

A linear dominance hierarchy among clones in chimeras of the social amoeba *Dictyostelium discoideum*

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Abstract

Amoebae from different clones of *Dictyostelium discoideum* aggregate into a common slug, which migrates towards light for dispersal, then forms a fruiting body consisting of a somatic, dead stalk, holding up a head of living spores. Contributions of two clones in a chimera to spore and stalk are often unequal, with one clone taking advantage of the other's stalk contribution. To determine whether there was a hierarchy of exploitation among clones, we competed all possible pairs among seven clones and measured their relative representation in the prespore and prestalk stages and in the final spore stage. We found a clear linear hierarchy at the final spore stage, but not at earlier stages. These results suggest that there is either a single principal mechanism or additive effects for differential contribution to the spore, and that it involves more than spore/stalk competition.

Introduction

In animals, dominance hierarchies often form when individuals compete with each other over resources. The predicted result of conflict can be based either on individual recognition and memory of past encounters, or it can be based on a simple cue, like relative size of the interactants. Dominance hierarchies are an important feature of social groups and have been described for diverse social organisms from the earliest studies on bumblebees and chickens to *Polistes* wasps and baboons (reviewed in Wilson, 1975). Here, we examine a very different social situation for the presence of a dominance hierarchy: the social, multicellular stage of the cellular slime mould, *Dictyostelium discoideum*.

Amoebae of *D. discoideum* are predators on bacteria in forest leaf litter and are most common at high altitudes or northern latitudes in eastern North America (Raper, 1984). When bacteria supplies are depleted, the amoebae aggregate, moving up cAMP gradients to form a multicellular slug or grex of 10^3 – 10^6 cells (reviewed in Kessin, 2001). This slug may migrate to a better location for dispersal, towards light, away from cold and ammonia,

up through the leaf litter, before differentiating into a fruiting body or sorocarp (Raper, 1984). When cells in the slug differentiate, about 20% die, vacuolate and form strong cellulose walls aggregated into a stalk, and the remaining cells form spores in a spherical structure called a sorus at the top of the stalk (Raper, 1984). The spores are hardy and hatch only when conditions improve, which may happen either because conditions change at the same location, or because they have been carried to a more favourable area. The stalk increases the spores' chances for dispersal. Dispersal may occur by adhesion to a passing insect or other invertebrate, ingestion by an invertebrate, or simple desiccation and rupture of the sorus, spraying the spores nearby (Raper, 1984; Suthers, 1985; Huss, 1989; Stephenson and Landolt, 1992).

Because individuals of *D. discoideum* achieve multicellularity by aggregation of cells that may be genetically different, clones may have evolved exploitative mechanisms to increase the odds that they will contribute to living spore cells, not to sterile stalk cells (Strassmann *et al.*, 2000). If a clone succeeds in reducing its contribution to dead stalk cells against a genetically different partner, these interactions could be described as dominance. If the interactions between different pairs are transitive, then they could be described as a dominance hierarchy.

To investigate this possibility we built on our earlier study, which demonstrated that genetically distinct

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clones of *D. discoideum* readily join in chimeras, and that there are unequal contributions by different clones to spore and stalk (Strassmann *et al.*, 2000). To assess precise relative contributions to spore and stalk of two genetically distinct clones, we began each interaction with equal numbers of amoebae of each clone, mixed equally and placed on a Petri plate lacking food, which induces immediate aggregation and fruiting. We then measured who predominated in the prespore (posterior) and prestalk (anterior) zones of the slug and who predominated in the final sorus of living spores. We used DNA microsatellite markers to identify clones, and quantified the relative presence of the two clones using a phosphorimager (Strassmann *et al.*, 2000). Our general result was that there was a clear linear dominance hierarchy among clones in which some clones consistently predominated in numbers in the spores after aggregation and fruiting, though they began with equal numbers of cells.

