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Clonal yeast biofilms can reap competitive advantages through cell differentiation without being obligatorily multicellular

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How differentiation between cell types evolved is a fundamental question in biology, but few studies have explored single-gene phenotypes that mediate first steps towards division of labour with selective advantage for groups of cells. Here, we show that differential expression of the *FLO11* gene produces stable fractions of $Flo11^{+}$ and $Flo11^{-}$ cells in clonal *Saccharomyces cerevisiae* biofilm colonies on medium with intermediate viscosity. Differentiated $Flo11^{+/-}$ colonies, consisting of adhesive and non-adhesive cells, obtain a fourfold growth advantage over undifferentiated colonies by overgrowing glucose resources before depleting them, rather than depleting them while they grow as undifferentiated $Flo11^{-}$ colonies do. $Flo11^{+/-}$ colonies maintain their structure and differentiated state by switching non-adhesive cells to adhesive cells with predictable probability. Mixtures of $Flo11^{+}$ and $Flo11^{-}$ cells from mutant strains that are unable to use this epigenetic switch mechanism produced neither integrated colonies nor growth advantages, so the condition-dependent selective advantages of differentiated *FLO11* expression can only be reaped by clone-mate cells. Our results show that selection for cell differentiation in clonal eukaryotes can evolve before the establishment of obligate undifferentiated multicellularity, and without necessarily leading to more advanced organizational complexity.

1. Introduction

Multicellularity has evolved many times, both in the prokaryotes and the eukaryotes, but most lineages have not progressed beyond the facultative expression of multicellular phenotypes with no or very limited differentiation of cell types [1,2]. Examples are unicellular *Myxococcus* bacteria and *Dictyostelium* slime moulds that form non-clonal aggregations upon starvation to cooperate for spore dispersal [3,4]. This involves differentiation between spore-forming cells and altruistic sacrifices by non-reproducing cells [5,6], but without affecting that unicellularity remains the standard life form. Reproductive altruism is favoured by high relatedness between donor and recipient cells, which is best secured in clonal aggregations, but cell-type differentiation remained very limited in cyanobacteria [7] and volvocine algae [8,9] in spite of clonality being secured. This suggests that synergistic, fitness-enhancing differentiation benefits normally evolve with clonal multicellularity or after it is established [8,10–12] rather than being already present as preadaptation in unicellular progenitors; in other words, cells must first stick to each other in undifferentiated form before they can reap any selective benefits of dividing labour between differentiated cell types [1,13].

Comparative data reconstructions of evolutionary transitions in multicellularity and division of labour among cell types usually rely on sister-lineage comparisons [2,7,8,14,15] between extant clades where entire life-history syndromes have been modified. This implies that the multicellular phenotypes that are compared often represent secondary elaborations rather than very first origins

of multicellularity [13,16], precluding formal tests of whether multicellularity always became obligate before cell differentiation evolved. Studying the selective benefits of genetic mutations that initiate multicellularity is therefore most feasible in lineages where evolution does not progress to more advanced stages. *Saccharomyces cerevisiae* baker's yeast is a eukaryotic microorganism that has these characteristics as it can switch between different unicellular and multicellular growth forms via the expression and regulation of flocculin (*FLO*) genes [17–23]. Flocculation phenotypes normally involve adhesion of undifferentiated cells in response to resource limitation [22], when clumping may provide protection against toxic ethanol challenges [19], but *FLO11* has a gene-expression polymorphism with the potential to induce cell differentiation benefits. This FLO gene is only distantly related to other cell adhesion genes, such as *FLO1*, *FLO5*, *FLO9* and *FLO10* [17,24,25], and has one of the most complex promotor regions in the genome of *S. cerevisiae* [22], encoding a Flo11p cell-wall glycoprotein that is essential for development of surface spreading biofilm phenotypes [26]. These biofilms can both be haploid or diploid [26], but their selective advantages have not been evaluated as possible examples of incipient multicellularity driven by cell differentiation benefits without prior adhesion as undifferentiated cells.

For an incipient facultative differentiation trait in a clonal unicellular microorganism to be maintained by natural selection, its expression would need to (i) depend on specific and thus predictable habitat (medium) conditions, (ii) offer unambiguous growth benefits under these conditions and (iii) be immune to invasion by unrelated cheater cells whose chimeric exploitation of enhanced colony growth rates would annihilate these fitness gains. *FLO11* expression has several aspects suggesting that these conditions might be fulfiled. First, the *FLO11* gene is periodically turned on and off by two oppositely acting transcription factors Slf1p and Flo8p [27–29], a stochastic expression switch that differentiates cells and creates mixtures of clonal $Flo11^+$ and $Flo11^-$ cells in several growth forms of *S. cerevisiae* [18,21,22,29]. Second, the *FLO11* gene becomes expressed only in specific environments such as low glucose medium, so that any multicellular phenotype initiated by this gene is likely to be predictably condition dependent [30]. Third, protein-level (Flo11p-Flo11p) adhesion interactions are strongly homophilic, providing a very direct ligand-to-ligand self-recognition system [25], which might better preserve clonal integrity in growing colonies than the heterophilic adhesion mechanisms via oligo/polysaccharides on the surface of neighbouring cells that other Flo proteins have [22].

