Emergent myxobacterial behaviors arise from reversal suppression induced by kin contacts

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Abstract

A wide range of biological systems – from microbial swarms to bird flocks, display emergent behaviors driven by coordinated movement of individuals. To this end, individual organisms interact by recognizing their kin and adjusting their motility based on others around them. However, even in the best-studied systems, the mechanistic basis of the interplay between kin recognition and motility coordination is not understood. Here, using a combination of experiments and mathematical modeling, we uncover the mechanism of an emergent social behavior in *Myxococcus xanthus*. By overexpressing cell surface adhesins, TraA and TraB, involved in kin recognition, large numbers of cells adhere to one another and form organized macroscopic circular aggregates that spin clockwise or counterclockwise. Mechanistically, TraAB adhesion results in sustained cell-cell contacts that trigger cells to suppress cell reversals, and circular aggregates form as the result of cells' ability to follow their own cellular slime trails. Furthermore, our *in-silico* simulations demonstrate a remarkable ability to predict self-organization patterns when phenotypically distinct strains are mixed. For example, defying naïve expectations, both models and experiments found that strains engineered to overexpress different and incompatible *traAB* allelles nevertheless form mixed circular aggregates. Therefore, this work provides key mechanistic insights into *M. xanthus* social interactions and demonstrates how local cell contacts induce emergent collective behaviors by millions of cells.

Importance

In many species, large populations exhibit emergent behaviors whereby all related individuals move in unison. For example, fish in schools can all dart in one direction simultaneously to avoid a predator. Currently, it is impossible to explain how such animals recognize kin through brain cognition and elicit such behaviors at a molecular level. However, microbes also recognize kin and exhibit emergent collective behaviors that are experimentally tractable. Here, using a model social bacterium, we engineer dispersed individuals to organize into synchronized collectives that create emergent patterns. With experimental and mathematical approaches we explain how this occurs at both molecular and population levels. The results demonstrate how the combination of local physical interactions triggers intracellular signaling, which in turn leads to emergent behavior on a population scale.

Introduction

Living systems display remarkable spatial organization patterns from molecules to cells to populations (1, 2). These patterns are a hallmark of emergent behaviors whereby complex functions arise from simple local interactions. For instance, at the cellular level, we have a relatively good understanding of neuron function, but how a collection of neurons integrates into a functional brain is poorly understood. In other cases, emergent behaviors are driven by the coordinated movement of system parts, as seen in the collective motion of insect swarms or bird flocks (3). Inherent in these processes is the ability of individuals to recognize their kin through brain cognition and adjust their movements relative to others around them. Despite much interest in emergent behaviors, the molecular and mechanistic basis of the interplay between kin recognition and the coordination of movements is poorly understood.

The Gram-negative gliding bacterium *Myxococcus xanthus* is a leading model for studying the molecular basis of microbial kin recognition and, separately, for understanding how cells coordinate their movements (4, 5). These microbes are unusually social and exhibit numerous emergent behaviors. Among these are the formation of traveling wave patterns, termed ripples, in which millions of cells self-organize into periodic, rhythmically moving bands (6-8) and, under starvation conditions, aggregate into multicellular fruiting bodies (9, 10). Notably, these emergent social behaviors form from incredibly diverse microbial populations in soil (11), where *M. xanthus* employs kin discrimination to assemble clonal populations and fruiting bodies (12-14). Central to these social behaviors is the ability of cells to control their direction of movement. These long rod-shaped cells tend to align in dense populations (9, 15) and move along their long axis periodically reversing their motion polarity – head becomes tail and vice versa. Cellular reversals are in turn largely controlled by the Frz chemosensory signal transduction pathway (5). Although much progress has been made in myxobacteria biology, a comprehensive and broadly accepted model that explains their self-organization behaviors and kin discrimination is lacking.

