing experiments. We grew cells separately and developed mixtures, essentially as described¹⁰. We collected spores and counted them as described for the GFP-labelled strains¹⁰. For the mix between LAS1 and *rccA*, we plated out the spores, transferred cells from individual plaques into HL5 liquid medium in 96-well plates, prepared genomic DNA, and tested for the mutant LAS1 and *rccA*

alleles by PCR. For the other mixes, we grew cells in HL5 containing blasticidin S in 96-well plates as described earlier, and scored for drug-resistance. In the three-way mix experiment, we scored AX4–GFP on the basis of fluorescence, and *rccA* on the basis of drug-resistance.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Author Contributions A. Khare conducted the experimental work and wrote the paper, L.A.S. isolated the original LAS5 and LAS1 cheater strains. All of the authors conceived the study, discussed the results and commented on the manuscript.

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METHODS

Strains. We generated all of the mutations in the laboratory wild-type AX4 strain¹⁹. The AX4-GFP strain⁷ was used for mixing experiments. The *chtC* mutant has the same insertion site as LAS5 (ref. 5). We cloned the region of homology from the original rescue plasmid pLAS5, and inserted it into the ClaI restriction endonuclease site of pLPBLP²⁰. We used the resulting vector to re-create the insertion in AX4 by homologous recombination. We then generated the *chtC* mutant by transforming the cells with the pDEX-NLS-Cre plasmid²⁰ to remove the BSR cassette.

Cell growth and transformation. We grew cells in shaking suspension in HL5 liquid broth with the necessary supplements, or on SM-agar plates in association with *Klebsiella aerogenes*¹⁰. Plasmid transformation was carried out as described¹⁰, with the following modifications: cells were resuspended at a final density of 3×10^7 cells ml⁻¹ before transformation, electroporated twice, and the transformants were recovered in HL5 with 10% FBS for 24 h before the addition of either blasticidin S (10 µg ml⁻¹) or G418 (5 µg ml⁻¹).

Mutagenesis, selection and identification of the mutated gene. We performed REMI mutagenesis, pooled 1,000 mutants, and plated the pool on SM-agar plates as described²¹. We grew the *chtC* mutant on SM-agar plates in association with bacteria. We collected spores from the pool and from *chtC* as described²¹, mixed them at a ratio of 1:4, and plated a total of 2×10^6 spores on each of two SM-agar plates in association with bacteria. We collected cells to prepare genomic DNA from one of the plates after 44–48 h. We selected spores from the second plate after 4 days, germinated them, and grew the amoebae in submerged cultures in HL5 with 10 µg ml⁻¹ blasticidin S to eliminate *chtC*. The mutant pool, and a fresh culture of *chtC* were then grown separately in suspension culture, mixed and plated on SM plates as above to repeat the selection cycle. This was done individually on seven pools of mutants generated by independent REMI mutagenesis experiments. We carried out plasmid rescue using ClaI to obtain the plasmid pRccA and identified the insertion site by sequencing the flanking regions²². The gene names in the manuscript (*DDB_G0290959* and *DDB_G0271758*) refer to the accession numbers in dictyBase (<http://dictybase.org>).

Nucleic acid analysis. We prepared genomic DNA as described²³ and performed Southern blot analysis by standard methods²⁴. The blots were hybridized with radioactive DNA probes made by random-primer labelling²⁵. We used linearized pBSR1 (ref. 21) as a probe for the BSR cassette, and a PCR fragment as a probe for the *rccA* gene (primers: 5'-TCGTTGTCATCTTGGTTTG-3' and 5'-GATTT CAGTTTACCCACCG-3'). We performed qPCR as described²⁶ using primers for the *rccA* mutant allele: 5'-TGCTGAAAATGTATTACCACC-3' and 5'-ATTTAGGTGACACTATAG-3' and primers for the unrelated *DDB_G0276785* gene to normalize for the amount of genomic DNA: 5'-TGATTGCCAA TGGATCATC-3' and 5'-ATAGTTTACTTCTACCAACATTAGG-3'.

Mixing experiments. For mixing experiments, we grew the cells separately and mixed them before development on nitrocellulose filters. We developed cells as described¹⁰ with the following modifications: cells were washed with KK2 buffer (16.3 mM KH₂PO₄, 3.7 mM K₂HPO₄, pH 6.2), resuspended at a density of 1×10^8 cells ml⁻¹, and 5×10^7 cells were deposited per filter. We collected all the spores (after 36–48 h), and in the case of GFP-labelled strains, we counted them as described¹⁰. For all of the other mixes (except that between LAS1 and *rccA*), we plated out the spores clonally on SM-agar plates in association with *K. aerogenes*, transferred cells from individual plaques into HL5 containing 10 µg ml⁻¹ blasticidin S in 96-well plates, and scored for drug-resistance. For each mix, we counted spores from either one or two 96-well plates (96–192 spores). In the three-way mix experiment, we scored AX4-GFP spores based on fluorescence, and *rccA* spores based on blasticidin S resistance. For the mix between LAS1 and *rccA*, we grew cells in 96-well plates as above. We prepared genomic DNA from the cells in these wells by washing the cells once with water, lysing them by incubation in lysis buffer (67.7 mM Tris-HCl, pH 8.9, 16.6 mM (NH₄)₂SO₄, 2 mM MgCl₂, 10 mM dithiothreitol (DTT), 0.5% glycerol, 0.5% Tween 20, 0.5% NP40, 50 µg ml⁻¹ proteinase K) at 56 °C for 45 min, and then inactivating the proteinase K by incubating at 95 °C for 10 min. We tested for the presence of both the mutant LAS1 allele and the mutant *rccA* allele by PCR (LAS1 primers: 5'-TAATACGACTCACTATAGGG-3' and 5'-GTAGAAATTG TAACATTACAGG-3', and *rccA* primers: 5'-ATTTAGGTGACACTATAG-3' and 5'-CACTAATAACTGAAAATCAACTACC-3').