

1 **Bacterial growth in multicellular aggregates leads to the emergence of complex lifecycles**

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3 Julia A Schwartzman^{1*}, Ali Ebrahimi¹, Grayson Chadwick², Yuya Sato^{1,3}, Victoria Orphan², Otto
4 X Cordero^{1*}
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6 ¹ Department of Civil and Environmental Engineering, Massachusetts Institute of Technology,
7 Cambridge, MA 02139, USA.

8 ² Division of Geological and Planetary Sciences, California Institute of Technology, Pasadena,
9 CA 91125

10 ³ Environmental Management Research Institute, National Institute of Advanced Industrial
11 Science and Technology, 16-1 Onogawa, Tsukuba, Ibaraki, 305-8569, Japan
12

13 *Corresponding authors: Julia A Schwartzman (julia5@mit.edu), Otto X Cordero
14 (ottox@mit.edu)
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16 **ABSTRACT**

17 In response to environmental stresses such as starvation, many bacteria facultatively aggregate
18 into multicellular structures that can attain new metabolic functions and behaviors. Despite the
19 ubiquity and relevance of this form of collective behavior, we lack an understanding of how the
20 spatiotemporal dynamics of aggregate development emerge from cellular physiology. Here, we
21 show that the coupling between growth and spatial gradient formation leads to the emergence of
22 a complex lifecycle, akin to those known for multicellular bacteria. Under otherwise carbon-limited
23 growth conditions, the marine bacterium *Vibrio splendidus* 12B01 forms multicellular groups to
24 collectively harvest carbon from the brown-algal polysaccharide alginate. This is achieved during
25 growth on dissolved alginate polymer through formation of spherical, clonal clusters of cells that
26 grow up to 40 μm in radius. Clusters develop striking spatial patterning as they grow due to
27 phenotypic differentiation of sub-populations into a ‘shell’ of static cells surrounding a motile ‘core’.
28 Combining *in situ* measurements of cell physiology with transcriptomics, we show that shell cells
29 express adhesive type IV pili, while motile core cells express carbon storage granules. The
30 emergence of shell and core phenotypes is cued by opposing gradients of carbon and nitrogen
31 that form within cell clusters due to local metabolic activity. Eventually, the shell ruptures,
32 releasing the carbon-storing core, and we show that carbon-storing cells more readily propagate
33 on alginate than non-carbon storing cells. We propose that phenotypic differentiation promotes
34 the resilience of 12B01 groups by enabling clonal groups to grow larger and propagate more
35 effectively. Phenotypic differentiation may be a widespread, but overlooked, strategy among
36 bacteria to enhance resilience in the context of resource limitation.
37

38 **INTRODUCTION**

39 Clonal groups of microbes often form transient multicellular structures such as biofilms and fruiting
40 bodies (Claessen et al., 2014; Shapiro, 1998). The emergent behaviors of these structures
41 underly ecologically important functions such as antimicrobial resistance (Høiby et al., 2010),
42 immune evasion (Arciola et al., 2018), and the mobilization of complex nutrients (Koschwanetz et
43 al., 2013; Ratzke and Gore, 2016). We typically consider only the benefits of life in a group.
44 However, life at high cell density is also an inevitable source of cellular conflict. Even where cells

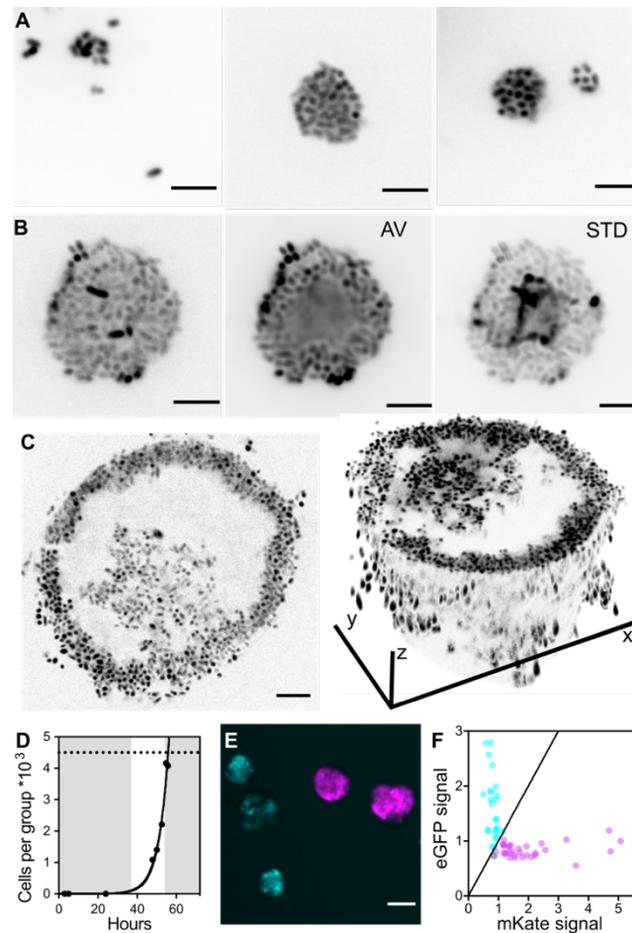
45 cooperate by sharing metabolic tasks such as resource mobilization or detoxification, they
46 compete for access to other growth-essential nutrients. Thus, cells in transient multicellular states
47 must balance cooperation and conflict for the population to derive and ecological benefit. One
48 possible mechanism through which this is achieved within clonal populations is the emergence of
49 phenotypic heterogeneity. There is a growing understanding that phenotypic heterogeneity is a
50 ubiquitous aspect of microbial populations (Dar et al., 2021; Vlamakis et al., 2013). However, we
51 have little understanding of when heterogeneity provides an ecological benefit (van Gestel et al.,
52 2015). Here, we explore how the emergence of phenotypic heterogeneity in transient multicellular
53 structures formed by a marine bacterium enhances resource partitioning and gives rise to
54 complex reproductive cycles.

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56 The focus of our study is transient multicellular behaviors that emerge in the context of
57 decomposition. Decomposers have an ecological advantage in resource poor environments
58 because they can access otherwise recalcitrant nutrients (Sichert and Cordero, 2021). Because
59 decomposition takes place outside of cells, the ability to pool enzymes accelerates the rate of
60 decomposition, and in turn, enhances the recovery of breakdown products. It is common for this
61 behavior to be positively dependent on cell density; strong positive-density dependence results in
62 critical population thresholds where cells that cannot create local density die off (Ratzke and Gore,
63 2016). In this context, the ecological benefit of local cell density can exert strong evolutionary
64 selection for multicellular behavior (Koschwanetz et al., 2011), suggesting that multicellular
65 behaviors are likely widespread among degraders in nature (D'Souza et al., 2021).

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67 Here, we demonstrate how phenotypic heterogeneity within multicellular groups shapes resource
68 allocation, growth, and reproduction in marine polysaccharide decomposer. The
69 Gammaproteobacterium *Vibrio splendidus* 12B01 grows on the brown-algae derived
70 polysaccharide alginate through the formation of cooperative multicellular clusters. Cells in the
71 clusters exist in a mix of phenotypic states and form reproducible spherical structures that grow
72 up to 40 μm in radius and develop mixing in their cores. In previous work, we have shown that
73 growth of 12B01 is strongly dependent on local population density (Ebrahimi et al., 2019a;
74 Hehemann et al., 2016). Using a combination of transcriptomics, quantitative light microscopy
75 and single cell stable isotope probing we characterized the phenotypic differentiation that occurs
76 within 12B01 groups. We show that phenotypic differentiation allows groups to form self-
77 organized structures that promote sharing of polysaccharide-derived carbon and mitigate
78 competition for other growth essential nutrients. Our results highlight the role of phenotypic
79 differentiation in the context of polysaccharide decomposition: a common microbial group
80 behavior.

