Experimental evolution of selfish policing in social bacteria

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Cooperative organisms evolve within socially diverse populations. In populations harboring both cooperators and cheaters, cooperators might adapt by evolving novel interactions with either social type or both. Diverse animal traits suppress selfish behaviors when cooperation is important for fitness, but the potential for prokaryotes to evolve such traits is unclear. We allowed a strain of the bacterium *Myxococcus xanthus* that is proficient at cooperative fruiting body development to evolve while repeatedly encountering a non-evolving developmental cheater. Evolving populations greatly increased their fitness in the presence of the cheater, both relative to their ancestor and in terms of absolute spore productivity. However, the same evolved lineages exhibited a net disadvantage to the ancestor in the cheater's absence. Evolving populations reversed a large ancestral disadvantage to the cheater into competitive superiority and also evolved to strongly suppress cheater productivity. Moreover, in three-party mixes with the cheater, evolved populations enhanced their ancestor's productivity relative to mixes of only the ancestor and cheater. Thus, our evolved populations function as selfish police that inhibit cheaters, both to their own advantage and to the benefit of others as well. Cheater suppression was general across multiple unfamiliar cheaters but was more pronounced against the evolutionarily familiar cheater. Also, evolution generated three new mutually beneficial relationships, including complementary defect rescue between evolved cells and the selection-regime cheater. The rapid evolution of cheater suppression documented here suggests that coevolving social strategies within natural populations of prokaryotes are more diverse and complex than previously appreciated.

cheater resistance | myxobacteria | social conflict | social evolution

Cooperative behaviors benefit individuals other than those who perform them and are prevalent in both animals and microbes (1), but cheaters that selfishly exploit cooperators are often favored by selection within social groups (2–4). In microbes, such cheaters are often defective at the trait they exploit and thereby impose a cheating load (4) that lowers group productivity when cheaters are frequent (2, 5). The persistence of high levels of cooperation among microbes therefore requires mechanisms that limit cheaters (1,6)—which can increase through mutation, within-group selection, and intergroup migration—and thereby allow cooperators, on average, to preferentially interact (1, 7). Beyond physical barriers to dispersal between groups and inherent frequency-dependent fitness disadvantages (2, 5, 8–11), the frequencies of microbial cheaters can be constrained by several additional forces. These potential forces include negative pleiotropic effects of social defection (6, 12), traits that promote between-group selection (6) by limiting migration [e.g., cell–cell adhesion and territorial kin discrimination (13, 14)], horizontal transfer of cooperative genes (15), and behaviors that specifically suppress resident cheaters (6, 16). The relative contributions of these various forces to limiting cheater frequencies in natural populations of social microbes remain unresolved.

Cooperative eukaryotes have evolved a wide range of behaviors that reduce the fitness of selfishly behaving individuals and thereby mitigate their negative effect on cooperator fitness (16–18). Little is known, however, about the adaptive pathways along which cooperative prokaryotes evolve in the presence of cheaters (19). In particular, the potential for bacteria to evolve the ability to suppress socially defective cheaters is unclear.

Some instances of prokaryotic cooperation are relatively simple and involve production of a single public good molecule, such as an iron-chelating siderophore (20). Other microbial traits are complex and require multiple cooperative acts governed by distinct genetic loci (21). For example, cells of the bacterium *Myxococcus xanthus* respond to starvation by using multiple intercellular signals to construct multicellular fruiting bodies within which a minority of cells differentiate into stress-resistant spores (22). In such complex systems, diverse cheater genotypes might evolve that compete with cooperators and with each other and might even exploit one another. Cooperators may also evolve to suppress cheating competitors. Interference competition, exploitation of both cooperators and distinct cheaters and cheater suppression may significantly shape the dynamics of cooperation and conflict in natural populations.

We allowed replicate populations of a cooperative, developmentally proficient *M. xanthus* genotype ("ANC" for ancestor) to evolve while repeatedly encountering the same developmental cheater ("CH" for cheater) during starvation, when fitness depends greatly on cooperation. Strain CH (aka LS523 and DK5208, refs. 8, 23) is an obligate defector from cooperation (4) that cheats due to a mutation that prevents production of an extracellular developmental signal (CsgA) and thereby causes a partial defect at spore production in clonal groups. Similar developmental cheaters can be generated by mutations in a variety of developmental genes and evolve readily in experimental populations (8, 24). Evolving populations alternated between development during starvation on agar plates and vegetative growth in liquid culture (Fig. 1) and were mixed at a 1:1 ratio with a fresh culture of CH at the onset of each developmental cycle. Growth of CH was inhibited during each phase of vegetative growth with a selective antibiotic (Fig. 1). After 20 cycles of development, we tested whether the evolved populations (EV1 to EV4) had adapted by increasing their intrinsic developmental fitness (i.e., increasing their absolute spore productivity in a manner unaffected by social environment) or by evolving novel social interactions with the cheater CH, their own cooperative ancestor ANC, or both.