2. Material and methods

(a) Strains

The *S. cerevisiae* Σ1278b YS-11 (*MAT***a** *can1Δ*::*STE2p-SpHIS5 lyp1Δ*::*STE3p-LEU2 his3*::*HisG leu2Δ ura3Δ*) was used as wild-type strain, and our *flo8* strain was a *flo8*::*KanMX* deletion mutant in the Σ1278b wild-type background, whereas our *sfl1* strain was a *sfl1*::*KanMX* deletion mutant in the Σ1278b wild-type background. All three strains were described in detail previously [20]. A green fluorescent protein (GFP)-labelled wild-type strain was constructed by inserting a P_{TEF1}-*GFP* at chromosome IX into Σ1278b (provided by Rasmus K. Bojsen). The natural isolates YJM269, CLIB219, 114, M22, T73, UC8, 273614X, WE372, Y9 J, Y55, YJM978, YJM981, T7, NC_02, IL_01, UC1, YPS1009, Y3, Y9, Y10, Y12, CLIB413, CLIB294, YPS163, YPS1000, EM93, K12, DBVPG6861, DBVPG4651, DBVPG3591, DBVPG1794, DBVPG1788, CECT10109, CBS7960, YJM678, YJM653, YJM454, YJM440, YJM436, YJM434, YJM428, YJM421, YJM413, YJM326, YJM320, YJM280, YJM145, CLIB326 and CLIB192 were provided by Joseph Schacherer.

(b) Surface spreading biofilms and their biomass

Biofilm colonies on yeast extract peptone dextrose (YPD) medium were made with 0.3% agar as described previously [31] unless specified otherwise. Colonies were inoculated with 500 cells and grown at room temperature for 7 days (or as indicated). Mixtures of $Flo11^-$ (*flo8*) and $Flo11^+$ (*sfl1*) mutants were made by placing 500 cells on the centre of semisolid complex medium plates in different inoculation proportions, and colony biomass (g dry weight; DW) was subsequently measured across YPD media of different viscosity (% w/v agar) (see electronic supplementary material, figure S2 for representative images). Details were as in [32] except that biofilm colonies were soaked in water, loosened with a Drigalski spatula and transferred to filters by pipette. All experiments were conducted in triplicate.

(c) RNA FISH

RNA FISH was conducted as described in [21] except that RNA FISH from 7-day-old biofilm colonies was made by soaking and washing plates with the same fixation medium and continuing fixation at 4°C as described in [21]. *ACT1* served as a positive control and only *ACT1* mRNA-positive cells were investigated for the amount of *FLO11* mRNA using images similar to those in figure 2*c* that were blinded to avoid observer bias. Error bars are s.d. based on three replicates.

(d) Glucose measurements

Glucose measurements were conducted on 5 µl medium collected right below the rim of biofilm colonies when these had reached a diameter of 3.5 cm on YPD, 0.3% agar. The medium was diluted in 45 µl H_2O and heated for 2 min at 90°C to kill cells after which the glucose concentrations were measured with a Contour Blood glucose meter (Bayer). Error bars are s.d. based on three replicates.

(e) Switching rate from non-adhesive to adhesive cells

Thirty-four non-adhesive mother cells were isolated by collecting solitary cells from the rim of a wild-type colony and placing them on YPD plates with a dissection needle. Cells were allowed to divide, after which daughter cells were removed from the mother cell with a micromanipulator at every cell division. The cell divisions in which a mother cell could be separated from her daughter cell were counted as non-adhesive divisions, and when the daughter cell could no longer be removed from the mother cell, we inferred that a switch to an adhesive cell had taken place.

(f) Images

Pictures of colonies were taken with a Canon EOS 1100D camera and microscope images were obtained with a Nikon Eclipse E600 microscope mounted with an Optronics camera at 400× magnification.