Materials and methods

Clone selection and growth conditions

We used seven different clones (NC4, NC28.1, NC34.1, NC60.1, NC85.2, NC98.1 and NC105.1) that were collected from Little Butt's Gap, North Carolina (Francis & Eisenberg, 1993). The type clone from which nearly all the laboratory clones have been derived, NC4, came from Little Butt's Gap, but was collected considerably earlier, by Raper in 1939 (Raper, 1984). For the other clones, we use the clone numbers assigned to them by Francis & Eisenberg (1993). We chose these seven clones from the larger set used in our previous study of mixing and unequal spore/stalk representation. We had previously competed only NC105.1 and NC34.1, and found that the former dominated the latter (Strassmann *et al.*, 2000). To evaluate potential conflict between these clones and to construct a hierarchy we mixed equal numbers of amoebae from each clone with each of the others in a round robin tournament for a total of 21 different pairwise mixtures.

We grew each clone in two different Petri plates, each prepared with SM/5 medium (glucose 2 g, Oxoid Bacto peptone 2 g, yeast extract 2 g, MgSO₄·7H₂O 0.2 g, KH₂PO₄ 1.9 g, K₂HPO₄ 1 g, Bacto agar 20 g, H₂O to 1000 mL for 40 Petri plates) at room temperature (23 °C) in metal cabinet drawers. This means the clones developed in the dark. We supplied the bacterium, *Klebsiella aerogenes*, as prey for *D. discoideum*. About 36 h after plating the spores and before the cells started to aggregate, we washed the medium surface with water to collect the amoebae in the vegetative phase. We collected this suspension of amoebae and bacteria in tubes and filled these to 20 mL. We centrifuged the tubes for 5 min at 2400 g to separate the amoebae from bacteria. We repeated this procedure three times to remove the

bacteria completely. Then we resuspended the *D. discoideum* cells in 300 µL of buffer.

To estimate the number of cells for each clone, we counted the amoebae in liquid suspension using a haemocytometer under a light microscope at 400×. We vortexed the cell suspension slightly before counting. Using a 200-µL pipette with a tip whose opening had been cut so it was slightly enlarged, we filled the chamber of a haemocytometer with the cell suspension. We waited for about a minute to allow the cells to settle so that they were on the glass surface and not in suspension. If the cells were too clumped to count accurately, we repeated the process. We counted five random squares of the haemocytometer. At the density we counted, in the seven clones there was an average of 138 ± 28 cells per square. There were no significant differences in cell numbers from different clones, indicating equivalent growth under our conditions (ANOVA $F = 2.1$, d.f. 6, 108, $P > 0.05$).

To avoid any systematic biases due to growing conditions at a single time, or counting errors, we did the experiment in six batches, separated in time, with separate plating and counting bouts. On average each clone had 3.7 independent trials of its six pairings (SD 0.95, range 2–5).

Round robin competitions among clone pairs

After growing and counting the cells, we mixed together two clones using the same number of cells for each clone, thus ultimately creating 21 different pair combinations. We plated out two different numbers of cells, 3.4×10^6 and five times fewer, 6.8×10^5 , in equal volumes of water in two different Petri plates made with starving medium (Na₂HPO₄ 0.356 g, KH₂PO₄ 1.98 g, 15 g Bacto agar, 1000 H₂O for 40 Petri plates). We pipetted the cells onto the centre of the plate and did not spread them out past the area 1.5 cm in diameter that they naturally covered, so some were five times as dense as the others. As a control for determining that the cells from each clone were still alive, we also plated each clone separately in two Petri plates made with the same starving medium. These conditions cause the amoebae to start aggregating immediately, and to form collectible slugs after 24–30 h.

To determine which clone dominated in each mixture, we collected 16 migrating slugs from 16 pairs and eight migrating slugs from five pairs (NC28.1/NC98.1, NC85.2/NC34.1, NC105.1/NC34.1, NC105.1/NC85.2 and NC105.1/NC60.1) and, after the formation of fruiting body, 16 sori for each experiment. During the slug phase, cells have started differentiation into prespore and prestalk cells (Kessin, 2001). These cells are located in different parts of the slugs. Thus, we could analyse the prespore and prestalk cells separately. From each slug we analysed the anterior 10% of the slug (the prestalk cells). We also analysed 10% of the slug towards the rear of the posterior 80% (the prespore cells), but avoiding the very

rear of the slug that can contain stalk-like rearguard cells (Kessin, 2001). From the fruiting body, we analysed the whole sorus, which is the spore cell mass on top of the stalk.