81 82 **RESULTS**

83 Alginate decomposition is a common trait among marine *Vibrio* (Hehemann et al., 2016), and the
84 cellular mechanisms by which 12B01 decomposes alginate are well characterized (Badur et al.,
85 2017, 2015; Jagtap et al., 2014; Wargacki et al., 2012). A striking feature of 12B01 growth on
86 dissolved alginate is the emergence of motility within cell clusters (Ebrahimi et al., 2019a). To
87 better understand the process leading to the emergence of motility, we monitored the



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90 **Figure 1. 12B01 growth on alginate involves formation of phenotypically distinct sub-populations by clonal**
91 **groups. A-C)** Stages of 12B01 growth on alginate. Scale bars are 5 μm . **A)** In stage i, single cells are suspended in
92 medium containing soluble alginate polysaccharides, and slowly form undifferentiated groups. Panels show three
93 examples of stage i groups. **B)** In stage ii, groups grow and differentiate into layered structures, with a solid shell and
94 a mobile core. Panels show mixing in the center of groups. AV; average intensity projection of time-lapse, showing
95 populations of cells that are motile and not motile (Movie S1); STD, standard deviation projection, showing
96 differences in pixel intensity between frames. **C)** Rupture of clusters leads to hollowing in stage iii. Left: optical section
97 through a cluster, showing absence of cells in center; right: 3D projection, showing structure of hollowed cluster. **D)**
98 Growth of 12B01 in groups, expressed as cells per group. Grey shaded regions delineate growth stages. Exponential
99 growth rate of cells in stage ii (fit line): $\mu_{\text{max}}=0.19 \pm 0.03 \text{ h}^{-1}$. Dashed line indicates size at which growth of clusters
100 ceased due to rupture. **E)** Formation of 12B01 groups tracked using isogenic strains that express fluorescent proteins
101 (Figure S2A). Representative images show distribution of fluorescent protein among eGFP (cyan) or mKate
102 (magenta)-labeled groups at stage ii. Scale bar= 20 μm . **F)** Quantification of fluorescence intensity within 12B01
103 groups. Each point represents the total intensity per group, normalized by the mean intensity of groups in each
104 channel. The black diagonal line represents identify (equal proportion of both strains).
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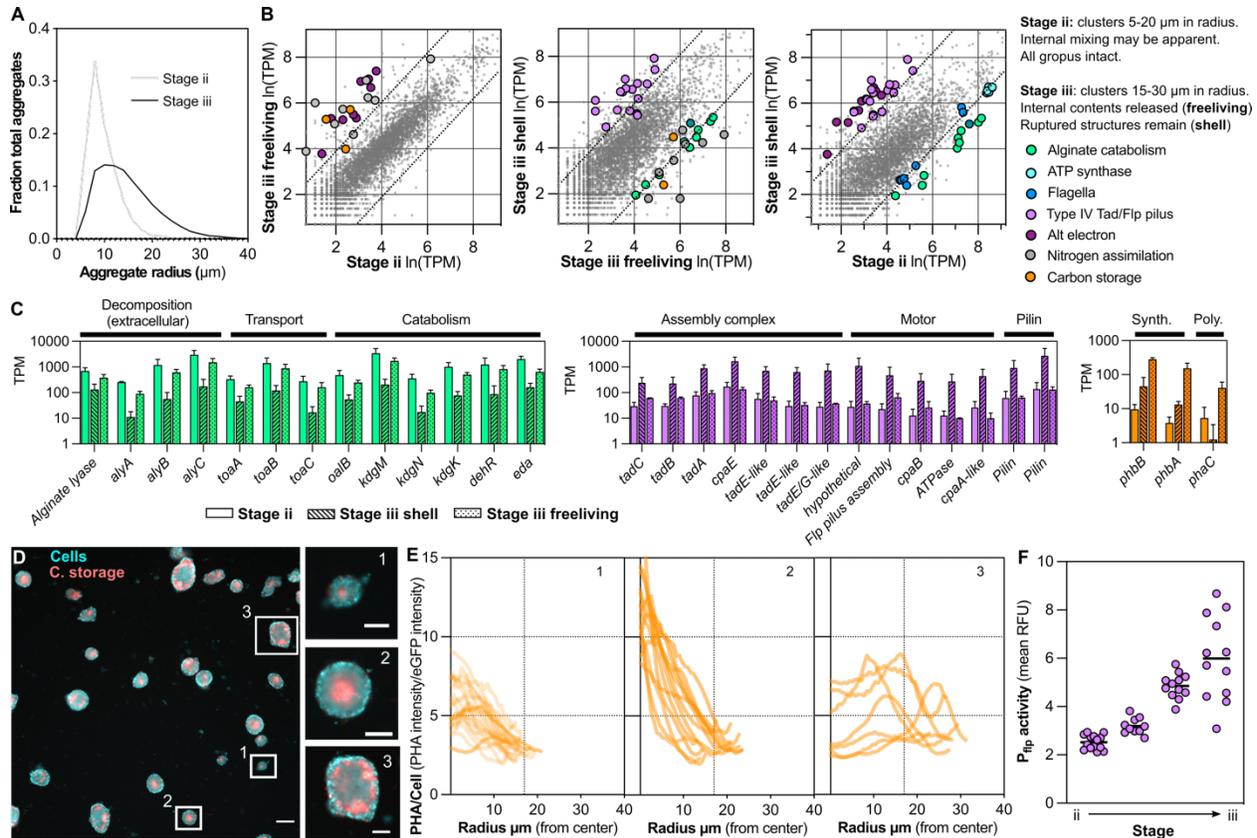
106 development of clusters during growth of 12B01 on alginate in shaking flasks. The population
107 (composed initially of solitary cells) underwent a long lag phase before tightly packed clusters of
108 cells started to form and population growth was detectable. Clusters underwent three distinct
109 stages as they grew: i) initial growth as structurally homogenous clusters (Figure 1A), ii) self-
110 organization of clusters into layered structures composed of an outer sub-population of cells with
111 clear cell-cell adhesion, and an inner motile sub-population of cells (Figure 1B, Movie S1), and iii)

112 rupture of layered structures, releasing motile internal contents (Figure 1C). This behavior is
113 reminiscent of the hollowing and seeding dispersal observed in diverse biofilm-forming bacteria
114 (Kaplan, 2010). Stages corresponded to changes in cluster growth rate: clusters in stage i grew
115 slowly compared to stage ii (Figure 1D), suggesting that the amount of cellular cooperation
116 increases from stage i to stage ii, and then ceases upon rupture.

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118 Self-organization emerges from local cellular interactions. Both physical interactions with alginate
119 polysaccharide, and physiological changes resulting from the availability of alginate-derived
120 carbon, could contribute to self-organization of 12B01. We investigated these ideas by growing
121 cells on alginate polysaccharide, uronic acids mannuronate and guluronate (metabolizable break-
122 down products of alginate), or a mix of polysaccharide and breakdown products. Although the
123 presence of alginate polysaccharide was sufficient to induce cellular aggregation, aggregates
124 forming in the presence of the breakdown products differed greatly in their morphology from the
125 compact clusters formed when only polysaccharide was available (Figure S1A). Staining with a
126 cell-impermeant fluorescent dye specific for DNA revealed that the loose biofilm-like aggregates
127 contained dead cells and extracellular DNA, whereas the clusters formed during growth on
128 alginate polymer only did not (Figure S1B). These results indicated that, while cellular interactions
129 with alginate polysaccharide are likely to take place, alginate polysaccharide must be the only
130 available carbon source for 12B01 to develop morphological complexity.

