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Developmental cheating in the social bacterium *Myxococcus xanthus*

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Cheating is a potential problem in any social system that depends on cooperation and in which actions that benefit a group are costly to individuals that perform them[1–5]. Genetic mutants that fail to perform a group-beneficial function but that reap the benefits of belonging to the group should have a within-group selective advantage, provided that the mutants are not too common. Here we show that social cheating exists even among prokaryotes. The bacterium *Myxococcus xanthus* exhibits several social behaviours, including aggregation of cells into spore-producing fruiting bodies during starvation. We examined a number of *M. xanthus* genotypes that were defective for fruiting-body development, including several lines that evolved for 1,000 generations under asocial conditions[6] and others carrying defined mutations in developmental pathways[7–10], to determine whether they behaved as cheaters when mixed with their developmentally proficient progenitor. Clones from several evolved lines and two defined mutants exhibited cheating during development, being over-represented among resulting spores relative to their initial frequency in the mixture. The ease of finding anti-social behaviours suggests that cheaters may be common in natural populations of *M. xanthus*.

The myxobacteria are soil-dwelling prokaryotes that exhibit a social life cycle similar to that of the eukaryotic slime mould *Dictyostelium discoideum*[11,12]. Like the slime moulds, myxobacteria undergo multicellular development in response to starvation that yields fruiting bodies of remarkable morphological variety[13]. This process involves multiple intercellular signals specific to distinct stages of development, and it results in a minority of the original population becoming stress-resistant spores that germinate under favourable conditions[14]. Myxobacteria also exhibit social motility and social predation, swarming as a 'wolf pack' toward prey, which they kill and degrade by the secretion of extracellular compounds[15].

A developmentally defective mutant of *M. xanthus*, when mixed with a developmentally proficient wild type, has five possible fates during development. First, the defective strain may sporulate with the same efficiency as it does in pure culture. This outcome corresponds to null hypothesis H_1 here. Second, a partially defective genotype's sporulation in the presence of wild type may be inhibited even below its efficiency in pure culture. The third and fourth potential fates are partial and complete rescue (relative to wild type), respectively, of the defective genotype by extracellular complementation in the presence of wild type. Complete rescue to the wild-type sporulation efficiency corresponds to null hypothesis H_2 here. Fifth, a developmentally defective genotype may produce more spores in the presence of wild type than would a neutrally marked wild type introduced at the same initial frequency. This last outcome constitutes evolutionary cheating, because the defective mutant obtains disproportionate reproductive success.

We first compared the developmental performance, in pure culture, of six experimentally evolved clones with that of their wild-type ancestor DK1622 (ref. 16). In pure culture, all six clones, to varying degrees, showed defects in spore production relative to DK1622 (Fig. 1). Thus, these evolved clones were all defective for this group function. We then measured spore production of these evolved defective clones when they were each mixed with their ancestor at an initial frequency of 0.01. The performance of each minority genotype was then contrasted with the two distinct hypothetical outcomes, H_1 and H_2 (Table 1). For five out of the six

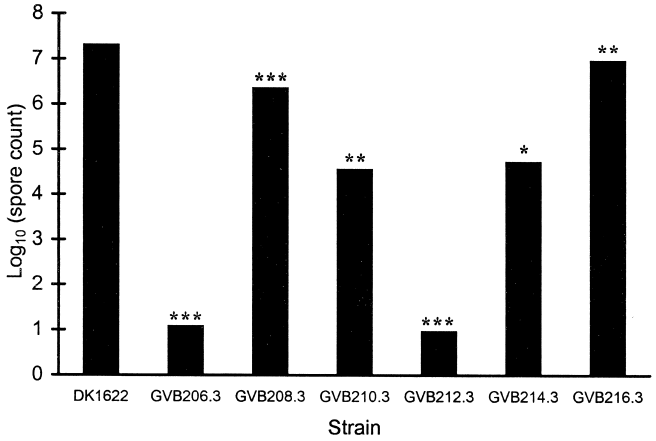

Figure 1 Spore production for six independently evolved clones[6] of *M. xanthus* and their common ancestor (DK1622). Asterisks indicate significant defects in an evolved clone's spore production relative to that of DK1622, calculated using Welch's approximate *t*-test which does not assume homogeneity of variance. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, all one-tailed.