Assessment of clone representation after contests

We extracted DNA from prespore, prestalk and spores using 150 μ L of 5% Bio-Rad Chelex-100 (after crushing the spores in a microfuge tube using a slightly melted, cooled 100- μ L pipetmen tip as a pestle). We then ran the samples in a PTC-100 programmable thermal controller (step 1: 56.0 °C for 4 h; step 2: 98.0 °C for 30 min; step 3: End). We preserved the solution of genomic DNA in Chelex at 4 °C for short-term storage and at -80 °C for long-term storage. Using PCR, we amplified one of three microsatellite loci, Dict13CAT, Dict19AAC and Dict25AAC, with annealing temperatures of 49, 48 and 49 °C respectively (Table 1). For each competition we chose a locus at which the clones being competed differed in allele lengths. To minimize the effects of differential amplification of short and long alleles, we chose loci for a given competition that differed as little as possible in allele lengths, but whose stutter bands did not overlap (more detail in Results). Because we used three different loci across the study, the overall results are not dependent on the vagaries of a specific locus.

We ran PCR reactions, incorporating S-labelled dATP in the PCR product (40 cycles: 92 °C for 30 s, annealing temperature for 30 s, 72 °C for 30 s, 72 °C for 45 s). We then ran the product on 6% denaturing polyacrylamide gels and quantified the relative amounts of the two alleles of the same locus using a phosphorimager. To do this we exposed the radioactive acrylamide gel to an imaging plate (IP) for 3 h and then transferred the data to a computer using an IP reader scan plate. We quantified the amount of PCR product using the FUJIX Bio-Imaging Analyzer System. We rescaled the raw data by subtracting the image background radiation on the IP during sample exposure. This was done by choosing a rectangle of the same size as the rectangle containing the PCR product from a region just above or below that containing the PCR product. We kept overall exposures low to avoid saturation effects.

Statistical methods and tests of hierarchy linearity

To evaluate contributions to spore, prespore or prestalk alone, we calculated the deviation of a clone from the 50% that is expected since we started with equal numbers of cells of each clone. To evaluate the relative contributions to prespore vs. prestalk, we tested for a significant difference between the two. The statistics done on proportions were first arcsine square root transformed to normalize them (Sokal & Rohlf, 1981). We used $P < 0.05$ as the cut-off for significance. All statistics were performed with Statview 5.0 for Mac.

To evaluate whether or not the entire hierarchy was significantly linear, we used Kendall's technique from Appleby (1983). This method is based on calculating the number of circular triads in the hierarchy. An example of a circular triad is when A dominates B, B dominates C, but C dominates A. A circular triad is sometimes referred to as 'rock, paper, scissors' after the child's game (Sinervo & Lively, 1996; Kerr *et al.*, 2002). The null hypothesis for any test of a dominance hierarchy is that the relationship between any two individuals (or in our case clones) predicts nothing about the relationships that each has with others (Appleby, 1983). To apply this test it is not necessary to arrange the matrix of individuals in any particular order initially. Statistical significance is derived from a chi-square test. The degree of linearity is K , and ranges from 0 to 1. A value of 1 means that there are no circular relationships, and that dominance is completely linear. Individuals can then be ranked in order of dominance from most dominant to least dominant, and there will be no reversals. In general, this test is quite conservative. For K to be significant with a group of seven individuals, only two circular relationships are permissible. We did not use the modifications proposed by de Vries (1995) because there were no missing cells and one or no tied values.

Results

Hierarchies and competition among clones

In all pairs, the two genetically distinct clones mixed together, forming chimeric slugs and fruiting bodies, confirming earlier results (Strassmann *et al.*, 2000).

Table 1 Microsatellite loci used in this study.