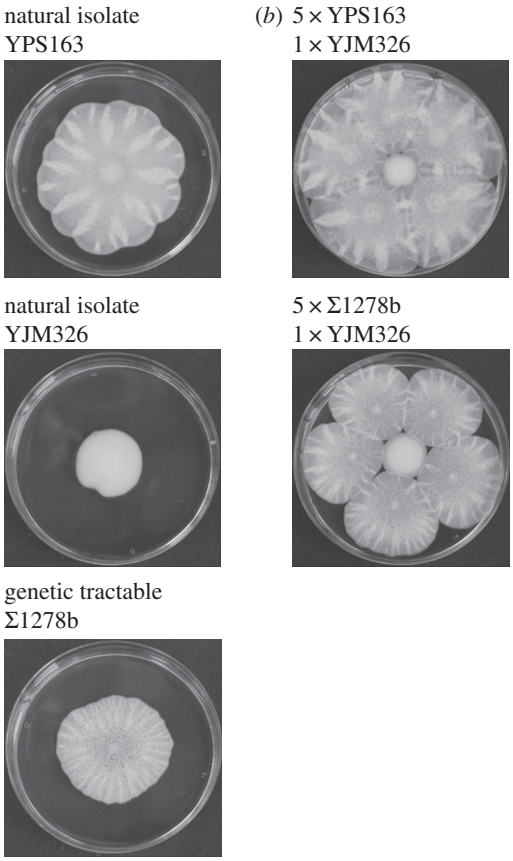

Figure 1. Growth phenotypes and competition between biofilm-forming and non-biofilm-forming isolates of *Saccharomyces cerevisiae*. (*a*) Morphology of the three isolates used in our study: the biofilm-forming YPS163 isolate from soil, the non-biofilm-forming clinical isolate YJM326, and the biofilm-forming genetically tractable isolate Σ1278b. (*b*) Competition experiments between non-biofilm-forming YJM326 (centre) and biofilm-forming YPS163 and Σ1278b, showing that biofilm-forming strains constrain the growth of a non-biofilm strain. All colonies were initiated on 0.3% agar from 500 cells, and growth was recorded after 7 days at room temperature (22°C–25°C). Pictures are representative for three independent experiments.

3. Results

(a) *Saccharomyces cerevisiae* biofilm colonies are competitively superior and differentiated in two cell types

We first tested whether natural isolates of *S. cerevisiae* formed surface spreading colonies similar to the biofilms of the genetically tractable haploid isolate Σ1278b in which *FLO11* is the only expressed *FLO* gene [17]. Six out of 49 natural isolates formed large morphologically structured biofilms (electronic supplementary material, figure S1) while 41 isolates formed smaller smooth colonies and two grew smaller rough colonies (electronic supplementary material, figure S1). Σ1278b biofilms thus appeared to be representative of natural biofilms (e.g. YPS163; figure 1*a*) and to grow larger than natural non-biofilm-forming colonies such as the clinical isolate YJM326. Large biofilm colonies were also competitively superior when we grew Σ1278b and YPS163 together with the non-biofilm-forming isolate YJM326 (figure 1*b*), as both biofilm-forming isolates inhibited the growth of smooth non-biofilm-forming YJM326 colonies.

More than 60% of cells in the growing rim of biofilm colonies formed small clumps of three or more yeast cells while the remaining 38% were single yeast cells or cells in the process of dividing (figure 2*a*,*b*). To test whether differentiation between cells expressing *FLO11* mRNA ($Flo11^+$) or not ($Flo11^-$) affected colony size and morphology, we compared these biofilm colonies with two recessive undifferentiated biofilm mutants, *sfl1* and *flo8*, that are known to have very high proportions of $Flo11^+$ and $Flo11^-$ cells, respectively, when grown in liquid medium [29]. We used RNA FISH to investigate whether differential *FLO11* expression could be responsible for the mixture of adhesive and non-adhesive cells, which showed that *FLO11* expression was restricted to a similar subset (59%) of the biofilm-forming cells (figure 2*c*,*d*). Differential expression of *FLO11* thus appears to explain why some cells formed small clumps whereas others remain free living.

The *sfl1* mutant, depleted of the *FLO11* repressor, formed small wrinkled colonies (figure 2*a*) with *FLO11* mRNA being expressed in 98% of the cells, confirming highly adhesive $Flo11^+$ phenotypes (figure 2*c*,*d*). The *flo8* mutant, depleted of the Flo8p transcriptional activator of *FLO11*, formed small smooth colonies (figure 2*a*) that did not produce *FLO11* mRNA, similar to the smooth unstructured colony morphology of the *flo11* mutant (electronic supplementary material, figure S2). Colonies of *flo8* were thus 100% $Flo11^-$ (figure 2*c*,*d*) and had much higher proportions of free-living cells than wild-type colonies (figure 2*b*). Median cell cluster sizes were five (wild-type), seven (*sfl1*, $Flo11^+$) and two (*flo8*, $Flo11^-$), differences that were statistically significant ($H = 341.14$; d.f. = 2; Crit-$H_{0.05} = 5.99$) and positively correlated with *FLO11* mRNA expression levels (figure 2*c*,*d*) ($H = 7.26$; d.f. = 2; Crit-$H_{0.05} = 5.99$).

(b) Cooperation in differentiated clonal biofilm colonies at intermediate medium viscosity

Biomass in differentiated $Flo11^{+/-}$ wild-type colonies was up to four times higher than the biomass of separate $Flo11^+$ (*sfl1*) and $Flo11^-$ (*flo8*) colonies (figure 3*a*), and these growth advantages were restricted to intermediate medium viscosities (0.25–1.0% agar; $H = 7.54$; d.f. = 2; Crit-$H_{(0.05)} = 5.99$ for 0.25% agar; figure 3*a*; electronic supplementary material, figure S3). Differentiated colonies with both $Flo11^+$ and $Flo11^-$ cells thus obtain condition-dependent growth benefits by some form of synergistic cell-type division of labour compared with undifferentiated colonies that were purely $Flo11^+$ or $Flo11^-$. Validation of this result in liquid medium (0% agar) and on high-viscosity medium (2% agar) confirmed that $Flo11^{+/-}$ differentiation does not affect clonal biomass under these growth conditions, and also showed that the *sfl1* and *flo8* mutants are not generally compromised in their growth ($H = 2.66$ (0% agar) and 2.11 (2% agar); d.f. = 2; Crit-$H_{(0.05)} = 5.99$; figure 3*a*; electronic supplementary material, figure S3). $Flo11^{+/-}$ induced biofilm formation thus appears to represent a lifestyle to rapidly colonize favourable habitat patches on semisolid substrate.