Table 1 Observed spore production of evolved clones when mixed at 1% with the wild-type ancestor compared with production expected under two hypotheses

Evolved clone	Observed	H_1 expected	Observed − H_1 expected	Observed − H_2 expected
GVB206.3	–0.31	–7.30	+6.99***	+1.69***
GVB208.3	–0.98	–2.96	+1.98***	+1.02***
GVB210.3	–3.32	–4.75	+1.43**	–1.32**
GVB212.3	–3.27	–7.30	+4.03**	–1.27*
GVB214.3	–0.93	–4.59	+3.66***	+1.07**
GVB216.3	–3.49	–2.35	–1.14***	–1.49***

All values are $\log_{10}$-transformed; an observed value of –3 thus indicates that an evolved clone produced 0.1% of the pure-culture wild-type spore count. According to H_1, a clone produces spores with the same efficiency when mixed with the ancestor as it does when alone. For example, a pure culture of GVB208.3 produces ~10% as many spores as does a pure culture of the wild type (Fig. 1). If GVB208.3 makes spores with the same 10% relative efficiency when mixed with its ancestor at a frequency of 1%, then it will produce 0.1% of the total spores. Expected values under H_1 vary among the evolved clones; a $\log_{10}$-transformed value of –7.30 represents the limit of detection. A positive difference between the observed spore production of an evolved clone and its expectation under H_1 indicates at least partial complementation of the evolved clone by the wild-type. According to H_2, an evolved clone behaves as would a neutrally marked variant of the wild-type; that is, it produces 1% of the total spores when mixed at 1% with the wild-type ancestor. The expected value under H_2 is thus –2 ($\log_{10}$ 0.01) for all clones. A positive difference between an evolved clone's observed spore production and the uniform expectation under H_2 demonstrates developmental cheating by the evolved clone. Asterisks denote that contrasts are significantly different from zero based on *t*-tests: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, all two-tailed.

defective clones, spore production was higher than predicted under H_1, thus demonstrating at least partial complementation of their developmental defects. All five of these clones were rescued more than 10-fold above the expectation under H_1, and in three cases the effect was greater than 1,000-fold. Sporulation of the sixth clone (GVB216.3) was hindered by the wild-type majority.

Out of the five clones exceeding the sporulation expected under H_1, two showed partial complementation, because they performed worse than expected under H_2; however, three clones exhibited cheating, as they performed significantly better than expected even under H_2 (Table 1). Two of these cheaters (GVB208.3 and GVB214.3) showed moderate sporulation efficiencies as pure genotypes, but they had an advantage over wild type when they were rare. Clone GVB206.3 showed the most marked cheating: it was almost completely defective at sporulation in pure culture, but as a 1% minority it produced ~50-fold more spores than would a neutrally marked wild type.

To investigate more closely the frequency-dependent behaviour of these interactions, we measured the sporulation efficiencies of two evolved cheater clones (GVB206.3 and GVB208.3) in mixes with their wild-type progenitor at nine different initial frequencies (Fig. 2). Both clones cheated by performing better than expected for a neutrally marked wild type (H_2) over a wide range of initial frequencies (Fig. 2a, b). Notably, as the initial frequency of each cheater genotype increased, the total spore production of the mixed culture fell below that of the pure wild type; this result indicates that cheaters indeed harm their group's performance. Both cheaters sporulated much more efficiently than wild type at low initial frequencies, but their efficiencies dropped below the wild-type efficiency at high initial ratios (Fig. 2c, d). The relative sporulation efficiency dropped below 1 at a lower initial frequency for GVB206.3 than for GVB208.3.

The evolved defective clones may have several mutations that distinguish them from their wild-type ancestor[6]. It is therefore unclear whether the cheating phenotype can arise by a single mutation or whether it requires multiple mutations. To test whether a single mutation can produce cheating behaviour, we examined three genotypes that differ from their wild-type progenitor by a defined mutation. The production of *M. xanthus* fruiting bodies involves several extracellular signals that are expressed at specific stages of development[17,18], and the defined mutants that we examined are defective in production of signal molecules. Sporulation of these mutants is defective (at least partially) in pure culture, but can be rescued (at least partially) by extracellular complementation when mutants are mixed at a 1:1 ratio with wild type. Our reanalysis of published data[9,10,19] from such mixes suggested that some mutants may even exhibit cheating behaviour. Therefore, we mixed a defined mutant that is defective in the production of C-signal with its wild-type progenitor at 1:1 and 1:99 ratios, and we carried out corresponding experiments with two different mutants defective in A-signal production.