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132 Biofilms, swarms, and other bacterial social structures often form by the coalescence of many
133 individual cells. Unlike clonal structures, conflict can emerge within aggregative social structures
134 due to the presence of multiple independent genotypes (Grosberg and Strathmann, 2007;
135 Márquez-Zacarías et al., 2021; Nadell et al., 2016; Pentz et al., 2020). This led us to investigate
136 whether 12B01 clusters were aggregative or clonal. We mixed isogenic populations of 12B01
137 each expressing a fluorescent protein and monitored the proportion of clusters that developed
138 with each fluorescent protein, or with a mix of both (Figure 1E, Figure S2A). Strikingly, clusters
139 expressed either one or the other fluorescent protein rather than forming mixed assemblages
140 (Figure 1F, Figure S2B), demonstrating that the emergent cooperation of 12B01 clusters on
141 alginate polymer is achieved by division of a progenitor cell into a clonal collective. The clonality
142 of 12B01 cooperation indicates that individual progenitor cells can support initial divisions to
143 create local cell density, and that cell clusters do not merge during development. One
144 consequence of the clonality of 12B01 clusters is that cooperative resource sharing and
145 phenotypic heterogeneity emerge from interactions among individuals that share high genetic
146 relatedness: on average, the population undergoes fewer than 10 rounds of division prior to
147 rupture.

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149 To define the physiological differences between sub-populations, we profiled global transcription
150 at different stages of 12B01 growth. The synchronous timing of cluster development in our
151 experimental setup enabled collection of RNA from cells in early stage ii, cells released from late-
152 stage iii clusters, and cells remaining in the shells of late-stage iii clusters (Figure 2A). These
153 samples were chosen to maximize the homogeneity within the sampled population, and to capture
154 populations from distinct stages of growth. Only 3% of the 12B01 transcriptome was differentially
155 expressed between stage ii and stage iii free-living populations (Figure 2B, Table S1). About 10%



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Figure 2. Transcriptional and physiological differences that define cluster sub-populations. A) Histograms showing the change in the distribution of cluster radii at different timepoints of 12B01 growth on alginate. 'Stage ii' represents a timepoint when most clusters are in the first stage of development, and 'Stage iii' represents a timepoint when most clusters are in stage ii of development. Clusters measured per timepoint: Stage ii=1085, Stage iii=6105. **B)** Pair-wise comparisons of normalized gene expression, showing patterns of differentially expressed genes that characterize each sample type. Points represent individual genes, plotted as the mean $\ln(\text{TPM})$ for all replicates; stage iii free-living samples, $n=2$, stage iii shell and stage ii samples, $n=3$. Diagonal lines indicate threshold 2-fold differential expression. Highlighted genes are discussed in the text, and differentially expressed in the comparison. See Table S1 for detailed information on all differentially expressed genes, including P-values for fold change expression, and TPM (Transcripts per Kilobase Million) values for differentially expressed genes. **C)** Normalized transcript abundance (TPM) for gene clusters encoding key metabolic functions: alginate catabolism (left), type IV *tad/flp* pili (middle), carbon storage (right). Error bars are the standard deviation of 3 replicates (stage ii and stage iii shell samples), or 2 replicates (stage iii free-living samples). **D)** Accumulation of polyhydroxyalkanoate (PHA) in a subset of cells making up 12B01 clusters. Composite image showing localization of PHA (C. storage) in eGFP-labeled 12B01 (cells). Three distinct patterns of carbon storage granule formation are shown inset, right. Scale bar main image= 50 μm , scale bar inset=20 μm . **E)** Quantification of image in 2D, showing radial profiles of carbon storage intensity, normalized by cellular expression of GFP. Each trace represents a measurement from an individual cluster. Measurements are made from the cluster center (zero) to the periphery. Vertical dashed line notes 17 μm radial distance. Traces are grouped by their pattern of PHA accumulation: numbers indicate the patterns represented by inset images in Figure 2D. First panel, $n=22$, second panel $n=13$, third panel $n=7$. **F)** Transcriptional activity of *tad/flp* pili within clusters at timepoints during growth of 12B01 on alginate. Each data point represents the mean activity measured within an individual cluster. Timepoints were taken in the transition from most clusters being in stage ii to most clusters being in stage iii.

181 of the transcriptome was differentially expressed more than 2-fold between samples and those
182 collected from stage iii clusters and either of the other sample types (Figure 2B). Although the
183 transcriptional state of free-living stage iii populations is likely to differ from that of cells belonging

184 to the motile core of 12B01 clusters, samples were collected no more than 30 minutes after the
185 first signs of rupture, as assessed by microscopy. Thus, the free-living population is likely to reflect
186 some aspects of the transcriptional state of the 'core' subpopulation directly prior to rupture. We
187 compared all pairs of samples to define sets of genes differentially more than 2-fold between
188 conditions (Table S1).

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190 Genes differentially expressed in stage ii pointed to cellular processes underlying the fast growth
191 and mixing observed in this stage. Relative to stage iii shell samples, stage ii samples more highly
192 expressed genes encoding flagella (V12B01_05395-V12B01_05425), F1F0 ATP synthase
193 (V12B01_01872-V12B01_01902), and the metabolic enzyme Eda (V12B01_24199): a key
194 aldolase in the conversion of alginate-derived uronic acids into glycolytic metabolites via the
195 Entner-Doudoroff pathway (Figure 2B, Figure 2C). Relative to either stage iii sample type, stage
196 ii samples also transcribed lower levels of genes involved in alternative electron acceptor
197 utilization, such as fumarate reductase (*frd* V12B01_23345-V12B01_23360), and nitrate
198 reductase (*nrf*, V12B01_23499-V12B01_23529) (Figure 2B). In *Escherichia coli*, ATP synthase
199 genes are subject to growth-rate dependent transcriptional regulation (Kasimoglu et al., 1996),
200 and the *eda* gene is transcribed in response to uronic acids (Murray and Conway, 2005). By
201 analogy to *E. coli*, the transcriptional activation of F1F0 ATP synthase and *eda* in stage ii relative
202 to the stage iii shell could reflect more active growth on alginate oligosaccharides. In addition, the
203 lower transcription of alternative electron acceptor genes in stage ii suggests that cells in this
204 stage have more access to oxygen than cells in stage iii. Culturing 12B01 on alginate under anoxic
205 conditions revealed that a consequence of oxygen limitation was diminished yield after 24 h of
206 growth (Figure S1C). Together, the comparison of growth phenotypes and transcriptional
207 signatures in stage ii suggest that the rapid growth and visible motility within stage ii clusters may
208 be due to availability of both alginate and oxygen.