Growth rate benefits at intermediate viscosity are consistent with social synergies being most likely to arise under intermediate spatial structure [33,34]. Yeast cells in low-viscosity liquid medium are less likely to interact with cells of their own clone so differentiation will be selected against when non-relatives benefit, whereas high-viscosity medium will maintain clonal population structure but physically

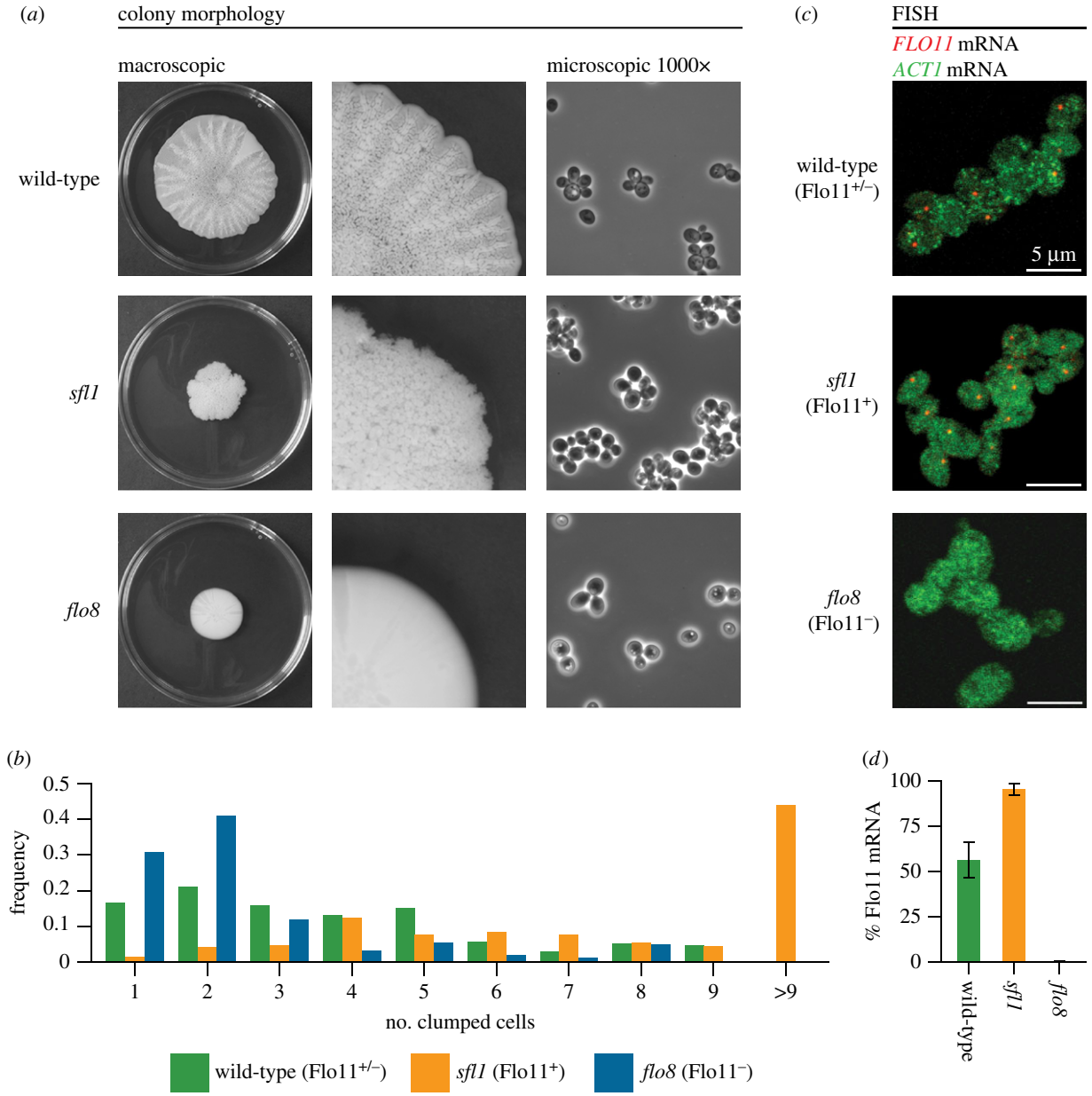

Figure 2. Differentiation of *S. cerevisiae* biofilms in Flo11^{+} and Flo11^{-} cells. (*a*) Colony morphology at different magnifications of the genetically tractable wild-type strain Σ1278b, and isogenic *sfl1* and *flo8* mutant strains. (*b*) The average cell cluster sizes of wild-type ($n = 1350$), *sfl1* ($n = 1844$) and *flo8* ($n = 1490$) colonies. (*c*) *FLO11* transcripts visualized after RNA FISH for wild-type Flo11$^{+/-}$, and *sfl1* and *flo8* strains, hybridized with *FLO11* (red) and *ACT1* (green) probes. (*d*) Percentage of cells expressing *FLO11* mRNA in wild-type ($n = 756$), *sfl1* ($n = 574$) and *flo8* ($n = 509$) colonies.