One system *M. xanthus* uses to discriminate against non-kin is based on outer membrane exchange (OME) (13). Here, cells recognize their siblings through cell-cell contacts mediated by a polymorphic cell surface receptor called TraA and its cohort protein TraB. TraAB functions as an adhesin, and cells that express identical TraA receptors adhere to one another by homotypic binding, while cells with divergent receptors do not (16, 17). Following TraA-TraA recognition cells bidirectionally exchange outer membrane proteins and lipids (18). The exchange of diverse cellular cargo, including polymorphic toxins, plays a key role in kin discrimination and facilitating cooperative behaviors (19-21). For these, among other reasons, TraAB-mediated OME in myxobacteria serves as a promising model for emergent behavior control; however, whether and how this actually occurs is unknown.

63 In this study, we investigate the interplay between TraAB-mediated cellular adhesion and motility
64 coordination. Specifically, elevated cell-cell adhesion forces through overexpression of TraAB drive
65 emergent behaviors involving coordinate movements of thousands to millions of cells. To mechanistically
66 understand this emergent behavior, we recapitulated these behaviors in agent-based simulations that
67 mathematically and mechanistically elucidate how these new behaviors emerge. Specifically, we deduced
68 that an intracellular signal arising from sustained cell-cell contacts, mediated by the TraAB adhesins, results
69 in suppression of cellular reversals and thereby allows millions of cells to move as a uniform collective.

70 Results

71 TraAB overexpression creates emergent circular aggregate behavior.

72 TraAB cell surface receptors govern allele-specific cell-cell adhesion. When TraAB was overexpressed,
73 cells adhered both end-to-end and side-by-side during shaker flask cultivation (Fig. 1) (16, 19). As
74 myxobacteria are motile on surfaces by adventurous (A) and social (S) gliding motility (22), we sought to
75 understand if TraAB-mediated adhesion affects their collective movements. To clearly assess the impact of
76 cellular adhesion on emergent group behaviors, TraAB adhesin was overproduced from a single copy
77 chromosomal locus in an A^+S^- background (Δ*pilA*), since S-motility promotes extracellular matrix
78 production that complicates analysis. When these cells (hereafter TraAB OE cells) were placed on agar,
79 they displayed an emergent behavior, where thousands of cells self-organized into macroscopic circular
80 aggregates (CAs) (Fig. 1). Initial signs of CAs were easily seen 4 h after cell plating and were prominent
81 by 8-12 h (Fig. S1A and the corresponding Movie S1). Following extended incubation periods, CAs
82 enlarged to millimeters in diameter with each containing millions of cells. In contrast, the parent strain
83 (A^+S^-, here referred to as wild type (WT)) does not form CAs. Using a different strain with inducible *traAB*
84 expression, CAs were only seen when cells were grown with an inducer (Fig. S1B). In prior work, smaller
85 and simpler versions of CA-like structures were seen in certain mutant backgrounds and were frequently
86 referred to as swirls (23-26). While CAs superficially resemble precursor aggregates that form into fruiting
87 bodies upon starvation-induced development (27), we emphasize that in our experiemnts TraAB OE cells
88 were grown on nutrient medium that blocks development. Therefore, without engaging in a complex
89 developmental lifecycle, TraAB overexpression provides a simple and tractable system to assess the
90 impacts of local cell-cell interactions on emergent group behaviors.

91 The biophysical model reveals CAs only arise from non-reversing agents

92 To understand mechanistically how CAs emerge in a TraAB OE strain, we attempted to replicate this
93 behavior *in silico* using a biophysical modeling framework that can properly account for forces between
94 cells. To this end, we started with the biophysical model developed by Balagam et al. (28). In this model,

95 to simulate flexible rod-shaped cells, each agent was represented by 7 nodes connected by springs. Agents
96 align with one another on collisions (15) and follow paths left by other agents. These biologically relevant
97 paths are called slime trails composed of poorly characterized material consisting of polysaccharides and
98 lipids that are deposited by gliding *M. xanthus* cells (29-33). Previously, this model was shown to result in
99 CA formation when the slime-trail following was strong and cells did not reverse (15). Notably, physical
100 adhesion between agents was not required in that model of CA formation. However, in light of our
101 experimental findings (Fig. 1), it seemed that TraAB adhesive forces directed CA formation.