Name of locus	No. of repeats*	Accession number	Primer sequence	Annealing temp.	Allele size (bp)*
Dict13CAT	18	JAX4a15a09	5'-CCCCTTTTTACTTTTTGCAC-3' 5'-CCAACAACTATAACCACCTCATC-3'	49	157
Dict19AAC	14	JAX4a12f12	5'-GCTTGATTTGCCAATAGTTC-3' 5'-TCAAAACCTGATCCATTACC-3'	48	173
Dict25AAC	27	JAX4a49g10	5'-AGAGCCACTGATTATCTATTCC-3' 5'-CACAAACCTATCACTAGAAACTG-3'	49	210

*Repeat number and allele size refers to sequence from which the primers were designed. Other clones will vary in these characteristics.

Table 2 Dominance of one clone over the other in spores in the sorus. Data represent mean \pm SE of the clone listed on the left in competition with the clone listed on top. For the hierarchy, degree of linearity, $K = 0.93$, $\chi^2_{23,3} = 45.3$, $P < 0.005$.

Clone	NC28.1	NC105.1	NC4	NC60.1	NC85.2	NC98.1	NC34.1
NC28.1	–	0.85 \pm 0.06*	0.81 \pm 0.04*	0.94 \pm 0.06*	0.83 \pm 0.02*	0.72 \pm 0.04*	0.59 \pm 0.06
NC105.1		–	0.99 \pm 0.01*	0.63 \pm 0.09	0.92 \pm 0.02*	0.99 \pm 0.004*	0.89 \pm 0.02*
NC4			–	0.62 \pm 0.06	0.62 \pm 0.06	1.00 \pm 0.00*	1.00 \pm 0.00*
NC60.1				–	0.79 \pm 0.06*	0.46 \pm 0.08	0.60 \pm 0.07
NC85.2					–	0.85 \pm 0.03*	0.86 \pm 0.07*
NC98.1						–	0.94 \pm 0.06*
NC34.1							–

*Means clone at left significantly overexpressed in sorus (sig. above 0.5).

Table 3 Dominance of one clone over the other measured in the prespore. Data represent mean \pm SE of the clone listed on the left in competition with the clone listed on top. For the hierarchy, degree of linearity, $K = 0.29$, $\chi^2_{23,3} = 21.7$, NS.

Clone	NC28.1	NC105.1	NC4	NC60.1	NC85.2	NC98.1	NC34.1
NC28.1	–	0.96 \pm 0.02*	0.63 \pm 0.09	0.96 \pm 0.03*	0.82 \pm 0.02*	0.64 \pm 0.05*	0.50 \pm 0.07
NC105.1		–	1.00 \pm 0.00*	1.00 \pm 0.00*	0.84 \pm 0.04*	0.13 \pm 0.03†	0.82 \pm 0.02*
NC4			–	0.92 \pm 0.02*	0.03 \pm 0.01†	0.80 \pm 0.06*	0.28 \pm 0.08†
NC60.1				–	1.00 \pm 0.00*	0.13 \pm 0.02†	0.34 \pm 0.09
NC85.2					–	0.19 \pm 0.07†	0.76 \pm 0.07*
NC98.1						–	0.95 \pm 0.04*
NC34.1							–

*Means clone at left is significantly over-represented in prespore (sig. above 0.5).

†Means clone at left is significantly underrepresented in prespore (sig. below 0.5).

In the sorus, where the differentiation of cells is complete and irreversible, we found a strong asymmetry between the clones in their representation in the spores, with 15 of these pairs having one clone significantly dominating the other ($K = 0.93$, $\chi^2_{23,3} = 21.7$, $P < 0.005$; Table 2). This asymmetry was transitive, with only one reversal allowing us to construct a clear hierarchy among the seven clones. The hierarchical order among these clones from the most dominant to least dominant is: NC28.1, NC105.1, NC4, NC60.1, NC85.2, NC98.1, and NC34.1 (Table 2). We use this spore contribution hierarchy to order the matrix of clones in all tables.