preclude differentiated clones from growing faster. However, at intermediate medium viscosity (figure 3*a*) cheater clones that fail to invest in Flo11p proteins could invade fast-growing Flo11$^{+/-}$ biofilms because both adhesive and non-adhesive cells were found in the rim (figure 2*a*,*b*). We therefore tested whether non-clonal mixtures of Flo11^{+} (*sfl1*) and Flo11^{-} (*flo8*) cells could produce the structured biofilm phenotype with the ensuing higher biomass and found that this was not the case (figure 3*b*). Such combined *sfl1*–*flo8* colonies always formed a central structured hub that appeared to be composed of adhesive Flo11^{+} cells surrounded by a smooth zone of non-adhesive Flo11^{-} cells (electronic supplementary material, figure S4), consistent with Flo11^{+} cells not offering resource acquisition advantages to unrelated Flo11^{-} cells. This lack of cooperation between Flo11^{+} (*sfl1*) and Flo11^{-} (*flo8*) cells underlines that clonality is essential for the expression of synergistic division of labour in differentiated Flo11$^{+/-}$ biofilms and appears to exclude any green beard explanations for the cooperative Flo11$^{+/-}$ biofilms [19].

(c) Cell-type switching ensures stable mixtures that monopolize local resources

Results so far indicated that synergistic growth benefits in Flo11$^{+/-}$ colonies need to be generated via differential gene expression within clones and that Flo11^{-} cells in wild-type colonies generate Flo11^{+} cells de novo in the actively growing periphery to ensure a stable beneficial mixture of the two cell types. To simulate this process, we sampled non-adhesive peripheral cells of wild-type colonies, dissecting them from their daughter cells, and followed their trajectory (figure 3*c*,*d*). This showed that non-adhesive cells produced only 1.8 non-adhesive daughters on average before they generated an adhesive daughter cell (figure 3*d*), supporting previous data for epigenetic switching of the