Results

Evolving Lineages Adapted to the Experimental Regime. During the evolution experiment, spontaneous mutants competed directly with the ancestor (at least early in the experiment) and one another in the presence of the non-evolving cheater CH. Thus, evolved lines should outcompete their ancestor in three-party mixes with CH if adaptation occurred. Strain ANC* is

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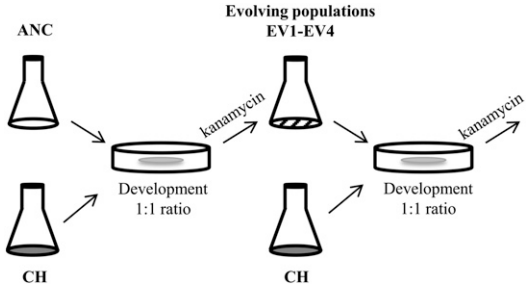

Fig. 1. Experimental evolution. Four founding populations of the cooperator ANC were mixed with the cheater CH at 1:1 ratios and subjected to 5 d of starvation-induced development. After heat selection, viable spores germinated in liquid medium that selectively allowed growth of cells descended from ANC, which were again mixed with a fresh culture of CH at the initiation of each round of development. Twenty cycles of development and intermittent growth were carried out.

the immediate parent of ANC but has a distinct antibiotic resistance phenotype. This distinct marker state allowed us to distinguish ANC* from both ANC and the ANC-derived populations EV1–EV4 in direct competition experiments. In three-party competition experiments with CH (EV:ANC*:CH) the evolved populations strongly outcompeted ANC* (Fig. 2*A*, Table 1, 12-fold average relative advantage, $P = 0.009$, one-sample t test), thus demonstrating that substantial adaptation took place.

Adapting lineages might have increased their relative fitness by (*i*) suppressing the productivity of ANC-derived competitors without increasing their own productivity or (*ii*) increasing their own productivity (i.e., reducing or eliminating the negative effect of CH on their ancestor ANC) to be greater than that of ANC-derived competitors regardless of their absolute effect on those competitors. The latter scenario occurred, as increases in relative fitness corresponded with extremely large evolved increases in productivity. (Productivity was measured as the number of spores produced per 5×10^8 cells entering development; Figs. 2 and 3.) ANC productivity in two-party mixes with CH was only ~0.66% of ANC productivity in pure culture (Table 2), whereas the average productivity of the four EV lines in pairwise mixes with CH was increased 24-fold to 17% of ANC productivity in pure culture (Figs. 2*B* and 3*A*; $P = 0.002$ for difference between EV and ANC in pairwise mixes with CH, paired t test). In three-party EV:ANC*:CH mixes, evolved productivity was higher than that of ANC in pure culture ($P = 0.002$, one-sample t test), whereas ANC productivity in ANC:ANC*:CH mixes is much lower by comparison (Figs. 2*A* and 3*A*).

Evolved Populations Outcompete and Suppress the Environmental Cheater CH. Adaptation by evolved populations was associated with altered fitness and social relationships between the evolving lineages and CH. CH exhibited 251-fold and 380-fold advantages over ANC in three- and two-party competitions, respectively

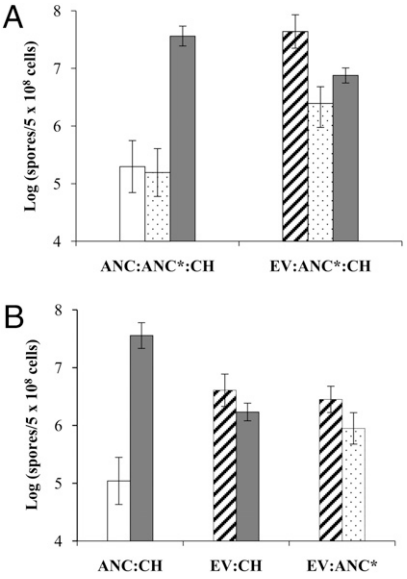

Fig. 2. Spore production in three-party and two-party competitions. (*A*) ANC:ANC*:CH (white:speckled:gray) and EV:ANC*:CH (hatched:speckled:gray) three-party mixes at 1:1:2 ratios. (*B*) Two-party mixes ANC:CH (white:gray), EV:CH (hatched:gray) and EV:ANC* (hatched:speckled) at 1:1 ratios. Bars show the sporulation efficiency of each strain as the number of spores produced per 5×10^8 cells present at the onset of development to allow direct comparison with pure culture values. Error bars indicate 95% confidence intervals.