Results were qualitatively the same in both the prestalk and prespore regions. Whichever clone dominated in one region also dominated in the other ($r = 0.94$, $P < 0.0001$, $n = 21$; Tables 3 and 4). In the prespore sample of the slug, one clone significantly dominated its partner in 18 of 21 chimeric mixtures (Table 3). In the prestalk sample, one clone dominated its partner in 16 of 21 chimeric mixtures (Table 4). However, neither of these hierarchies was significantly linear (prespore: $K = 0.29$, $\chi^2_{23,3} = 21.7$, NS, Table 3; prestalk: $K = 0.09$, $\chi^2_{23,3} = 14$, 23.3, Table 4). Investment in prespore was not correlated with investment in spore ($r = 0.19$, $P > 0.41$, $n = 21$).

We then compared dominance in prespore samples to that in prestalk samples by taking the difference between them for specific slugs and found that one clone was

proportionately more abundant in the prespore region of the slug as compared to the prestalk region in only six pairs (Table 5). The significant differences varied between 0.09 and 0.25. In four of these pairs the dominant clone in the spores also dominated in prespore relative to prestalk; in the other two the reverse was the case. The hierarchy of differences is not linear ($K = 0.30$, $\chi^2_{23,3} = 22$, NS, Table 5). The paucity of differences between prespore and prestalk representation combined with the large differences in each region indicates that there is dominance of one clone over the other that is not only due to differential contribution to the prespore region relative to the prestalk region.

It is interesting that there were some reversals between the prespore and prestalk cells of the slug stage, and the final spore stage. In five experiments, NC98.1 vs. NC105.1, NC98.1 vs. NC85.2, NC85.2 vs. NC4, NC34.1 vs. NC4 and NC34.1 vs. NC60.1, the first-listed clone has more cells in the prespore region of the slug but after the completion of differentiation the second clone prevails with more spores in the sorus (Tables 2–5). In the other experiments the results from the prespore and prestalk stages mirrored that at the spore stage.

Effect of cell density

Overall, the number of cells plated out over a given area did not have a significant effect on the competition

Table 4 Dominance of one clone over the other measured in the prestalk. Data represent mean \pm SE of the clone listed on the left in competition with the clone listed on top. For the hierarchy, degree of linearity, $K = 0.09$, $\chi^2_{23,3} = 14$, NS.

Clone	NC28.1	NC105.1	NC4	NC60.1	NC85.2	NC98.1	NC34.1
NC28.1	–	0.97 \pm 0.01*	0.84 \pm 0.05*	0.997 \pm 0.00*	0.85 \pm 0.02*	0.5 \pm 0.03	0.38 \pm 0.06
NC105.1		–	1 \pm 0*	0.97 \pm 0.02*	0.63 \pm 0.09	0.27 \pm 0.02*	0.51 \pm 0.03
NC4			–	0.84 \pm 0.04*	0.03 \pm 0.01†	0.82 \pm 0.06*	0.03 \pm 0.01†
NC60.1				–	1 \pm 0*	0.15 \pm 0.02†	0.27 \pm 0.06†
NC85.2					–	0.11 \pm 0.06†	0.62 \pm 0.1
NC98.1						–	0.997 \pm 0.00*
NC34.1							–

*Means clone at left significantly over-represented in prestalk (sig. above 0.5).

†Means clone at left significantly underrepresented in prestalk (sig. below 0.5).

Table 5 Difference between prespore and prestalk investments. Data represent mean \pm SE of the clone listed on the left in competition with the clone listed on top. For the hierarchy, degree of linearity, $K = 0.3$, $\chi^2_{23,3} = 22$, NS.

Clone	NC28.1	NC105.1	NC4	NC60.1	NC85.2	NC98.1	NC34.1
NC28.1	–	–0.01 \pm 0.01	–0.21 \pm 0.07†	–0.04 \pm 0.03	–0.03 \pm 0.02	0.07 \pm 0.07	0.12 \pm 0.07
NC105.1		–	0.00 \pm 0.00	0.01 \pm 0.02	0.11 \pm 0.06*	–0.14 \pm 0.04†	0.150*
NC4			–	0.09 \pm 0.05*	–0.01 \pm 0.01	–0.02 \pm 0.03	0.25 \pm 0.07*
NC60.1				–	0.00 \pm 0.00	–0.01 \pm 0.03	0.07 \pm 0.10
NC85.2					–	0.08 \pm 0.10	0.07 \pm 0.07
NC98.1						–	–0.04 \pm 0.04
NC34.1							–

*Means clone at left significantly over-represented in prespore – prestalk differences (sig. >0).