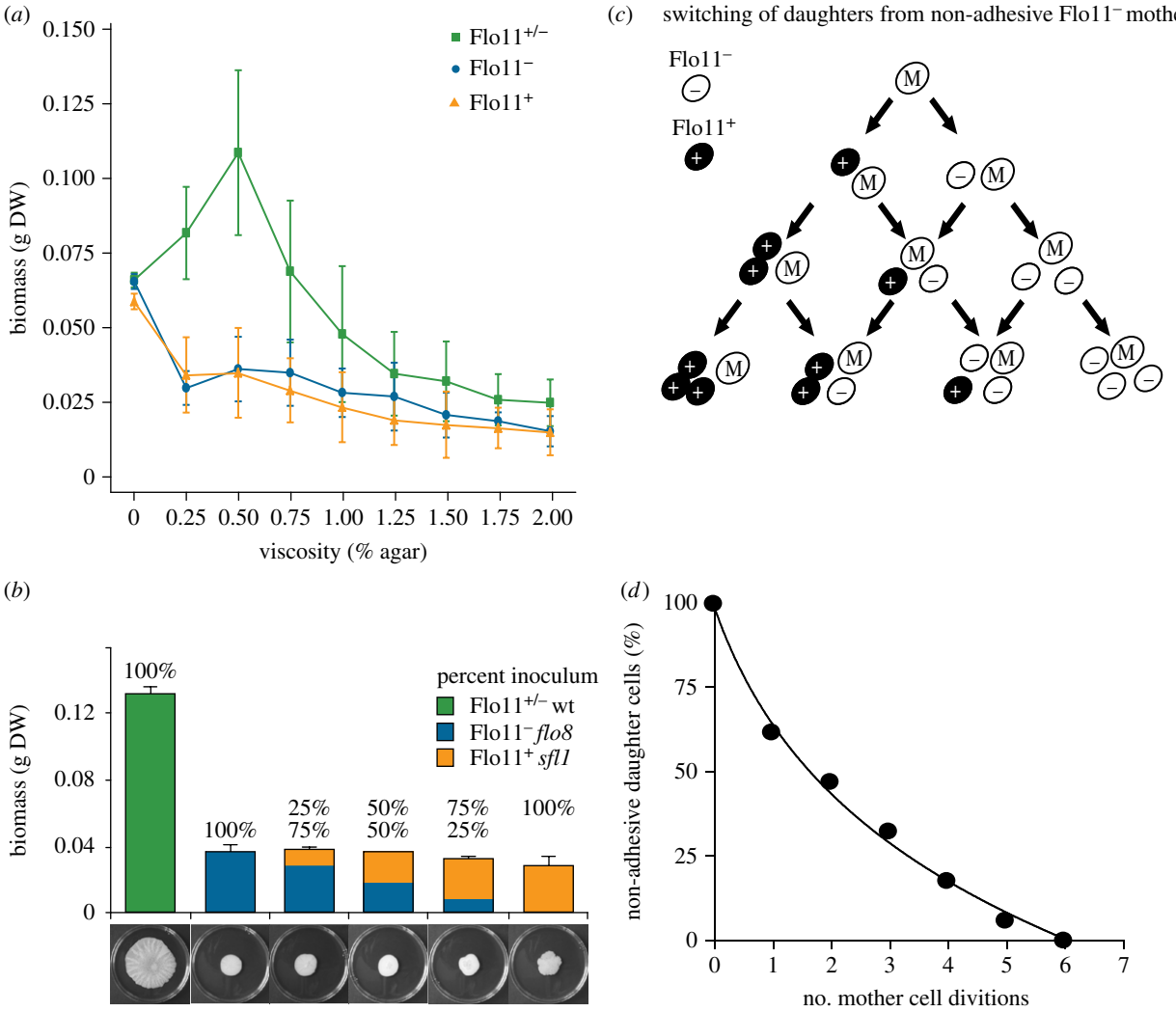

Figure 3. Condition-dependent growth advantage of biofilm colonies relies on cell-type switching. (*a*) Dry weight biomass (g DW) of wild-type Flo11$^{+/-}$ (green), *sfl1* (orange, Flo11^{+}) and *flo8* (blue, Flo11^{-}) colonies $\pm$ s.d. on medium of different viscosity. (*b*) Biomass of differentiated wild-type (Flo11$^{+/-}$) clones and mixtures of mutants that are either *flo8* (Flo11^{-}) or *sfl1* (Flo11^{+}). (*c*) The expected trajectory of Flo11^{-} mother cells (M) in a wild-type colony, assuming that Flo11^{+} (black) and Flo11^{-} (white) can either produce daughter cells of their own type or daughter cells of the opposite type so that a mixed population of Flo11$^{+/-}$ cells arises de novo. The observed trajectories of non-adhesive Flo11^{-} mother cells producing non-adhering Flo11^{-} daughter cells are plotted in (*d*), showing that Flo11^{-} daughter cell production exponentially decays with a half-life of 1.8 cell divisions. Flo11^{-} mother cells were recorded by microdissection until they produced an adhesive daughter cell.

FLO11 promoter in conjunction with the reporter gene YFP [28]. Stochastic switching thus allows biofilm clones to maintain stable proportions of adhesive and non-adhesive cells in the growing rim to reap differentiation benefits without risking invasion by free-riding Flo11^{-} cells from other clones that would require costly kin-discrimination mechanisms to eliminate.

To resolve how clonal Flo11$^{+/-}$ colonies obtain a threefold to fourfold increase in growth rate, we reared a series of them in competition with same-phenotype colonies. This confirmed the growth rate advantages of Flo11$^{+/-}$ colonies (figure 1): differentiated Flo11$^{+/-}$ biofilms covered plates almost entirely within a week, whereas undifferentiated Flo11^{+} and Flo11^{-} colonies never reached each other and never overgrew entire plates (figure 4*a*), suggesting that they access nutrients in the medium in a fundamentally different way. Fast-growing Flo11$^{+/-}$ biofilms created contact zones where neighbouring colonies overgrew each other, but apparently without merging (figure 4*b*).

Further measurements showed that glucose was almost depleted in the periphery of undifferentiated Flo11^{-} colonies and natural smooth isolate colonies (mean 4.5 $\pm$ 2.2 mM glucose compared with the original 111 mM glucose; $n = 7$), but that glucose concentrations were up to 15 times higher (mean 33.2 $\pm$ 11.0 mM; $n = 7$) in the periphery of differentiated Flo11$^{+/-}$ colonies and similar natural biofilm isolates (figure 4*c*; ANOVA: $F_{(14,35)} = 68.6$; $p < 0.0001$). Flo11$^{+/-}$ biofilms thus appear to monopolize territories on agar plates before depleting them, whereas smooth Flo11^{-} colonies use glucose in direct proportion to immediate availability as yeast colonies normally do [35] (figure 4*c*,*d*). This unusual growth pattern offers consistent opportunities for enhanced cell division within Flo11$^{+/-}$ biofilms relative to what can be achieved by Flo11^{-} colonies (figure 4*d*). The epigenetic *FLO11* expression switch thus enables clonal biofilms to practise a form of pre-emptive contest competition by optimizing the frequency of adhesive and non-adhesive cell production. Under specific growth conditions, this must provide significant fitness advantages relative to the scramble competition strategies that are practised by unicellular or adhesive growth forms that are unable to produce Flo11^{+} and Flo11^{-} cells simultaneously.