One of the A-signal mutants (MS2021)[10], which has a mutation in the *asgE* gene, showed partial complementation but no cheating (data not shown); that is, it fell between the expectations under H_1 and H_2. The C-signal mutant (LS523)[8]and the other A-signal mutant (DK4312)[7], which has a mutation in the *asgB* gene, both showed strong cheating behaviour when mixed with their respective wild-type progenitors. DK4312 was severely defective for sporulation in pure culture, but performed much better than expected for a neutrally marked wild type (H_2) at an initial frequency of 0.01 (Fig. 3a). LS523, which also sporulated poorly in pure culture, showed a similar degree of cheating at an initial frequency of 0.01 (Fig. 3b). In 1:1 mixes, both mutants dominated the wild type and the total spore counts fell below wild-type levels, with LS523 having a greater negative effect on total spore yield than DK4312 (data not shown). These defined mutants failed to contribute normal amounts of a particular developmental signal to the group, yet they performed better than wild type when rare. Therefore, even single mutations can produce cheating behaviour in *M. xanthus*.

Our experiments with both the evolved clones and defined mutants indicate that developmental cheaters are readily obtained in *M. xanthus*. Cheater genotypes presumably also appear in nature. (Although cheaters have not been studied in natural populations of *M. xanthus*, developmental cheating has been reported in a natural isolate of the eukaryotic slime mould *Dictyostelium mucoroides*[20].) Once present, cheaters have the potential to invade social groups and persist indefinitely as a parasitic subpopulation. Our results therefore suggest that cheaters of myxobacteria are common in

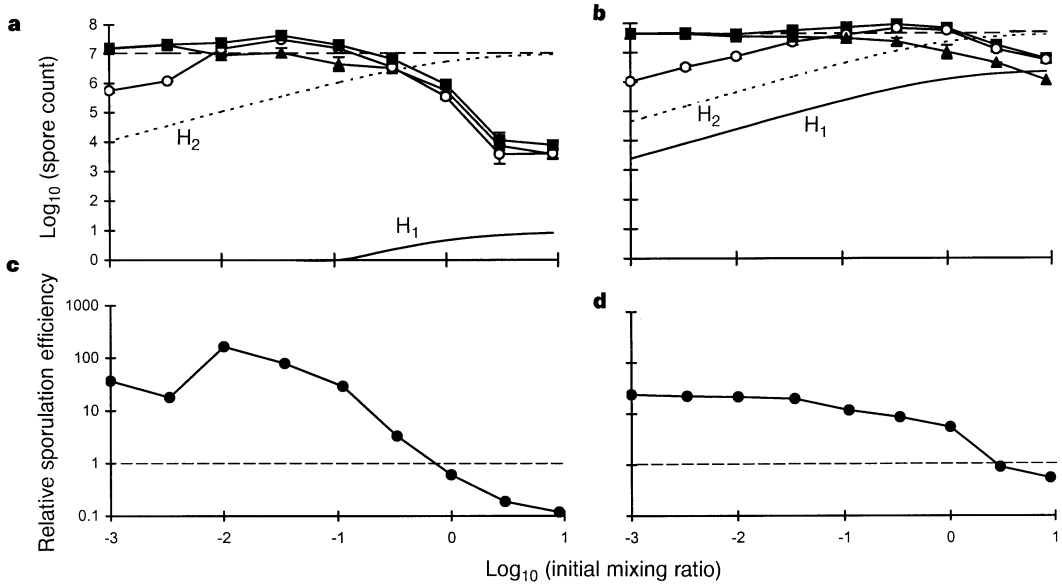

Figure 2 Spore production of two evolved clones when mixed with their wild-type progenitor at nine initial ratios, and the corresponding relative sporulation efficiencies. **a**, Mixtures of GVB206.3 with DK1622. **b**, Mixtures of GVB208.3 with DK1622. Squares, triangles and circles indicate total, DK1622 and evolved clones, respectively. The expected production of the evolved clones under H_1 (solid lines), the expected production of evolved clones under H_2 (dotted lines) and the spore production of DK1622 in independent pure cultures (dashed lines) are shown. Error bars indicate 95% confidence intervals. **c**,**d**, Sporulation efficiencies of GVB206.3 (**c**) and GVB208.3 (**d**), relative to that of DK1622 for these same initial mixing ratios. Dashed lines indicate a relative efficiency of 1.