†Means clone at left significantly underrepresented in prespore – prestalk differences (sig. <0).

between clones. With one exception, the relative representation of a clone in a mixture with another clone was the same at both cell numbers (data not shown). However, one clone, NC60.1, was more likely to dominate at the final spore stage when fewer cells were plated out. In three different experiments NC60.1 performed better in the fruiting body phase at the lower cell number (*t*-test: NC60.1 vs. NC105.1, 14 d.f., $t = 3.585$, $P = 0.003$; NC60.1 vs. NC4, 14 d.f., $t = 4.121$, $P = 0.001$; NC60.1 vs. NC85.2, 14 d.f., $t = 2.542$, $P = 0.0235$). There were no cell number effects for NC60.1 when paired with the other three clones. No cell number effects were apparent in the prespore region at the slug stage.

Ruling out possible artefacts

A concern about any PCR-based test is that amplification differences of longer and shorter alleles in competition will influence the outcome. Longer alleles sometimes lose in competition to shorter alleles in PCR, particularly if the lengths vary greatly (Wattier *et al.*, 1998). We attempted to minimize this effect by choosing loci with alleles as close as possible in size for the clones in each pairwise competition. Our allele lengths differed by an average of 16.3 (SD 8.1) bases. We examined our hierarchy to see if dominant clones consistently had

shorter alleles and found that they did not. In fact, the dominant clone had a longer allele in all but five cases. Therefore, the pattern we observed cannot be due to short allele dominance. Furthermore, we used three different loci and did not get results that varied according to which locus we used to evaluate differences between two clones. Variation among the replicates was very low, leading to the small standard errors within pairs. Differential PCR amplification is less of a concern for analyses of the difference in investment between prespore and prestalk because both tissues are amplified, and the alleles are the same for a given clone in each area, and so would affect them the same. Allele length differences did not correlate with any measures of exploitation or differences in spore/stalk measures (>0.16 in all cases).

We also ruled out another artefact that could have given a spurious hierarchy: errors in cell counting. If one clone was counted and used only once, and the count did not represent the true number of cells, then that clone could be over- or underrepresented in all the mix experiments. To avoid this problem we did the experiment in six batches separated in time, so that the counting of any given clone was done on average 3.7 times. For example, the winning clone, NC28.1, was counted and used in four separate batches, as was the losing clone, NC34.1.

Discussion

Relation to previous study

These experiments support the key findings of our earlier study (Strassmann *et al.*, 2000). Amoebae from genetically different clones readily join together in the same fruiting body. Some clones manage to become over-represented in the spores. This study extends our previous results in important ways. First, we demonstrate that there is a linear dominance hierarchy of representation in spore cells among a set of seven clones. Second, we demonstrate that the monopolization of spores can be much greater than that explained by competition to become either spore or stalk alone.

The current study differed from our previous one in experimental set-up. Previously we plated uncounted spores from five fruiting bodies of two clones on nutritive plates, allowing the amoebae from each clone to divide many times, then starve and form fruiting bodies. Because we did not assure that we had equal numbers of amoebae from each clone we could only compare relative prespore to prestalk investment. The study reported on here took equal numbers of amoebae of each clone, placed them on non-nutritive plates so that they would aggregate immediately. This allowed us to compare relative prespore to prestalk investment as before, and also to look at absolute allocation at the final spore stage. Under these different conditions (and using different clone pairs in all but one case), we found 20% of pairs had significant levels of exploitation based on prespore/prestalk differences (Table 5), and that 71% of pairs had significant levels of exploitation at the final spore stage (Table 2). These results obtained under more controlled conditions are qualitatively similar to those of our earlier study (Strassmann *et al.*, 2000).