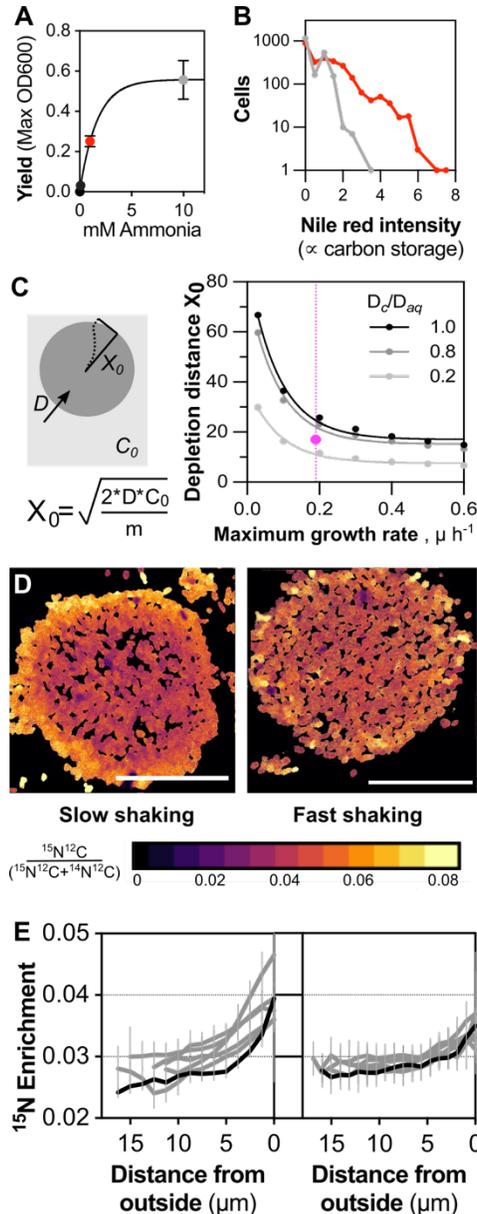
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210 Genes differentially expressed at least 2-fold in the stage iii shell pointed to differences in alginate
211 catabolism and cellular adhesion. Cells remaining in clusters during stage iii transcribed lower
212 levels of alginate catabolism genes (V12B01_24194-V12B01_24214 and V12B01_24234-
213 V12B01_24274) relative to other samples (Figure 2B, Figure 2C). Additionally, stage iii shell
214 samples highly expressed a cluster of genes encoding a putative type IV Tad/Flp pilus
215 (V12B01_22446- V12B01_22511, Figure 2C). Type IV pili are known to mediate adhesion in a
216 variety of bacteria (McCallum et al., 2019), including social behaviors such as twitching motility in
217 *Pseudomonas aeruginosa* (Burrows, 2012) and gliding in *Myxococcus xanthus* (Wu and Kaiser,
218 1995). To validate the type iii shell-specific transcription of *tad/flp* genes, we localized cellular
219 transcription within 12B01 clusters using a fluorescent reporter construct (Figure S3A,B).
220 Consistent with the transcriptional profiles, we found that transcription of *tad/flp* pilus genes were
221 elevated in cells associated with late-stage iii clusters, relative to stage ii (Figure 2F). Together,
222 the decreased transcription of alginate catabolism genes and increased transcription of *tad/flp*
223 pilus prompted us to ask whether transcriptional regulation of this type IV pilus was growth-rate
224 dependent. Transcriptional activity of *tad/flp* increased in cells as growth in batch culture entered
225 stationary phase, a pattern not shared by the synthetic *tac* promoter (Figure S3C). The specific
226 activation of the *tad/flp* promoter during stationary phase growth suggests that the transcriptional
227 regulation of this gene cluster is responsive to cellular growth state. Together, these results

228 suggest that the increase in transcription of the *tad/flp* pilus genes during development of 12B01
229 clusters may be linked to decreased availability of alginate-derived carbon and slower growth in
230 the shell sub-population of stage iii clusters.

231
232 Differential gene expression specific to stage iii free-living samples highlighted genes related to
233 nitrogen assimilation and carbon storage. These genes included ammonium transport
234 (V12B01_25139), urease (V12B01_09226, V12B01_09231) glutamine synthase *glnA*
235 (V12B01_02370), and nitrogen responsive regulator *glnGL* (V12B01_02360, V12B01_02355)
236 (Table S1). In *E. coli*, transcription of *glnG* and *glnL* is activated by nitrogen limitation, and this
237 regulator activates transcription of nitrogen assimilation genes (Pahel et al., 1982), suggesting
238 that stage iii free-living cells may be nitrogen limited. A polyhydroxyalkanoate (PHA) polymerase
239 (*phaC*, V12B01_15111), and associated PHA biosynthetic genes (*phbA* V12B01_15121, *phbB*
240 V12B01_15126), were also highly transcribed by stage iii free-living samples (Figure 2B, Figure
241 2C). Together, *phbA*, *phbB*, and *phaC* encode machinery to synthesize carbon storage organelles
242 called carbonosomes (Greening and Lithgow, 2020; Jendrossek, 2009). While *phbA* and *phbB*
243 expression were highest in stage iii free-living samples, expression of these genes was also
244 elevated in stage iii shell samples (Figure 2C). In contrast, *phaC* expression was found to be
245 significantly elevated only in stage iii free-living samples (Figure 2C). Together, these results
246 suggest that the ability to polymerize carbonosomes is a specific attribute of a sub-population of
247 stage iii cells. We experimentally validated the prediction that carbon storage granules
248 differentiate subpopulations within the cluster by staining clusters with the lipophilic fluorescent
249 dye Nile Red (Figure 2D). We found that cells stained brightly by Nile Red, the presumptive PHA-
250 storing sub-population, were indeed localized within the cluster core. Carbon storage was
251 detectable only when clusters grew to a size larger than 17 μm in radius (Figure 2E),
252 corresponding to late stage ii, as predicted from the global transcriptional profiles (Figure 2C).
253 Together, these observations suggest that the microenvironment in the core of cell clusters, in
254 which alginate-derived carbon is available in excess but ammonia-derived nitrogen is limiting,
255 induces accumulation of PHA as clusters grow larger.

256
257 The accumulation of PHA within cells in the core of clusters, combined with transcriptional
258 signatures hinting at nitrogen limitation in stage iii free-living cells, led us to hypothesize that
259 nitrogen availability cues cells to accumulate carbon storage polymer. We tested this idea by
260 measuring the yield of 12B01 during growth on alginate oligomers under differing concentrations
261 of ammonium to define nitrogen-limited growth conditions (Figure 3A). Cells grown under nitrogen
262 limitation and carbon excess accumulated more PHA, measured by intensity of Nile Red staining
263 (Figure 3B). Numerical estimates and direct measurement of ammonium assimilation using stable
264 isotope probing combined with nanoscale secondary ion mass spectrometry (NanoSIMS) showed
265 that N gradients emerged within 12B01 cell clusters, and that the emergence of gradients
266 occurred at a size predicted by cellular consumption rate. Importantly, our numerical estimate
267 assumed that cells in clusters were phenotypically undifferentiated and did not move, a
268 simplification that allowed us to approximate the length scale over which cellular consumption
269 depleted ammonium supplied by diffusion from outside the cluster (Figure 3C). For clusters
270 growing at the maximum observed rate of 0.2 h^{-1} , radial cluster size reached 15-20 μm before
271 cellular consumption depleted ammonium from the center of clusters (Figure 3C). Using

272 NanoSIMS, we quantified the incorporation of heavy-labeled nitrogen into cellular biomass
 273 following four hours of incubation with ^{15}N -ammonium (see methods, Figure S4). The NanoSIMS
 274 measurements revealed that cellular uptake of ammonium decreased towards the center of cell
 275 clusters (where PHA accumulated). The outer layers of cells assimilated more than twice the
 276 amount of ammonium than cells in the core of clusters (Figures 3D-E). The qualitative agreement
 277 between the gradient estimates and direct measurements of ammonium assimilation into biomass
 278 support the idea that cellular consumption creates local ammonium limitation within the center of
 279 growing clusters, favoring PHA accumulation.