(Fig. 2 and Table 2; $P = 0.001$ and $P < 0.001$, respectively, one-sample t tests). In stark contrast, the evolved populations exhibited 5-fold and 2-fold average advantages over CH in three- and two-party competitions, respectively (Fig. 2 and Tables 1 and 2; $P = 0.004$ and 0.057, respectively, one-sample t tests). Although the average EV advantage over CH in two-party mixes was not quite significant, the individual advantages of two specific populations (EV3 and EV4) were significant ($P = 0.03$ and 0.021, respectively, one-sample t tests). Thus, evolved lineages reversed the ancestral condition of severe inferiority to CH into competitive superiority over this cheater in both three-party (Fig. 2*A*) and two-party (Fig. 2*B*) competitions (Tables 1 and 2). In other words, populations evolved immunity to cheating by CH.

Evolved populations accomplished this reversal of fitness ranks with the cheater not only by increasing their own productivity but also by suppressing CH productivity. In three-party EV:ANC*:CH competitions, CH productivity was only ~27% of CH productivity in ANC:ANC*:CH competitions (Fig. 2*A*, $P < 0.001$, paired t test). Also, CH productivity in two-party mixes with evolved populations was only ~5% of CH productivity in two-party mixes with ANC (Fig. 2*B*, $P = 0.003$, paired t test).

Table 1. Relative fitness values and interactant effects in EV:ANC*:CH three-party competitions

	Relative productivity	*P* value	Absolute effect of:	Compared with:	Sign	Effect factor	*P* value
EV/ANC*	12	**	EV on ANC*	ANC* in ANC:ANC*:CH mix	+	47	**
			ANC* on EV	EV in EV:CH mixes	+	16	***
EV/CH	5.5	**	EV on CH	CH in ANC:ANC*:CH mix	–	3.7	***
			CH on EV	EV in EV:ANC* mixes	+	19	***
ANC*/CH	0.6	NS	ANC* on CH	CH in EV:CH mixes	+	8.7	*
			CH on ANC*	ANC* in EV:ANC* mixes	+	14	**

Comparisons involving evolved populations reflect averages across the four populations. Asterisks indicate significant deviation from 1. NS > 0.05, *$P \le 0.05$, **$P \le 0.01$, ***$P \le 0.001$.