Dominance cannot be explained by spore/stalk ratios alone

Our results suggest that dominance is sometimes too strong to be explained completely by unequal division of the cells into stalk and spores. If the stalk uses 20% of the cells and all of those come from the loser, the loser still contributes 60% of its cells to spores, to go with 100% of the other clone's cells. Thus, the winner would get 100/(100 + 60) or 5/8 of the spores. Working the other direction, in order to explain 90% dominance in the spores (seen in seven of 21 mixes) purely by loser cells going to the stalk, the stalk would need to be about 45% of total cells, which it is not (Bonner, 1967; Kessin, 2001; Rafols *et al.*, 2001). This might not be the case if spore/stalk ratios were different, or if there were other arenas for conflict than spore/stalk ratios. But we have seen no differences in spore/stalk dimensions in chimeras vs. pure clones (Foster *et al.*, 2002), indicating that other arenas for competition are involved.

Other factors that might affect final representation in the spores include failure to aggregate, being dropped out of the slug in the slime trail, cell division in the slug stage and cannibalism in the slug stage. We did not see any nonaggregated cells. All clones aggregated properly when grown up by themselves. Since *D. discoideum* does not make microcysts, nonaggregating starving cells would simply die, an unlikely strategy. Starving *D. discoideum* in dense populations respond reliably to the cAMP signal to aggregate.

The other possible processes that might contribute to differential spore/stalk allocation all occur in the slug stage. Early in the slug stage, engulfment, and a single round of cell division have been reported, both processes that could change relative cell numbers (Ross, 1960; Huffman & Olive, 1964). As slugs migrate, they continually lose cells at the rear (Kessin, 2001). They could preferentially lose one clone over the other if one clone's cells have become concentrated there. The rearguard cells are a group of cells that do not make it into the fruiting body spores, and so staying out of this region could be an arena for competition. It would be good to know the exact ages in hours of the slugs we chose to evaluate, but we do not have that information. Slugs were harvested between 24 and 30 h after we placed the amoebae on the starving plate. The slugs had probably been migrating several hours since a clear slime trail was used in determining which part of the slug was the front prestalk region and which part was the rear prespore region. That we had small standard errors in our slug assays argues that there was not a great deal of variability in slug age within treatment, and indicates the robustness of our techniques (Tables 3 and 4).

Cannibalism in the slug stage could preferentially affect one clone over the other if clones have some means of discriminating cells in the chimeric slug. *D. caveatum* is a species that is predatory on other species both as amoebae and in the slug stage when it co-aggregates with other species (Waddell, 1982). The aggregation stage is extended in time until the victim clone is entirely consumed. Whether this goes on in natural chimeras of *D. discoideum* is not known. There is one report of cannibalism in the NC4 clone of *D. discoideum* that indicates about 20% of cells are cannibalized at the early aggregation stage (Huffman & Olive, 1964). *D. discoideum* has a much longer slug stage with migration before irreversible commitment to stalk or spore as compared to nearly all other dictyostelid species (Raper, 1984). Clearly, something must occur in the slug stage that goes beyond 80% spore/20% stalk allocation to explain the extreme dominance of one clone over the other. These processes could also explain the lack of correlation in dominance between the prespore stage and the spore stage, and particularly those cases where differences were great between the two stages. Until we have more detailed information on exactly what goes on in the slug stage, it is hard to know exactly which

processes will prove to be important. What we have shown clearly is that something is occurring.

Our analysis of relative prespore/prestalk investment may not have been ideal because of the structure of the prestalk region. After we did our experiment, it was discovered that the rear region of the prestalk area, closest to prespore cells, might be most sensitive to manipulation by cheater clones. This is the pstO region. The most forward region of the prestalk area is the pstA and pstAB regions (Williams, 1997; Kessin, 2001). The cells of the pstO region are most sensitive to DIF, an alkyl phenone produced by some cells that induces neighbouring cells to become prespore (Kay & Thompson, 2001). If this were the region most subject to manipulation, it would be most vulnerable to exploiting clones. Unfortunately, we probably did not sample much of this region since we took only the front 10% or so of the slug for prestalk in efforts to avoid taking any of the prespore region. Still, too high a percentage of some clones was left entirely out of the spore for spore/stalk competition to account for the differences in absolute representation in prespore, prestalk, and spore that we observed.