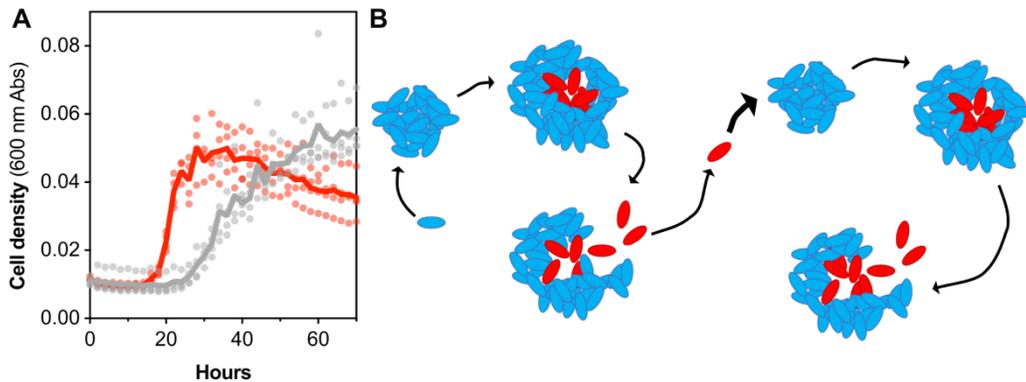
280 **Figure 3. Carbon-storing inner sub-populations are nitrogen limited.** **A)** Yield of 12B01 grown on alginate
 281 oligosaccharides and varying amounts of ammonium provided as the sole source of nitrogen. Grey dot indicates
 282 ammonia-replete growth, red dot indicates ammonia-limited growth. Error bars: standard deviation, $n=3$. **B)** PHA
 283 content under ammonia limitation. Measurements are of individual cells stained by Nile Red staining, made on
 284 populations analyzed in panel A. Grey: population grown under ammonia replete conditions; red: population growth
 285 under ammonia limited conditions. **C)** Estimates of gradients emerging within clusters due to cellular consumption of
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287 ammonium as it diffuses in from the bulk medium. Steady state approximation derived as in (Crank, 1975). X_0 =
288 distance from outer surface where no ammonium remains, D_{aq} = diffusion coefficient of ammonium in water, D_c/D_{aq} =
289 ratiometric difference between diffusion coefficient in water and in the extracellular space of cell clusters estimated
290 from (Stewart, 2003), C_0 =concentration of ammonium ion in bulk medium, m =per cell consumption of ammonium, μ =
291 cellular growth rate. Pink dot indicates the 17 μm radius size threshold from Figure 2E where cells inside clusters
292 begin to express carbon storage granules. Vertical pink dashed line indicates the growth rate of clusters with slow
293 shaking (pink). Cellular consumption was estimated from the yield of 12B01 grown on alginate oligomers under
294 ammonium limitation (Figure 1A), and from measurements of cell size in clusters. Length scale estimates were
295 calculated for cellular growth rates ranging from the maximum rate of exponential growth on alginate oligomers (0.6 h^{-1}),
296 to the maximum rate of 12B01 cluster growth (0.2 h^{-1} , Figure 1D). **D**) ^{15}N -ammonium assimilation within stage ii
297 clusters formed under slow shaking (left), or fast shaking (right) measured by nanoscale secondary ion mass
298 spectrometry. Representative secondary ion images show spatial patterns of ^{15}N enrichment in an aggregate
299 (fractional abundance ^{15}N), where warmer colors represent higher levels of enrichment. Cellular N assimilation is
300 defined from heavy ammonium uptake measured as fractional abundance ($^{15}\text{N}^{12}\text{C} / (^{14}\text{N}^{12}\text{C} + ^{15}\text{N}^{12}\text{C})$). Scale bars= 20
301 μm . **E**) Radial profiles of ^{15}N -ammonium enrichment within 12B01 clusters grown under slow (left, $n=6$) or fast (right,
302 $n=7$) shaking. Bars indicate standard deviation of pixel intensity. Black traces indicate profiles for images shown in
303 3D. Samples were incubated with ^{15}N -labeled ammonium for 4 h.

304
305 To test the idea that cooperation for carbon and competition for nitrogen create nutrient gradients
306 and cue self-organized formation of shell and core structures, we sought to perturb the
307 environmental context in which competition and cooperation take place. As we had already
308 established that the addition of alginate oligomers to the growth environment profoundly changes
309 cellular interactions, we instead sought to shift gradients more subtly. It follows from our prediction
310 of gradients emerging from diffusive supply and local cellular uptake (Figure 3C), that decreasing
311 cellular uptake will flatten gradients of ammonia within clusters. We found that increasing the
312 shaking speed at which we grew 12B01 clusters decreased the cellular growth rate and reasoned
313 that this decrease might also decrease cellular demand for ammonium. Consistent with this
314 expectation, NanoSIMS profiling of clusters formed under fast shaking revealed decreased ^{15}N -
315 ammonium assimilation across their radial profile of clusters and no detectable gradient in
316 assimilation (Figure 3E, Figure S5). Notably, clusters formed under fast shaking not only grew
317 slower, but they also failed to differentiate into motile 'core' and immobile 'shell' sub-populations.
318 Instead, only a much smaller sub-population of cells developed motility. Thus, shifting cellular
319 physiology by changing environmental context perturbed both gradient formation and the
320 emergence of complex self-organized structures during growth of 12B01 on alginate.

321
322 What does the ability to phenotypically differentiate in response to nitrogen limitation contribute
323 to 12B01 clusters? Previous work has suggested that the emergence of mixing in the core of
324 clusters can collapse resource gradients (Ebrahimi et al., 2019a). Mixing would thus homogenize
325 allocation of carbon and nitrogen among cells in the core sub-population. But this explanation
326 does not account for the accumulation of PHA. To determine whether the ability to store carbon
327 enhanced the ability of 12B01 cells to form new clusters on alginate, we derived populations with
328 high or low proportions of PHA-rich cells (Figure 3B) and compared the growth of these two
329 populations on alginate polysaccharide (Figure 4A). The high PHA population resumed growth on
330 alginate more readily than the population with fewer PHA-rich cells. Moreover, high PHA
331 populations grew at a faster rate than low PHA populations. Together, the faster resumption of
332 growth on alginate and faster population-level growth rate suggest that carbon storage
333 accelerates reproductive cycles during growth of 12B01 on alginate. Thus, phenotypic

334 differentiation of sub-populations may balance cellular conflict and cooperation within aggregates
335 through mixing and may enhance reproduction of clonal lineages by promoting carbon storage.
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337
338 **Figure 4. Carbon storage enhances propagation of 12B01 on alginate polysaccharide.** A) Growth on alginate
339 polymer by 12B01 populations with high or low proportions of PHA-rich cells. Populations were derived by growing
340 12B01 on alginate oligosaccharides with 1 mM or 10 mM ammonium chloride as the sole nitrogen source. The two
341 populations were then used to inoculate polymeric alginate cultures containing 10 mM ammonium. Blue, high PHA
342 population formed under ammonia-limited growth on alginate oligosaccharides; red, low PHA population formed
343 under ammonia-replete growth on alginate oligosaccharides. Proportion of PHA in each population quantified in
344 Figure 3B. All replicates are shown, solid line represents mean of replicates. B) Proposed 'reproductive cycles'
345 supporting cooperative growth of 12B01 growth on alginate. Cells form clonal clusters. Local density promotes growth
346 on alginate polysaccharide in a carbon-limited environment. As clusters grow, they phenotypically differentiate into
347 'shell' (grey) and 'core' (blue) sub-populations in response to resource gradients. Clusters rupture, releasing carbon-
348 storing 'core' sub-population. Cells carrying high levels of PHA exit stage i faster and initiate a new cycle of self-
349 organization.