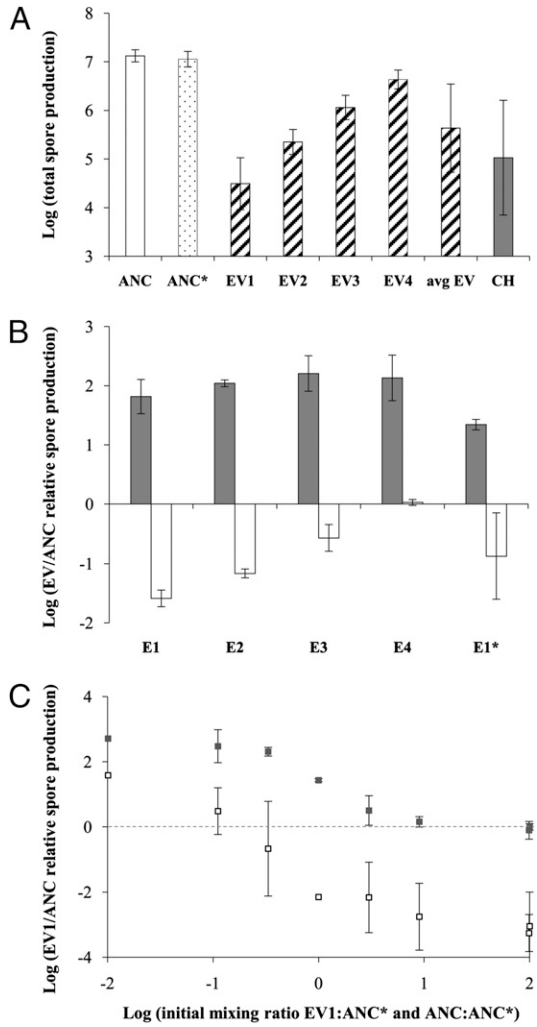

Fig. 3. Evolved performance with vs. without the cheater CH. (*A*) Pure culture spore productivity. avg EV is the average of the four evolved populations. (*B*) Spore productivity ($\log_{10}$ transformed) of evolved populations relative to that of their ancestor ANC across 1:0 and 1:1 EV:ANC* and ANC:ANC* mixing ratios combined both in the presence (gray) and absence (white) of CH. Positive and negative values indicate superiority and inferiority, respectively, of the evolved populations relative to their ancestor. EV1/ANC relative spore production summed across a greater number of EV1:ANC* and ANC:ANC* ratios is also shown (EV1*). (*C*) Spore productivity ($\log_{10}$ transformed) of EV1 relative to ANC at several EV1:ANC* and ANC:ANC* mixing ratios both in the presence (closed squares) and absence (open squares) of CH. EV1/ANC relative spore production at 1:0 EV:ANC* and ANC:ANC* ratios is shown by data points at the right end of the *x* axis. These data points are slightly elevated relative to the respective data points for 99:1 EV:ANC* and ANC:ANC* mixes. Error bars indicate 95% confidence intervals.

We also found that evolved populations exploit CH for an absolute gain in productivity. EV productivity is enhanced by the presence of CH relative to pure culture (Figs. 2*B* and 3*A* and Table 2; $P = 0.014$, one-sample t test). Thus, not only did the fitness ranks of the evolved lineages and the cheater reverse after adaptive evolution, but so did the direction of social exploitation.

Evolved Populations Benefit Their Cooperative Ancestor When the Cheater Is Present. Intriguingly, evolved populations benefit their cooperative ancestor in three-party mixes relative to the ancestor's productivity in mixes of ANC and ANC* with CH. The average productivity of the parent ancestor ANC* in EV:ANC*:CH three-party mixes was 47-fold higher than in ANC:ANC*:CH mixes (Fig. 2*A* and Table 1, $P = 0.002$, paired t test). The presence of evolved strains also increased the whole-population frequency of ANC* spores ~40-fold from 0.002 in the ANC:ANC*:CH competition to 0.09 in the EV:ANC*:CH competitions ($P = 0.003$ for difference in ANC* frequencies, paired t test). Thus, in these social groups, the evolved populations benefit a third party of cooperators when the cheater is present. Total group productivity did not differ between evolved vs. ancestral three-party mixes ($P = 0.838$, paired t test).

Evolved Advantages Specific to Cheater Presence. For each evolved population (EV), we compared its spore productivity to that of the ancestor ANC across two distinct metapopulations that differed in whether the cheater CH was absent or present within each treatment of developing cells (Fig. 3*B*). There were four treatments within each metapopulation. The metapopulation without CH consisted of two clonal treatments (EV and ANC) and two pairwise mix treatments (EV:ANC* and ANC:ANC* at 1:1 ratios). The metapopulation with CH consisted of two pairwise mix treatments (EV:CH and ANC:CH at 1:1 ratios) and two three-party treatments (EV:ANC*:CH and ANC:ANC*:CH at 1:1:2 ratios). Total initial cell number (5×10^8) was constant across all treatments. On average, the evolved populations showed a large net advantage over ANC in the presence of the cheater CH but a disadvantage in its absence (Figs. 2 and 3). Summed across all treatments in the metapopulation without CH, evolved cells produced fewer spores on average than did ANC cells (Fig. 3*B*, $P = 0.007$, one-sample t test). In contrast, the evolved lines strongly outperformed ANC in the presence of CH (Fig. 3*B*, $P < 0.001$, one-sample t test).

We used a similar approach to examine the productivity of evolved line EV1 relative to that of ANC across a broader range and finer gradation of EV1:ANC* and ANC:ANC* ratios (1:99, 1:9, 1:3, 1:1, 3:1, 9:1, 99:1, and 1:0) both in the absence and presence of CH (Fig. 3 *B* and *C*). In the absence of CH, ANC was more productive than EV1 at most ANC* frequencies, with an ~10-fold overall advantage across all ANC* frequencies (Fig. 3*B* and 3*C*). In contrast, EV1 productivity in the presence of CH was at least equal to, and in most cases greater than, that of ANC at each ANC* frequency, with EV1 showing an ~22-fold overall advantage over ANC across all ANC* frequencies (Fig. 3 *B* and *C*). The difference between relative EV1/ANC productivity across the metapopulations with vs. without CH was significant (Fig. 3*B*, $P = 0.046$, one-tailed paired t test, $n = 2$).