102 To further assess the role of physical adhesion on emergent behavior, we introduced end-to-end and side-
103 by-side adhesion into our model (see Material and Methods for details). The simulation results indicated
104 that the addition of adhesion forces by itself does not promote the formation of CAs when agents have
105 periodic reversals (WT cells reversal period ~8 min (6)). Instead agents self-organized into a network of
106 connected streams (Fig. 2A), with patterns resembling those without adhesion (15). These simulated
107 patterns also resemble experimental observations of the parent strain (Fig. 1, top middle panel). Notably, a
108 further increase in the strength of adhesive forces does not lead to CAs in the population of reversing agents.
109 Instead, excessive adhesion forces exceeding those generated by the agent's motors resulted in unrealistic
110 bending of agents (Fig. 2B). On the other hand, non-reversing agents in our simulations self-organized into
111 CAs either in the absence (Fig. 2C) or in the presence (Fig. 2D) of adhesion. By varying the reversal
112 frequency of agents we show that CAs only begin to appear when the reversal period exceeds ~70 min, i.e.
113 about 10-fold reversal suppression relative to WT was required for the emergence of CAs (Fig. 2E).
114 Comparing the emergent patterns in Figs. 2C-D, we conclude that in our model side-to-side and end-to-end
115 adhesions by themselves do not significantly affect the emergent patterns. On the other hand, in addition to
116 suppressed reversals, the ability of agents to lay and follow slime trails was critical (Fig. 2F). As groups of
117 cells move unidirectionally along such trails, the natural fluctuation in their trajectories leads these paths to
118 close on themselves so that swirling patterns efficiently reinforce trails to nucleate CAs. As other cells join
119 these swirling paths, CAs grow. Thus, our simulations predict that long reversal periods were necessary for
120 CA formation and, therefore, we predict that TraAB OE cells must somehow alter cellular reversals.
121 However, to date, no connection between TraAB levels and reversal control was known.

122 Cells in CAs suppress reversals

123 To experimentally test the model prediction, we tracked the movement of single cells within CAs. To this
124 end, a small fraction of TraAB OE cells were fluorescently labeled and mixed with isogenic unlabeled cells
125 (Fig. S2A). Cell movements were recorded by time-lapse microscopy (Movie S2) and the tracks and
126 reversals were quantified as in (9). Figure 3A shows the compiled trajectories of these cells with different
127 (random) colors assigned to individual cells. These trajectories reveal that inside CAs all cells move in the

128 same direction around the center of each aggregate. The CAs themselves rotated in either a clockwise or
129 counterclockwise direction (Fig. S2A and Movie S2). Importantly, when the reversal period was measured
130 for all 443 cells that remained trackable (i.e. in the field of view) for the duration of the movie (60 min),
131 only 12 reversal events were detected. This corresponds to an average frequency of one reversal per cell
132 every ~36 hrs. In other words, cells within CAs did not reverse (Fig. 3B). These results were consistent
133 with our simulation predictions that cell reversals were indeed inhibited within CAs.

134 Reversal suppression and CAs are dependent on cell-cell adhesion and independent of OME

135 To determine whether reversal suppression was dependent on cell-cell adhesion or simply due to the TraAB
136 proteins being expressed at elevated levels, the reversal frequencies of isolated cells were also tracked.
137 Here, isolated TraAB OE cells were found not to suppress their reversals compared to controls (Fig. S2B).
138 Additionally, given that TraAB mediates OME, whereby bulk protein and lipid cargo are bidirectional
139 transferred between cells (34), it raises the possibility that reversal suppression, and hence CA formation,
140 was the result of hyper-active OME. To address this possibility, the OmpA domain from TraB was deleted,
141 resulting in a strain producing functional TraAB adhesins, but defective in OME (Fig. S3A, B). Importantly,
142 this strain similarly formed CAs, albeit at reduced levels (Fig. S3C). We conclude that sustained cell-cell
143 contacts mediated by TraAB OE, but not OME, suppresses cell reversals.