350 351 **DISCUSSION**

352 Phenotypic heterogeneity is a property of most clonal populations of bacteria (Ackermann, 2015;
353 Dar et al., 2021; Nadell et al., 2016). Where this heterogeneity allows cells to perform specific and
354 synergistic tasks, it can give rise to a phenomenon called division of labor. We propose that the
355 phenotypic differentiation of 12B01 cells during cooperative growth on alginate falls into the
356 framework of a division of labor. We show that gradients of resource limitation arising from cellular
357 conflict and cooperation cue the expression cell-surface adhesins and the accumulation of
358 carbon-storage granules in distinct sub-populations within clusters (Figure 4B). These sub-
359 populations form a structural shell, and a motile, carbon-storing inner core. The ability of the inner
360 sub-population to store carbon enhances propagation of clonal lineages upon dispersal (Figure
361 4B). It is well known that bacteria integrate environmental cues and gradients into the regulation
362 of biofilm formation (Gerstel and Römling, 2001; Glick et al., 2010; Zacharia et al., 2021).
363 However, it remains challenging to demonstrate that heterogenous cell sub-populations interact
364 synergistically; outside of elegant experiments that have engineered such interactions in the lab
365 (Dragoš et al., 2018), multicellular behaviors (Geerlings et al., 2020; van Gestel et al., 2015) and
366 infection (Ackermann et al., 2008), few natural examples of bacterial division of labor are known.
367 Our work demonstrates that this barrier in understanding can be overcome by studying bacteria
368 in micro-scale ecological contexts that favor growth of collectives over individuals; such
369 environments are typically resource limited, in contrast to the nutrient rich growth environments
370 favored by laboratory studies.

371
372 The formation and rupture of the multicellular structures described in this work are reminiscent of
373 the formation and dispersal of surface-attached biofilms (Hunt et al., 2004; Kaplan et al., 2003;
374 Mai-Prochnow et al., 2004; Purevdorj-Gage et al., 2005; Rumbaugh and Sauer, 2020; Sauer et
375 al., 2002; Stewart et al., 2007). This similarity extends beyond the formation of convergent
376 multicellular structures: our work demonstrates that type IV pili are involved in the formation of
377 12B01 clonal groups, like the role that these dynamic cellular adhesins play in microcolony
378 formation. Interestingly, many type IV pilins have been shown to transfer cargo, such as DNA or
379 bacteriophage (McCallum et al., 2019). Further work will be needed to determine if the Tad/flp
380 type IV pilus expressed by 12B01 shell cells performs cellular functions other than adhesion. In
381 addition, the emergence of a motile core in 12B01 clonal groups recalls a well-known dispersal
382 phenotype of proteobacterial biofilms sometimes called hollowing (Rumbaugh and Sauer, 2020).
383 We show that hollowing emerges in 12B01 clonal groups in the context of a resource imbalance,
384 which promotes formation of carbon storage granules. Although this physiological adaptation is
385 likely to provide an ecological benefit only if cells disperse into carbon-limited environments,
386 hollowing may more generally promote the re-allocation of cellular resources towards a sub-
387 population of cells that will disperse as propagules. More work will be needed to determine
388 whether phenotypic differentiation is a conserved strategy of resource allocation in natural
389 bacterial populations.

390
391 The ability to form large, self-organized clusters and undergo reproductive cycles with coordinated
392 bursts of dispersal may shape the ecological dynamics of 12B01. We do not know whether this
393 strain can form similar structures in coastal seawater, however, the concentrations of alginate and
394 ammonium chloride used in our experimental model fall within what can accumulate in coastal
395 seawater during algal blooms (Martin-Platero et al., 2018). Blooms are also characterized by
396 waves of bacteriophage infection. Biofilms and other bacterial structures have been proposed as
397 barriers to phage infection (McDougald et al., 2011; Rumbaugh and Sauer, 2020; Vidakovic et
398 al., 2018). Thus, the highly structured clusters formed by 12B01 may provide a barrier to
399 bacteriophage infection, particularly of the cells in the interior of clusters. Blooms are also
400 accompanied by increases in bacterivores such as protists. The grazing efficiency of protists has
401 been shown to depend on the size and structural integrity of their prey, thus, the formation of
402 dense 40 μm groups may enhance resistance to predation (Pernthaler, 2005). Future work will
403 be needed to determine whether, in addition to accelerating cycles or reproduction, formation of
404 phenotypically differentiated multicellular groups also enhances resistance to bacteriophage or
405 protist predators.

406
407 The division of labor, emergence of complex self-organized structures, and clonality that
408 characterize 12B01 growth on alginate are attributes shared by simple multicellular organisms
409 (Szathmary and Smith, 1995). In this context, the ability of 12B01 to grow in resource-limited
410 environments as clonal structures is particularly intriguing: simulations have suggested that
411 microbial decomposition is constrained both by resource availability and by the evolution of cheats
412 (Allison, 2005). We speculate that 12B01's ability to form clonal, rather than aggregative,
413 structures that phenotypically specialize in response to resource limitation stabilizes this strain's
414 strong cooperation, much like clonality stabilizes the evolution of complex multicellularity by

415 minimizing cheating and evolutionary conflict (Márquez-Zacarías et al., 2021; Pentz et al., 2020).
416 This parallel suggests that 12B01 may be a useful experimental model in which to study the
417 evolution of multicellular behaviors such as reproductive specialization.

418

419 **METHODS**

420 **Strain information and culture conditions**

421 *Vibrio splendidus* strain 12B01 (Le Roux et al., 2009) was maintained in Marine Broth (Difco 2216),
422 as a liquid culture, or on medium solidified with 1.5% agarose. Unless otherwise specified,
423 incubation temperature was 25°C. For experimental measurements, precultures were established
424 in a defined minimal medium (Ebrahimi et al., 2019b) containing 10 mM ammonium as sole source
425 nitrogen, and an indicated carbon source, using a previously described protocol (Amarnath et al.,
426 2021). Precultures were grown with 10 mM glucose to an optical density of 0.4-0.6, as measured
427 at 600 nm with a 1 cm path cuvette on a Genesys 20 Spectrophotometer (Thermo Scientific,
428 Waltham, MA), rinsed by pelleting cells at 5000 rcf for 1 min in a tabletop microcentrifuge
429 (Eppendorf, Hamburg, Germany), and resuspended in carbon-free defined minimal medium such
430 that the final was 1.0. Experimental cultures were initiated by inoculating cells to an initial OD of
431 0.01 in 250 mL borosilicate glass Erlenmeyer flasks containing 70 mL of alginate minimal
432 medium. Low viscosity alginate (Sigma-Aldrich A1112, St. Louis, MO) was added as the medium
433 carbon source at 700 mg/L (0.07% w/v). 'Fast' shaking was established by orbital rotation of flasks
434 at setting 4 on an orbital shaker (Model 3500, VWR, Radnor, PA), which corresponds to ~200
435 rpm, 'Slow' shaking was established at a setting of 2, corresponding to ~100 rpm. To pre-digest
436 alginate polysaccharide, 0.01 µg/mL commercial alginate lyase (Sigma-Aldrich A1603, 0.75"
437 rotational orbit) was added to culture medium and incubated overnight with stirring at 37°C.
438 Alginate lyase was removed from culture medium prior to assay by filtering the medium through
439 3 kDa Amicon Ultra molecular weight cutoff filters (MilliPore Sigma, Burlington, MA). To maintain
440 plasmids in 12B01, 12.5 µg/mL chloramphenicol was added to the medium.