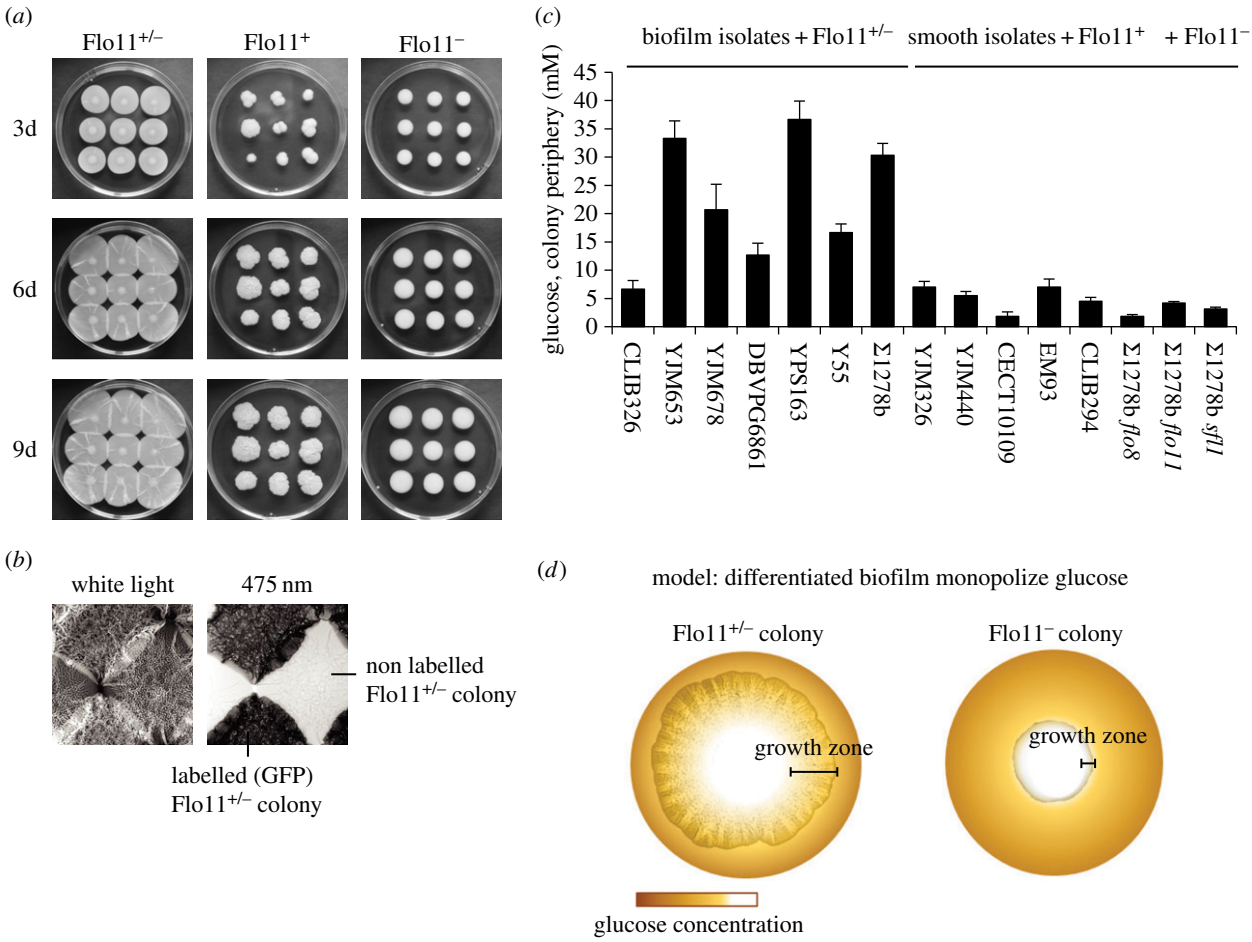

Figure 4. Differentiated biofilm colonies monopolize local resources before exhausting them. (*a*) Growth of wild-type Σ1278b, *sfl1* and *flo8* colonies after 3, 6 and 9 days. (*b*) Contact zones between four Flo11$^{+/-}$ colonies of which two expressed green fluorescent proteins (GFP) (white light, left; 475 nm light, right). (*c*) Glucose concentrations (means ± s.d.) below the rim of natural biofilm-forming isolates (CLIB326, YJM653, YJM678, DBVPG6861, YPS163, Y55) and Σ1278b compared with smooth (Flo11^{-}) colonies (YJM326, YJM440, CECT10109, EM93, CLIB294, *flo8*, *flo11*) and the *sfl1* (Flo11^{+}) mutant. (*d*) Schematic diagram reconstructing how differentiated biofilm colonies monopolize glucose resources. High glucose concentration is shown in dark brown and low glucose concentration in white. Scale bars indicated the growing edge of a differentiated biofilm colony (left) and an undifferentiated smooth colony (right).

4. Discussion

Our results show that clonal but differentiated Flo11$^{+/-}$ biofilm phenotypes fulfil the three conditions that should apply for a latent synergistic division of labour trait to be evolutionary stable: condition-dependent expression at low glucose concentration, unambiguous fitness benefits but only on medium of intermediate viscosity, and robustness against exploitation because undifferentiated strains that do not invest in the simultaneous production of both cell types cannot invade. Intermediate medium viscosity as a condition for realizing cooperative growth benefits (figure 3*a*) may be related to solid medium precluding niche pre-emption via fast surface growth and liquid medium having too much clonal mixing to favour Flo11$^{+/-}$ phenotypes. The mechanics by which the complementary cell types realize synergistic growth are unknown, but it would seem likely that Flo11^{+} cells form a kind of scaffold for the subsequent spread of clone-mate Flo11^{-} cells while generating the structured appearance of biofilm colonies. The stochastic gene-expression switch in clonal Flo11$^{+/-}$ biofilms [18,29] may thus function as a feedback system to maximize growth rate through somatic differentiation, but without realizing true multicellularity as biofilms remain fragmented mixtures of adhesive and non-adhesive cells.