Implications of the finding of a linear hierarchy

We found a single, linear hierarchy at the final spore stage. One of the implications of a single hierarchy is that there is either a single mechanism determining dominance or there are multiple mechanisms with additive effects. A single mechanism is consistent with a single gene, but does not require a single gene. A group of genes contributing to a specific exploitative behaviour, or to an attribute that favours becoming spores could also result in a hierarchy. If there are multiple mechanisms that are additive in their effects, a hierarchy could also be the end result. For example, it is known that cells that just divided are more likely to become stalk (Gomer & Firtel, 1987). If clones differ in how long they spend in that stage, they would correspondingly differ in their frequency in spore relative to stalk.

Dominance hierarchies in other systems

Dominance of the sort we report for *D. discoideum* has parallels elsewhere. Dominance of some clones over others was also found in chimeras of the prokaryote, *Myxococcus xanthus* (Velicer *et al.*, 2000). *Physarum polycephalum* is an acellular slime mould (the sister group to the cellular slime moulds that include *D. discoideum*). In *P. polycephalum*, there is a hierarchy that describes which parental clone will pass on its mitochondria and which will not (Meland *et al.*, 1991). All pairwise combinations of five distinct haploid clones with different mitochondrial genotypes were crossed. Only one parent passed on its mitochondria, and there was a clear linear hierarchy of mitochondrial genotypes predicting which one was passed on.

In *Botryllus schlosseri*, a colonial protochordate, colonies may fuse depending on their genotype at a single locus that is highly polymorphic in populations (Oka & Watanabe, 1960; Grosberg & Quinn, 1986). Although they must share at least one allele at this locus, they may be genetically different, and thus exploitation can occur (Stoner & Weissman, 1996). This can occur either by one clone totally dominating the other, often because it was older (Rinkevich *et al.*, 1993), or by one invading the germ line of the other (Stoner & Weissman, 1996). That fusion occurs at all in these systems may be because of the benefits of larger size that can be attained (Buss, 1982; Grosberg & Quinn, 1986). Rinkevich *et al.* (1993) demonstrated that there was a hierarchy of response, with some clones consistently dominating their partners. This is analogous to what we found in *D. discoideum*, although in our system there is no evidence for a recognition locus that ever precludes fusion into chimeras of genetically distinct genotypes.

Nonlinear, rock/paper/scissors competitions

A linear dominance hierarchy is not the inevitable outcome of competition. If A dominates B, and B dominates C, but A loses to C, the hierarchy is not linear. A nonlinear hierarchy can be described as a rock/paper/scissors game (Sinervo & Lively, 1996; Frean & Abraham, 2001). Nonlinear hierarchies are particularly common in systems with a spatial component to competition such as corals (Buss & Jackson, 1979) or bacteriocins (Riley & Gordon, 1999; Kerr *et al.*, 2002). Diversity is easily maintained in nonlinear systems.

The maintenance of diversity with a linear hierarchy

It is surprising to find a linear hierarchy among clones of *D. discoideum* in view of their clonal diversity over small scales. One might expect a single clone to dominate in nature if the hierarchy is linear. What might maintain diversity among clones in a trait that is so advantageous in chimeras of two clones? First, it could be that chimeras are uncommon in nature because clones do not often find themselves in close proximity to nonclonemates. We find that this is not likely to be the case because clones are dense even in micro-samples of 0.2 g of soil (Fortunato *et al.*, 2003). Another possibility is that clones at a disadvantage in chimeras have advantages at other stages in their life cycle. For example, they may grow and divide more rapidly as solitary amoebae. Another possibility is that the clones are not at equilibrium, that there is so much diversity at this micro-scale that the advantages of some clones have not yet driven the others out of existence. Future work in *D. discoideum* is necessary to elucidate exactly what processes are causing the hierarchy and, eventually, its genetic basis.

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