Evolved Populations Cheat On Their Ancestor. Interestingly, populations EV1 and EV4 both evolved the capacity to not only suppress exploitation by the cheater CH but also to cheat on their own ancestor. EV1 and EV4 both lose to ANC and ANC* in pure culture comparisons (Fig. 3*A*). However, EV1 outcompetes both ANC (indirectly across groups, Fig. 3*C*) and ANC* (directly within groups) at low EV1 frequencies in mixes with ANC* (e.g., EV1 makes 74% of spores when initially mixed at 25%, $P = 0.035$ one sample t test for difference between final and initial percentage). Also, EV4 was the only individual evolved population to perform significantly better than ANC* in 1:1 pairwise mixes (sevenfold difference, $P = 0.037$, one-sample t test). EV4 performed better in 1:1 mixes with ANC* than alone in pure culture ($P = 0.03$, one-sample t test).

New Mutually Beneficial Relationships Evolved. Our competition experiments reveal the evolution of new mutually beneficial interactions between conspecifics. First, evolved populations and their ancestor increase one another's productivity in three-party mixes with the cheater CH (Fig. 2*A* and Table 1) relative to their respective productivity in two-party mixes with CH (Fig. 2*B*, $P < 0.001$ and 0.002 for EV and ANC*, respectively, one-sample t tests). The interaction between evolved populations and their

Table 2. Relative fitness values and interactant effects in two-party competitions

	Relative productivity	*P* value	Absolute effect of:	Effect sign	Effect factor	*P* value
EV/ANC*	4.7	**	EV on ANC*	–	44	**
			ANC* on EV	+	24	**
EV/CH	2.3	NQS	EV on CH	+	260	NS
			CH on EV	+	16	*
ANC/CH	0.003	***	ANC on CH	+	1500	*
			CH on ANC	–	152	***

Comparisons involving evolved populations reflect averages across the four populations. Absolute effect values are from comparison with pure culture productivity. Asterisks indicate significant deviation from 1. NS: $P = 0.126$, NQS (not quite significant): $P = 0.057$, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

ancestor is mutually beneficial only in the presence of the cheater, as it is not observed in pairwise mixes of these parties relative to pure culture productivity (Figs. 2*B* and 3*A* and Table 2).

Second, the presence of evolved populations in three-party mixes generates a new mutually beneficial relationship between ANC* and CH relative to their respective productivity in pairwise mixes with evolved populations (Fig. 2 and Table 1). ANC* and CH spore production are 14- and 9-fold higher in three-party mixes than in two-party mixes with the evolved strains, respectively ($P = 0.003$ and 0.016, respectively).

Finally, our data also reveal the evolution of mutual defect rescue between the evolved populations and CH in two-party mixes (Figs. 2*B* and 3*A* and Table 2). The evolved populations performed significantly better in the presence of CH than alone (16-fold average effect, $P = 0.014$, one-sample *t* test). Reciprocally, all four estimates of the effect of evolved populations on CH were positive (ranging from 2- to 467-fold). Although the average effect (~260-fold) was not significant ($P = 0.124$, one-sample *t* test), CH significantly benefited from being mixed with the individual populations EV3 and EV4 ($P = 0.04$ for EV3 and 0.02 for EV4 from multiple comparisons on CH productivity after ANOVA). Correspondingly, populations EV3 and EV4 each benefited from pairwise mixing with CH ($P = 0.024$ and $P = 0.022$, respectively, one-sample *t* tests).