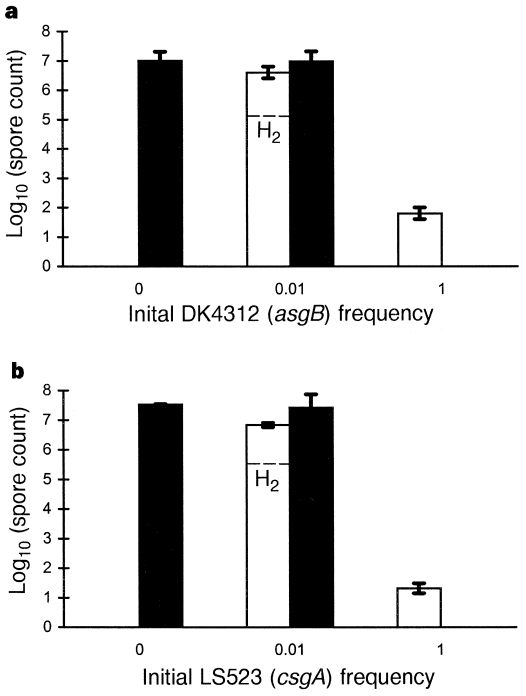

Figure 3 Spore production of mutants DK4312 (*asgB*) (**a**) and LS523 (*csgA*) (**b**), and their respective wild-type progenitors in pure and mixed culture. Dashed lines show the expected production of each mutant as a 1% minority under hypothesis H_2, a value above which indicates developmental cheating. Error bars indicate 95% confidence intervals.

nature, unless these bacteria have evolved strategies that oppose cheating. Such strategies may include reproductive cycles that are unfavourable to cheaters and policing functions that repress competition[21–27].

In principle, the myxobacterial reproductive cycle could involve the founding of new colonies by either single or multiple spores. With single-spore colonization, the outcome of competition between cheaters and cooperators will depend on the performance of genetically homogeneous groups (assuming no migration or appearance of new mutants), a situation in which the developmentally defective cheaters are inferior. Alternatively, if colonies are founded by clumps of many spores, competition will occur within fruiting bodies, a situation in which cheaters are superior when rare and will therefore persist as a parasitic subpopulation. Two features of the myxobacteria suggest they do not exclude cheaters by single-spore dispersal. First, *M. xanthus* spores adhere tightly to one another and require vigorous sonication to separate them in the laboratory. Thus, new colonies in nature are probably founded by multiple clumped spores. Second, myxobacteria are highly motile, promoting migration that opposes the homogenizing effect of single-spore colonization. Barring some unknown policing mechanism that represses cheater genotypes, we therefore predict that cheaters are common in natural populations of *M. xanthus*. □

Methods

Strains

Two wild-type clones of *M. xanthus* were used in this study, along with mutant genotypes derived from each of them. Wild-type strain DK1622 (ref. 16) is sensitive to the antibiotics used to distinguish competing genotypes. DK1622 is ancestral to the evolved genotypes GVB206.3, GVB208.3, GVB210.3, GVB212.3, GVB214.3 and GVB216.3, all of which are genetically marked by resistance to rifampicin[6]. These six clones were isolated from six populations that had evolved independently from DK1622 for 1,000 generations in a nutrient-rich, liquid habitat[6]. The C-signal defective mutant LS523 (*csgA* mutation) also derives from DK1622 through genetic manipulation and is resistant to oxytetracycline[8]. DK101 is another developmentally wild-type strain that carries a *pilQ* mutation that hinders S motility[28,29]; it is sensitive to kanamycin. DK101 is the progenitor of the A-signal defective mutants DK4312 (ref. 7) (*asgB* mutation) and MS2021 (ref. 10) (*asgE* mutation), both of which are resistant to kanamycin. All strains are stored as clones in an ultralow freezer.