441

442 **Constructs and validation**

443 Multicopy plasmids expressing eGFP or mKate2 from the synthetic P_{tac} promoter were
444 constructed in previous work (Pollak et al., 2020). These constructs encoded P_{tac} -eGFP/mKate,
445 the chloramphenicol resistance gene *cat* and the segment of plasmid pVSV208 released by
446 restriction digest by *SpeI* and *SphI*, which contains an origin of transfer for RP4 conjugation (Dunn
447 et al., 2006), as well as an origin of replication from a *Vibrio fischeri* plasmid that is stably
448 maintained in several species of *Vibrio* (Le Roux et al., 2011).

449 To construct a transcriptional reporter for the *tad/flp* locus, we mapped mRNA-Seq reads
450 onto the region of the 12B01 chromosome encoding the 17 open reading frames of the locus,
451 revealing coverage above background levels of read mapping across the cluster. We observed
452 elevated mapping of genes encoding the pilin and inner-membrane assembly complex (Figure
453 S2A). Tad/Flp are a family of type IV pili with broad distribution across bacteria and archaea
454 (Giltner et al., 2012). In the genus *Vibrio*, the genetics and biochemistry of Tad/Flp pili are best
455 characterized in *V. vulnificus* (Ellison et al., 2019; Pu and Rowe-Magnus, 2018). 12B01 encodes
456 a single *tad/flp* locus, which contains homologs of Flp pilin, the inner membrane assembly
457 complex Tad, and the outer membrane motor Rcp all encoded on the minus strand. Accordingly,
458 we mapped the ribosome binding site associated with the putative pilin ORF V12B01_22511 and

459 used 142 bp segment of DNA falling upstream of this site to promote transcription in our reporter
460 construct. The reporter was engineered to express the red fluorescent protein mKate2 from the
461 *tad/flp* promoter by cloning this segment of DNA into plasmid pLL103 through digest by KpnI and
462 SphI restriction enzymes (NEB, Ipswich, MA) to generate pJS2020.1.

463 The activity of the promoter was confirmed by measuring mKate fluorescence as a
464 function of growth on a Tecan Spark plate reader (Tecan). We performed titration experiments to
465 ensure that the amount of chloramphenicol used to maintain positive selection (12.5 µg/mL) was
466 sufficient to prevent plasmid loss and did not alter growth kinetics or self-organization relative to
467 a no-antibiotic control (Figure S2B). For these experiments, strains were grown in 10 mM glucose,
468 10 mM ammonium minimal medium with shaking at 25°C.

470 **Experimental manipulation of PHA expression in 12B01 populations.**

471 Nitrogen limitation was used as a cue to induce the expression of carbon storage granules in
472 12B01. Cells were cultured as described above, except that precultures were established in
473 0.07% alginate digest minimal medium containing 1 mM or 10 mM ammonium chloride. The
474 eGFP-expressing version of 12B01 (carrying plasmid pLL104) was cultivated under these
475 conditions. The fraction of cell populations expressing carbonosomes was quantified by Nile Red
476 staining.

478 **Light microscopy- staining conditions**

479 Self-organization of 12B01 was visualized using a ImageXpress high content microscope
480 equipped with Metamorph Software (Molecular devices, San Jose, CA), operating in widefield
481 mode. Images were acquired in widefield mode at 40x with a Ph2 ELWD objective (0.6 NA, Nikon),
482 and filter sets: Ex 482/35 nm, Em: 536/40 nm, dichroic 506 nm to detect eGFP; Ex: 562/40 nm,
483 Em:624/40 nm, dichroic 593 nm to detect mKate, Nile Red, and Propidium Iodide (Invitrogen);
484 Ex:377/50 nm Em: 447/60 nm dichroic 409 nm to detect TOTO-3 (Invitrogen). The excitation light
485 source was LED lines from a Lumencore light engine set to 100% of their maximum intensity.
486 Images were acquired with a sCMOS detector (Andor Zyla, Andor, Belfast, North Ireland). Unless
487 otherwise noted, raw images were the average of 8 frames, and were collected with exposure
488 times of 100 ms.

489 Higher resolution images of cells in 12B01 structures were acquired using a Yokogawa
490 CSU-22 spinning disk confocal light path, installed on a Zeiss AxioVert 200M inverted microscope
491 with DIC optics (Zeiss, Jena, Germany). Images were acquired using Metamorph software, with
492 63x or 100x 1.4 NA Plan Achromat oil objectives and illumination from a 488 nm 150 mW OPAL
493 excitation laser with a 525/50 nm bandpass emission filter and 488 nm dichroic. Unless otherwise
494 noted, images were collected with exposure times of 100 ms, and processed with ImageJ,
495 (Version 2.1.0/1.53h).

496 To visualize DNA, samples of live culture were stained with 5 µM of the DNA-binding dye
497 SYTO9 (Invitrogen), which emits green fluorescence when bound to DNA. Extracellular DNA was
498 stained as described previously (Jemielita et al., 2018) except that 1 µM TOTO-3 iodide was used
499 in place of TOTO-1 iodide (fluorescent molecules are both cell-impermeant DNA stains, but with
500 different excitation/emission properties). Dead cells were detected by staining with 20 µM
501 Propidium iodide. PHA accumulation within cells was stained with 0.5 µg/mL lipophilic fluorescent
502 dye Nile Red (Spiekermann et al., 1999). Dye was resuspended in DMSO at 1 mg/mL, and a fresh

503 10 µg/mL working solution was created for each experiment to enhance solubility in experimental
504 medium.

505

506 **Analysis of light microscopy images**

507 Custom analysis scripts were written to segment 12B01 aggregates to measure aggregate
508 growth, cell composition, expression of transcriptional reporters, and accumulation of PHA.
509 Scripts and raw data are available on the Github repository for this project
510 (github.com/jaschwartzman/12B01.git).

511 Aggregate growth (plotted in Figure 1D) was measured by segmenting images of
512 aggregates obtained by brightfield microscopy. An intensity-based threshold was used to define
513 the 2-dimensional area of aggregates, and this area was quantified. To convert from segmented
514 2D area to an estimate of cells per aggregate, cells were assumed to be cylinders of radius 0.5
515 µm and length 3 µm (volume 3 µm³), aggregates were assumed to be spherical with no space
516 between cells. A radius was derived from the 2D area and used to calculate aggregate volume
517 then convert to number of cells per aggregate.

518 Composition of aggregates (plotted in Figure 1F) was measured by segmenting images
519 of eGFP and mKate expressing cells. A brightfield image was used to create a mask that defined
520 aggregates, and the intensity of eGFP or mKate expression was quantified within each area.

521 The mean radial intensity profile of PHA accumulation (Figure 2E) was quantified
522 measuring mean intensity of Nile Red fluorescence, or cell-associated eGFP signal within
523 concentric bands spaced from the center of aggregates to the periphery. Accumulation of PHA
524 was defined as an increase in the ratio of Nile Red fluorescence to GFP fluorescence.