Evolved Populations Are Resistant to Several Cheater Genotypes. Evolved cheater suppression was found to be a qualitatively general trait that is effective against unfamiliar cheaters as well as against CH. The evolved populations suppressed two other cheaters that strongly exploit the ancestor. These two cheaters, GJV9 and GVB206.3, produced 84 and 90% of all spores in 1:1 pairwise mixtures with the ancestor ANC, respectively, with both values being significantly greater than the null expectation of 50% ($P = 0.005$ and 0.0001, respectively, one-sample *t* tests, $n = 5$). In contrast, these cheaters respectively accounted for only 39 and 49% of spores produced in 1:1 mixes with the evolved populations, with the fitness values for both strains being significantly reduced relative to their performance in mixes with the ancestor ($P = 0.001$ for both strains, paired *t* tests, $n = 5$). This result suggests that natural populations of bacteria may evolve modes of resistance that are effective against multiple distinct cheater genotypes. Despite the qualitative generality of cheater suppression, the average fitness increase of the evolved lines relative to evolutionarily familiar cheater CH (~700-fold, Fig. 4) was significantly greater than their improvement relative to the unfamiliar cheaters (Fig. 4, 20-fold and 26-fold relative to GJV9 and 206.3, respectively, $P < 0.001$ in post hoc multiple comparison test for differences in the degree of evolved increase against CH vs. against GJV9 and GVB206.3 after ANOVA ($F = 24.93$, df = 2, $P < 0.001$) to test for any difference in the degree of improvement against the three cheaters).

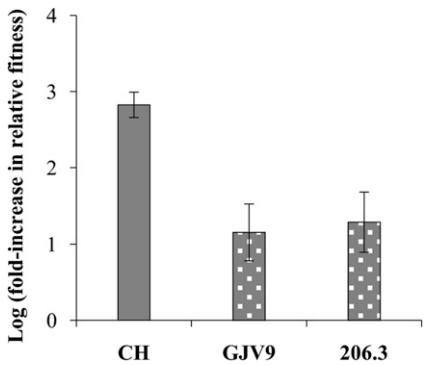

Fig. 4. Fitness improvements against the familiar cheater vs. unfamiliar cheaters. Evolved populations showed greater fitness improvement against the evolutionarily familiar cheater CH than against two unfamiliar cheaters (GJV9 and GVB206.3). Error bars indicate 95% confidence intervals.

Discussion

In this study, multiple lineages of cooperative bacteria rapidly evolved to outcompete their ancestor in groups harboring a non-evolving social cheater. This fitness advantage of evolved populations corresponded with strong suppression of the environmental cheater and was specific to the presence of that cheater.

Cheater suppression might be accomplished by a single mechanism or multiple mechanisms. Regardless of which evolved trait, or suite of traits, suppresses cheater spore production, it is uniquely detrimental to the cheater CH. This follows from the observation that sporulation by the ancestor ANC* in three-party EV:ANC*:CH mixes is elevated relative to ANC* in ANC:ANC*:CH mixes (Fig. 2*A* and Table 1). Thus, it is clear that cheaters are not suppressed by an indiscriminate anticompetitor trait.

First, evolved strains may actively produce a compound that hinders cheater sporulation. The compound might be evolutionarily altered in its composition in a manner uniquely harmful to a step in the cheater sporulation pathway. Alternatively, the compound might be ancestral in its composition but produced in greater amounts that are toxic to cheater sporulation but not to the ancestor. If not a developmental signal, the cheater-harming compound might be a secondary metabolite or peptide that serves as a predatory or anticompetitor compound in the ancestor.

Second, cheater suppression might be accomplished by withholding (partially or completely) a secreted compound required for normal development in the ancestor if the cheater is uniquely sensitive to the reduced signal level. For example, if an evolved mutation causes a slight reduction (or delay) in the production of a signal, CH could be highly sensitive to that reduction, whereas ANC* is unaffected. Such a withholding scenario (for a signal other than CsgA) could potentially explain several of our results, including the partial social defects of evolved lines, cheater suppression, the ability of some evolved lines to exploit their ancestor at some frequencies, and mutual defect rescue between CH and evolved cells. However, preliminary data suggest that

cheater suppression may have evolved before the origin of developmental defects in at least some evolved lines, suggesting a more active mode of cheater suppression.

Finally, the cheater might be suppressed by evolved changes in the temporal dynamics of developmental signal production and/or cell motility patterns to which CH, but not ANC*, responds dysfunctionally. Further experiments are required to test among these possible modes of cheater suppression. In all scenarios, elevated sporulation by the ancestor in three-party EV:ANC*:CH mixes compared with ANC:ANC*:CH mixes is presumably an indirect byproduct of cheater suppression.