Sporulation assay

Cultures growing vegetatively in CTT liquid[16] were harvested by centrifugation at 4,900*g* for 15 min at room temperature and resuspended in 0.5 ml TPM liquid[30] at $\sim 5 \times 10^9$ cells ml^{-1}. Resuspended cultures were mixed at appropriate ratios, and 100 μl of the mixtures were spotted on TPM agar plates. Development progressed at 32 °C for 68 h, when cells were collected into 0.5 ml of sterile H_2O, heated for 2 h at 50 °C, sonicated by microtip, diluted and plated by mixing samples with 10 ml CTT soft agar (0.5% agar). Collected samples containing rifampicin-resistant evolved clones were also plated in CTT soft agar containing 5 μg ml^{-1} rifampicin. Spore counts of the rifampicin-resistant genotypes were obtained from plates containing rifampicin, and total spore counts from mixed populations were obtained from plates without antibiotic; spore counts of the rifampicin-sensitive wild-type strains were then calculated as the difference between these plate counts. The plating efficiencies of the rifampicin-resistant genotypes were similar on selective and non-selective agar. The kanamycin- and oxytetracycline-resistant genotypes, however, show decreased plating efficiency during germination on selective relative to non-selective plates. Therefore, to estimate the marked genotype frequency for mixed cultures containing DK4312, MS2021 or LS523, numerous single colonies were transferred (4–5 days after plating) from the non-selective plates to CTT plates containing the appropriate antibiotic (40 μg ml^{-1} kanamycin or 12.5 μg ml^{-1} oxytetracycline) and the frequency of colonies growing on selective plates was determined after three additional days. Sporulation estimates are the mean of $\log_{10}$-transformed spore counts for at least three replicate assays. A marker-control experiment was run for the rifampicin resistance marker using DK1622 and a developmentally proficient, rifampicin-resistant derivative of DK1622. (The resistant clone is the proximate ancestor of the evolved genotypes used in this study[6].) This marker-control experiment indicated that rifampicin resistance caused a slight disadvantage during development; however, any handicap associated with this or other resistance markers is conservative with respect to the inferences of complementation and cheating among the evolved clones. Sporulation efficiency is calculated as the ratio of a genotype's spore production to its initial vegetative population size. In Fig. 2c, d, a relative sporulation efficiency of 1 means that an evolved clone and its progenitor produce spores with equal efficiency; values higher than 1 indicate that the evolved clone has a higher sporulation efficiency, whereas values less than 1 indicate higher efficiency of the wild-type progenitor, DK1622.

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A family of candidate taste receptors in human and mouse

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The gustatory system of mammals can sense four basic taste qualities, bitter, sweet, salty and sour, as well as umami, the taste of glutamate[1–6]. Previous studies suggested that the detection of bitter and sweet tastants by taste receptor cells in the mouth is likely to involve G-protein-coupled receptors[2,7,8]. Although two putative G-protein-coupled bitter/sweet taste receptors have been identified[9], the chemical diversity of bitter and sweet compounds leads one to expect that there is a larger number of different receptors[8,10,11]. Here we report the identification of a family of candidate taste receptors (the TRBs) that are members of the G-protein-coupled receptor superfamily and that are specifically expressed by taste receptor cells. A cluster of genes encoding human TRBs is located adjacent to a Prp gene locus[12], which in mouse is tightly linked to the SOA genetic locus that is involved in detecting the bitter compound sucrose octaacetate[13–15]. Another TRB gene is found on a human contig assigned to chromosome 5p15, the location of a genetic locus (PROP) that controls the detection of the bitter compound 6-*n*-propyl-2-thiouracil in humans[16,17].

To search for taste receptors, we devised a strategy that was based on four ideas: first, taste receptors would be encoded by a family of related genes; second, some taste receptor genes would be found at genetic loci associated with the ability to taste specific compounds in mouse or human; third, taste receptors would be G-protein-coupled receptors (GPCRs) that have limited sequence similarity to other members of the GPCR superfamily; and last, taste receptor genes might be found by using the resources of the Human Genome Project to look for GPCR-encoding genes in genomic regions implicated in taste perception.