525

526 **RNA isolation and RNA-Seq**

527 Cultures were grown an indicated stage of self-organization, as defined by microscopy. 30 mL
528 RNA Protect Bacterial Reagent (Qiagen, Hilden, Germany) was added to 15 mL cultures and
529 incubated at 4 °C overnight. Cells were collected by centrifugation at 5000 rcf and 4°C in a Sorvall
530 Legend XIR centrifuge (Thermo Fisher, Waltham, MA), equipped with a Fiberlite F15-6x100g rotor
531 for 30 min. Supernatant was decanted, leaving pelleted cell material which was frozen at -20°C.
532 A Qiagen RNeasy kit was used to isolate total RNA, following manufacturer's protocol, except for
533 the following modifications: cells were resuspended in 15 mg/mL lysozyme in TE buffer, and
534 incubated for room temperature for 30 min, prior to addition of buffer RLT. Samples were lysed
535 by mechanical disruption using lysing matrix B (MPBio, Santa Ana, CA). Samples in lysing matrix
536 shaken in a homogenizer (MPBio) for 10X 30 second intervals, taking care to keep samples from
537 heating up. Samples were treated with DNase digestion using a Turbo DNA free DNase kit
538 (Ambion, Austin, TX), following manufacturer's instructions. The integrity and purity of total RNA
539 was assessed running a sample on an Agilent 4200 Tapestation in a HS RNA screen tape
540 (Agilent, Santa Clara, CA). Depletion of rRNA was accomplished using the Ribominus Yeast and
541 Bacteria transcriptome isolation kit (Invitrogen, Waltham, MA) and included ethanol precipitation
542 with glycogen to concentrate remaining mRNA. Ethanol precipitated samples were resuspended
543 in FPF sample buffer from the TruSeq stranded mRNA-Seq kit reagents (Illumina, San Diego,
544 CA). mRNA abundance was quantified using a Quant-iT RNA Assay kit (Invitrogen) on a Tecan
545 Spark plate reader equipped with a monochromator, following manufacturer's instructions (Tecan,
546 Männendorf, Switzerland). The quality of the mRNA was further assessed using an Agilent HS

547 tape. We analyzed resulting samples on a Bioanalyzer to confirm mRNA depletion, and
548 concentrated samples using ethanol precipitation with glycogen. Samples were screened for
549 quality by requiring an RNA integrity number (RIN) or more than 8. mRNA remaining from rRNA
550 depletion of 2-10 µg total RNA was used as template to create sequencing libraries. Sequencing
551 libraries were prepared using an Illumina TruSeq Stranded mRNA library preparation kit
552 (Illumina). Libraries were assessed for quality using screen tapes and fluorescence-based plate
553 reader assays prior to pooling. Libraries were sequenced at the Whitehead sequencing core
554 (Whitehead Institute, MIT) on an Illumina HiSeq in 60x60 paired-end mode.

555

556 **RNASeq data analysis**

557 Paired-end Illumina reads were trimmed using Trimmomatic (v0.30) to remove sequencing
558 adapters and low quality reads (phred >30)(Bolger et al., 2014). The remaining paired reads
559 were checked for quality using FastQC (version 0.11.9) (Brown et al., 2017) and mapped to the
560 predicted coding regions of the *V. splendidus* 12B01 genome (assembly ASM15276v1) using
561 Bowtie2 (v 2.3.4.1)(Langmead and Salzberg, 2012), outputting SAM files. SAM files were sorted
562 by position using SAMTools (V 1.9) (Danecek et al., 2021), and mapping was parsed using
563 HTSeq (Anders et al., 2015) to obtain count tables. Differential gene expression was assessed
564 from count tables DeSeq2 (Love et al., 2014) (run in R Studio Version 1.2.5042). Briefly, tables
565 were processed using a variance stabilizing transformation to assess clustering of replicates,
566 after which the statistical significance of log fold change between pairs of samples was
567 assessed using a Wald test. P values were adjusted using the Benjamin-Hochberg method, to
568 account for multiple tests in followed by a Wald test to determine differential gene expression in
569 log₂ normalized abundance. Normalized transcript abundance was calculated from count tables
570 with custom analysis scripts to implement the transcripts per kilobase million (TPM) method
571 (github.com/jaschwartzman/12B01.git for code). Predicted 12B01 coding regions were further
572 annotated using Egnogg mapper 2.1 to make functional inferences (Cantalapiedra et al., 2021;
573 Huerta-Cepas et al., 2019).

574

575 **Stable isotope amendment experiments and nanoSIMS analysis**

576 To trace the assimilation of ammonium into cellular biomass, 10 mM of ¹⁵N-labeled ammonium
577 (99% ¹⁵N, Cambridge Isotope Laboratories, Tewksbury, MA, USA) was added to cultures of 12B01
578 growing on 0.07% alginate. The cultures initially contained 10 mM of unlabeled ammonium as the
579 sole nitrogen source which was depleted upon labelled ammonium amendment. Sample
580 collection and processing was conducted following previously established protocols (McGlynn et
581 al., 2018), with minor modifications: Samples were collected after a 4 h incubation with the heavy-
582 labeled ammonium. Incubation time includes a 1 h step in which aggregates settle out. After
583 removal of supernatant, an equal volume of 4% paraformaldehyde mPBS (50 mM sodium
584 phosphate pH 7.4, 0.4 M NaCl) was added to the concentrated aggregate mixture for 45 min. 30
585 min of this time was static, to allow aggregates to settle out. Three 1-hour rinses with 1xmPBS
586 were performed, in which aggregates were incubated with rotation, then allowed to settle for 30
587 min. Following the final wash step, concentrated aggregates were combined with 1 part molten
588 9% noble agar in phosphate buffered saline. The agar-aggregate mixture was allowed to solidify
589 in a 1.5 mL centrifuge tube. Samples were stored at 4°C prior to processing. Immobilized samples
590 were dehydrated in ethanol and stored at -20 °C prior to embedding in Technovit 8100 resin

591 (Heraeus Kulzer GmbH) and continuing with the manufacturer's standard protocol. Embedded
592 samples were sectioned into 1 μm thick slices with a glass knife using a Lecia microtome and
593 mounted onto poly-l-lysine-coated glass slides (Tekdon, Myakka City, FL), sputter coated with
594 40nm of gold using a Cressington sputter coater. Samples were analyzed on a CAMECA
595 nanoSIMS 50L in the Center for Microanalysis at Caltech Data acquisition began with a pre-
596 sputtering step using a 90-pA primary Cs⁺ ion beam with aperture diaphragm setting of D1 = 1
597 until the ¹⁴N¹²C⁻ ion counts stabilized. ¹⁴N¹²C⁻ and ¹⁵N¹²C⁻ ions were imaged along with
598 secondary electron images. Data were collected using a 0.5-pA primary Cs⁺ beam with aperture
599 diaphragm setting of D1 = 3 and the entrance slit set at (ES) = 2. Dwell times ranged from 1-
600 10ms/pixel with acquisition area between 10 and 40 microns.

601 Raw images were processed with Look@nanosims (Polerecky et al., 2012) analysis
602 scripts, to convert secondary ion count data output from the CAMECA into a format readable by
603 MATLAB. Converted ¹⁴N¹²C and ¹⁵N¹²C secondary ion count data were further processed with
604 custom analysis scripts to calculate the ¹⁵N fractional abundance and to calculate the average
605 radial ¹⁵N fractional abundance. Where aggregates were imaged with multiple fields of view,
606 images were stitched together in ImageJ (Version 2.1.0/1.53h) using the Grid collection/stitching
607 plugin (internal version 1.2) (Preibisch et al., 2009). Image analysis code and raw data are
608 available in the Github repository for this manuscript (github.com/jaschwartzman/12B01.git).
609

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624

625 **AUTHOR CONTRIBUTIONS**

626 J.S., A.E, and O.X. designed the study, with help from V.O. and G.C, about the design of stable
627 isotope experiments. J.S, A.E., Y.S. and G.C. performed experiments. All authors contributed to
628 data analysis and writing the paper.
629

630 **COMPETING INTERESTS STATEMENT**

631 The authors declare no competing interests.
632

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