The evolved lines are more productive in the presence of CH than in its absence (Figs. 2 and 3). This result implies that the partial defects of evolved populations in pure culture are mechanistically distinct from CH's defect at producing CsgA. Thus, social defects unique to interacting parties are partially rescued by the other party in mixed groups and a cooperator/cheater relationship evolved into a mutually beneficial relationship (at least in two individual populations). In this experimental system, one party's defect (the *csgA* mutation) was introduced experimentally whereas the evolved defects arose spontaneously. Such mutually beneficial relationships of complementary defect rescue might also evolve in natural populations through degradation of distinct social functions across interacting lineages that are cotransmitted within social groups across generations.

A consensus definition of policing has yet to emerge in the evolutionary literature (25–30). The term was initially extended beyond human behavior to describe suppression of worker-insect reproduction by fellow workers (25) but has since been applied to diverse empirical and theoretical scenarios (17, 18, 26–30), including instances in which the policing trait itself appears to be selfish rather than altruistic (29, 30). A common feature of traits labeled as policing is that they reduce the expression of—or benefit derived from—a cheating trait and thereby benefit policing alleles specifically when cheating individuals are present. The benefit derived from policing might be direct (i.e., selfish) and increase the individual fitness of policing organisms themselves (29, 30). Alternatively, policing may be altruistic and come at an individual fitness cost but nonetheless be maintained by a net positive effect on inclusive fitness (26). By harming cheaters, policing individuals can increase the fitness of nonpolicing cooperators and/or increase group productivity (27, 28).

Here we adopt a mechanism-independent definition of policing as a trait that eliminates a cheating advantage and confers a fitness advantage (directly or indirectly) to individuals with that trait that is specific to the presence of cheaters. Our evolved populations conform to this definition, as they (*i*) outcompete the cheater in pairwise competitions and (*ii*) have an overall advantage over their ancestor specifically in the presence of the cheater. Moreover, the presence of evolved strains actually elevates cooperator productivity in three-party groups compared with two-party groups of only the cooperator and cheater. Thus, our results conform to a scenario of selfish policing in which cheater suppression benefits both evolved populations and a third party of cooperators.

Cooperators encountering a new social defector might already be preresistant to exploitation by that defector, might be driven to extinction by it (2), or might evolve a new social strategy immune to exploitation (31, 32). Such cheater immunity will be inherently stable if it comes at no cost to social productivity. Previous studies identified a spontaneous mutant of *M. xanthus* and transposon mutants of *Dictyostelium discoideum* that resist being outcompeted by a socially defective cheater and a socially proficient competitor, respectively, at no cost to social productivity in clonal groups (16, 32).

In contrast, in this study we found that cheater suppression evolved spontaneously in association with partial social defects (Fig. 3*A*), although a causal link between these derived traits has not been established. The derived social defects are largely responsible for the net disadvantage of evolved populations to their ancestor in the absence of the cheater (Fig. 3*B*). Such an overall disadvantage suggests that cheater extinction events would lead to loss of evolved cheater suppressors as well. However, analysis of population EV1 revealed that it has an advantage over the ancestor when EV1 is rare. In other words, population EV1 is simultaneously a suppressor of CH and itself a frequency-dependent cheater that exploits its own ancestor. Such an advantage when rare over cooperators will prevent socially defective cheater suppressors from being driven to local extinction by within-group selection and thus prolong their opportunity to further adapt to their social environment.

Suppression of an obligately defecting cheater evolved rapidly in our experimental populations. In nature, evolution within cheater-infected lineage groups may proceed faster than the rate at which cheaters are passively lost from a population due to kin selection operating at the group level (33). Thus, local cheater frequencies might often be shaped by complex coevolutionary arms races in which diverse cooperators, cheaters, and policers cycle through phases of exploitation and resistance among socially cotransmitted lineages (19, 34). Our results suggest that such cotransmitted lineages might even evolve distinct signaling defects that are mutually complemented when the defective strains interact. Such diverse social strategies may be common in microorganisms (e.g., ref. 19) and should be increasingly incorporated into formal models of microbial social evolution (21).

It has been proposed that resistance to cheaters that defect from "fair" production of a public good may be difficult to achieve without converting the cheaters back into producers (16, 35). However, our experiments show that resistance to—and quantitative suppression of—microbial cheaters that defect from cooperative production of a social compound can readily evolve. In human pathogens, evolution of resistance to such cheaters would pose difficulties for the proposed clinical use of cheater genotypes to subvert virulent infections (35).