We first asked whether there are genes encoding new GPCRs in the region of the human genome corresponding to the mouse SOA locus, which is tightly linked to a Prp gene[13,14]. Using the Jackson Laboratory Mouse Genome Informatics website (http://www.informatics.jax.org), we determined that the Prp gene and the SOA locus are located on mouse chromosome 6 (63.6 centimorgans (cM)) and that the syntenic region in human is on chromosome 12p13. We then determined whether any of the genes that mapped close to the mouse SOA locus had been cloned in human and were deposited in the National Center for Biotechnology Information (NCBI) nr database (http://www.ncbi.nlm.nih.gov); we used the sequences of those genes to search the NCBI Human Genome Sequence (HGS) database, focusing on human chromosome 12. Among the genes we used for this search was a Prp gene, which we found on the chromosome-12 contig NT_001856. By examining the contig map of chromosome 12 in the NCBI HGS database, we were able to identify contigs that flanked NT_001856. This provided us with a focus set of contigs that might contain taste receptor genes.

To find genes encoding GPCRs in this focus region, we first searched the human chromosome 12 database with large sets of GPCR protein sequences that we compiled from a GPCR database (http://www.gpcr.org/7tm). Although we identified a few genes on human chromosome 12 that appeared to encode GPCRs, none was located in the focus set of contigs or their vicinity. We then searched the database with a member of the V1R family of candidate pheromone receptors (V1R5)[18,19], because members of this family are not in the GPCR database and therefore had not been included in our GPCR sequence sets. We identified two sequence stretches in contig NT_001856 (which contains the Prp gene) encoding protein sequences distantly related to V1R5. When we retrieved the DNA sequence in and around one of these DNA regions and translated it, we determined that it contained an intronless gene encoding a putative receptor protein of 311 amino acids (hTRB2 (for human taste receptor, family B, no. 2) with weak homology to V1R5 (Fig. 1).

We then asked whether hTRB2 belongs to a family of related receptors, as we expected would be the case for taste receptors. Using hTRB2 to search the chromosome 12 database, we identified eight related genes, all in the NT_001856 contig. Of the eight TRB genes, six encode receptors related to hTRB2 (Fig. 1) and two are pseudogenes. Using these TRBs, we were unable to find any members of this family in either the NCBI nr or expressed sequence tag databases, consistent with the idea that these receptors might be expressed exclusively in taste tissue. However, we did identify a gene encoding a TRB family member (hTRB7, Fig. 1) in a contig assigned to human chromosome 5p15, the location of PROP, the genetic locus that governs the ability of humans to taste 6-*n*-propyl-2-thiouracil, a bitter compound[16]. We also found a total of five TRB genes (one a pseudogene) on three chromosome-7 contigs, two of which are assigned to 7q31-32 (data not shown).

The candidate receptors encoded by the TRB genes on chromosomes 12 and 5 (and 7) share sequence motifs with one another, uniting them as members of the same receptor family. Although they have the seven-transmembrane domain structure characteristic of GPCRs, they are unrelated in sequence to both mGluR4, which detects glutamate[20,21], and the candidate taste receptors TR1 and TR2 (ref. 9). In addition, mGluR4, TR1 and TR2 have long extracellular amino-terminal domains that are proposed to bind ligand, whereas TRBs have very short N termini, suggesting that they use a different mode of ligand binding. Although TRBs are distantly related to V1Rs, TR1 and TR2 resemble V2Rs (refs 22–24), candidate pheromone receptors that are expressed, with V1Rs, in the vomeronasal organ. The TRBs that we have identified show high variability in protein sequence, suggesting that, like odorant receptors in the nose[25], different family members may recognize chemicals with very different structures, such as chemically diverse bitter tastants.

Are TRBs expressed in taste receptor cells, as they must be if they are truly taste receptors? To address this question, we turned to the mouse. We first asked whether we could isolate sequences encoding TRBs from either mouse genomic DNA or complementary DNA prepared from mouse taste tissue. We used polymerase chain reaction (PCR) with degenerate primers matching conserved sequences in TRBs to amplify related sequences, and then cloned