A central question is whether the low glucose concentration and intermediate viscosity conditions that might select for differentiated biofilm colonies and allow for preemptive contest competition exist in natural habitats. We believe this is likely to be the case. *Saccharomyces cerevisiae* is the dominant yeast in many spontaneous fermentation processes of domesticated and natural stands of grape, cacao and other types of fruits [36,37], and is known to be dispersed through insect vectors [38]. Thus, it seems likely that the semisolid surface of natural fruit pulp provides the advantageous environment and that insect vectors will inoculate clonal biofilm colonies soon after such patches reach the appropriate stage of decay. However, quantitative field studies of *S. cerevisiae* biofilm are lacking, so it is at present impossible to evaluate the extent to which reproductive fitness of differentiated *FLO11* gene expression is realized in natural environments.

The ability of Flo11$^{+/-}$ *S. cerevisiae* biofilms to maintain clonality in a highly efficient homophilic way (figure 1) may have been crucial for the evolution and maintenance of differentiation without proper multicellularity. This is consistent with a large comparative study of obligate and facultative multicellular organisms revealing that obligate multicellular lineages are all clonal and that the highest level of organizational complexity, measured as the number of different cell

types, is also found in clonal lineages [2]. Theoretical studies also predict that clonality is a key condition for the evolution of obligate multicellularity, because separation between germline and sterile somatic cells required clonality and reduced the mutation load from selfishly over-replicating mutant cell lines (cancer) in complex multicellular organisms such as the bilaterian Metazoa [1,16]. The importance of clonality has not been directly tested in the present experiments, but its importance can be inferred. Given the cohesion of the *FLO11* biofilms and our consistently negative results when attempting to create differentiated biofilms from non-clonal *FLO11* genotypes (figure 3), it must be true that differentiated biofilms maintain clonality. Even biofilm cultures of the same clonal stock that were inoculated separately overgrew each other rather than becoming mixed (figure 4*b*), suggesting that recognition mechanisms are present that even exclude subtle somatic mutations to be secondarily mixed.

It is important to realize that multicellularity in Flo11$^{+/-}$ *S. cerevisiae* biofilms differs fundamentally from several other model systems that have been used to study facultative multicellularity. First, the biofilms of baker's yeast differ from the multicellular structures in *Dictyostelium discoideum* slime moulds [3,5] and *Myxococcus xanthus* bacteria [6] in being clonal rather than arising via cell aggregation, making the latter much more vulnerable to chimeric invasion by free-riding cells. Second, *S. cerevisiae* biofilms differ fundamentally from the undifferentiated *S. cerevisiae* aggregates known as flocs that can form between both related and non-related yeast cells [17,19]. Flocculation protects *S. cerevisiae* cells from environmental stress [19] but does not provide a novel and potentially synergistic growth form as the *FLO11* biofilm phenotype does. Flocculation relies on the production of Flo proteins with a PA4 domain, such as Flo1p, Flo5p, Flo9p and Flo10p that form Ca^{2+}-dependent heterophilic interactions with mannose residues on the cell walls to adhere to neighbouring cells [24], whereas the biofilm phenotype of our present study relies on homophilic protein (Flo11p–Flo11p) interactions [25].

Finally, the organization of *S. cerevisiae* biofilms is also clearly different from any form of filamentous multicellularity such as found in *Streptomyces* [39], where clonal cell differentiation has evolved to enhance spore dispersal rather than resource acquisition. Yet any form of more advanced multicellularity is lacking in *Saccharomyces*, confirming that clonal integrity is a necessary but not sufficient condition for establishing obligate multicellularity [2]. We suspect that the condition dependence (intermediate viscosity media only) of synergistic growth advantages has constrained *S. cerevisiae* to remain unicellular by default, because its mode of dispersal and the media available throughout its natural niche would not select for irreversibly multicellular phenotypes. Overall, the incipient cell-type division of labour characteristics that our present study discovered underline that Flo11$^{+/-}$ biofilm is a highly suitable model for evaluating evolutionary trade-offs that likely applied to most single-gene very first steps towards differentiated multicellularity. Other recent experimental evolution studies of *S. cerevisiae* have also considerably advanced our understanding of the selection pressures and proximate mechanisms that can make differentiated multicellularity evolve [40]. The addition of Flo11$^{+/-}$ biofilms to this spectrum appears to further enhance the status of *S. cerevisiae* as a genetic and phenotypic model system for incipient multicellularity that recent authors of major evolutionary transitions reviews have found wanting [12,13,16].

Authors' contributions. K.S.A., K.E.H and B.R. performed experiments and analysed data; B.R. and J.J.B designed the study and wrote the paper. All authors discussed the results and commended on the manuscript.

Data accessibility. All supplementary data are presented in the electronic supplementary material for this article.

Competing interests. The authors declare no competing interest.

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