Materials and Methods

Strains. Strain ANC* (also known as "GJV2," or strain "R" in ref. 24), is a developmentally proficient, rifampin-resistant derivative of *M. xanthus* strain GJV1 (strain "S" in ref. 24, a derivative of strain DK1622). Strain ANC is GJV27, a kanamycin- and rifampin-resistant derivative of ANC* (GJV2) generated by integration of the plasmid pDW79 (36) and is also developmentally proficient (i.e., shows no sporulation defect relative to ANC*). Strain CH is DK5208, a yellow clonal isolate of LS523, which bears a Tn5 disruption of the *csgA* gene and is resistant to oxytetracycline (37). DK5208 exhibits low spore production in pure developmental cultures but shows a cheating phenotype in mixed cultures with the wild-type GJV1 (8) and was used as the non-evolving cheater in the evolutionary regime. DK5208 was the *csgA* mutant used in Velicer et al. (8) but it was referred to as LS523 as the strains were considered equivalent.

To test whether evolved cheater suppression was specific to CH, evolved strains were mixed with cheaters that they had never encountered: GVB206.3 (24) (rifampicin resistant) and GJV9 (this study), a spontaneous rifampicin-resistant mutant of GVB207.3 (24). The cheating phenotypes GVB206.3 and GVB207.3 have been previously demonstrated in Velicer et al. (8) for GVB206.3 and Fiegna and Velicer (2) for an alternative marked variant of GVB207.3.

Experimental Evolution. Four clones of ANC were used to establish four independently evolving lineages. After growth to mid-log phase, development was initiated as described previously (2). Briefly, ANC and CH grown in CTT liquid culture were each resuspended in a starvation medium to a density of 5×10^9 cells/mL. They were then mixed at a 1:1 ratio and mixed cultures were dispensed onto buffered TPM agar plates in 10×100 μL aliquots to induce development. Plates were incubated at 32 °C, 90% rH for 5 d, after which cultures were harvested in ddH_2O and heated at 50 °C for 2 h to select for viable spores. Spores were then grown in liquid medium with kanamycin (40 μg/mL), which allowed spores descended from the ancestor ANC to germinate and grow but which inhibited growth of CH spores. After evolving cultures reached adequate density, they were again mixed at a 1:1 ratio with a fresh culture of CH and spotted onto buffered agar plates to undergo the second cycle of development (Fig. 1). This procedure was re-

peated weekly for a total of 20 developmental cycles at the Max-Planck Institute for Developmental Biology in Tübingen, Germany in 2002. This design is similar to that adopted by Khare et al. (16) to select for transposon mutants of *D. discoidium* resistant to being outcompeted by a socially proficient competitor.

Sporulation Assays. Spore production by the four evolved populations was compared with that of the ancestor ANC in clonal development, in 1:1 pairwise mixes with ANC*, CH, GVB206.3, and GJV9 and in three-party mixes with both ANC* and CH (1:1:2 ratio). Population EV1 and ANC were also mixed with ANC* at 1:99, 1:9, 1:3, 1:1, 3:1, 9:1, and 99:1 ratios in both the absence and presence of CH. In the absence of CH, either EV1 and ANC* together or ANC and ANC* together constituted the entire competition population in these mixes. In the three-party mixes with CH, either EV1 and ANC* together or ANC and ANC* together constituted 50% of competition population and CH the other 50%.

Development was initiated and cultures were harvested as described above except that strains were mixed at appropriate ratios or remained unmixed for pure culture assays, and 100-μL aliquots of each culture (5×10^9 cells/mL) were spotted onto the buffered agar plates. Thus, for each development assay the total number of cells present at the onset of starvation was 5×10^8 and only genotype ratios were altered across treatments. After heat treatment, cultures were sonicated and diluted into CTT soft agar (0.5% agar) with the appropriate antibiotic treatments (40 μg/mL kanamycin, 5 μg/mL rifampicin, or no antibiotic). Spore production was estimated either directly from colony counts in agar medium with an appropriate antibiotic or from the difference between counts in nonselective vs. selective medium for strains sensitive to an antibiotic used to select a competitor. Experimental replicates represent temporally independent assays.

For all statistical tests involving an average of the four evolved populations, evolved values were averaged and compared with the respective ancestral value within each replicate block, with those comparisons serving as independent values that were then averaged across four temporally independent replicate blocks. $n = 4$ unless specified otherwise. Normality and homogeneity of variances, when applicable, were verified before using parametric tests. Statistical analyses were performed on $\log_{10}$-transformed data with Minitab 15.

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