144

145 Reversal suppression is required for CA formation

146 Cellular reversal control in *M. xanthus* is complex. Here a central decision making system is the
147 'chemosensory' signal transduction pathway called Frz (5), which influences the polar localization of the
148 master reversal switch MglA, a small Ras-like GTPase, which in turn determines the polarity of motor
149 function and direction of cell movement. To test the role of reversal suppression in CA formation we first
150 used a chemical inducer (isoamyl alcohol, IAA) of reversals, which acts as a repellant by activating the Frz
151 pathway (35, 36). Here IAA was added at low concentrations to agar media and the behavior of the TraAB
152 OE strain was assessed. Importantly, in a dose-dependent manner CA formation was abolished (Fig. S4A).
153 Secondly, based on our simulations (Fig. 2C) and prior work (24, 26), we confirmed that Frz non-reversing
154 mutants can form CAs in the absence of engineered adhesion (Fig. S4B), although these structures were
155 not as prominent as those in the TraAB OE strain. Additionally, another mutation (Δ*mglC*) that reduces
156 cellular reversal frequencies (25), and apparently functions independently of the Frz pathway (37, 38), also
157 forms CAs (aka swirls) (25), albeit infrequently. Taken together these results support the model that CA
158 formation requires reversal suppression.

159 Next, we tested whether CA formation, and hence reversal suppression, was signaled through the Frz
160 pathway. As background, similar to other chemosensory pathways in enteric bacteria (39), the Frz pathway
161 contains a methyl-accepting chemotaxis protein (MCP) called FrzCD. However, FrzCD is an atypical MCP
162 that localizes in the cytoplasm and lacks transmembrane and ligand-binding domains (40). Nevertheless, a
163 hallmark of Frz-dependent signaling, similar to other MCPs, are changes in its methylation state as judged
164 by western analysis (41, 42). As previously described (35, 36), in a control treatment with the IAA repellent
165 added to agar media the migration of FrzCD was retarded, indicating an unmethylated state as compared to
166 untreated (½ CTT only) cells (Fig. 4C, upper band). On a nutrient rich agar (CYE), which alters FrzCD
167 methylation and inhibits motility (36), a change in the FrzCD methylation state was also detected as
168 compared to the ½ CTT control. In contrast, when CA formation was induced in the TraAB OE strain by
169 IPTG addition, FrzCD methylation pattern did not change compared to growth in the absence of IPTG (no
170 CAs) or the parent strain grown on ½ CTT (Fig. 4C). However, we note that when SDS-PAGE was
171 conducted under standard conditions, which were not optimized for detecting FrzCD methylation migration
172 differences according to (42), we found minor changes in FrzCD mobility when cells were in CAs (data
173 not shown). Nevertheless, when gel conditions followed the established and optimized protocol for FrzCD
174 (42), we repeatedly found no difference in FrzCD mobility from cells in CAs as compared to controls.
175 Taken together, we conclude that under the optimized assay conditions for detecting FrzCD gel mobility
176 shifts, and hence methylation state, we did not detect appreciable changes when cells were assayed from
177 CAs.

178 The biophysical model suggests that contact-dependent reversal suppression leads to CA 179 formation

180 To reconcile the differences in reversal frequencies between cells in CAs (Fig. 3B) and individual cells
181 (Fig. S2B), we hypothesized that sustained cell-cell contacts mediated by TraAB result in an intracellular
182 signal that suppresses cell reversals. Consistent with this model, cell density and contact-dependent signals
183 are known to regulate reversal frequency during development (5, 9, 43, 44), and, additionally,
184 myxobacterial ripples originate from cell contact-dependent reversal modulation (8, 10). To implement this
185 mechanism in our model, we chose a phenomenological approach to simulate contact-dependent reversal
186 suppression inspired by Zhang et al. (10). To this end, at each time step when a given agent was in contact
187 with another agent, its reversal clock was reset backward by a fixed amount. Given that TraAB stimulates
188 both end-to-end and side-to-side adhesion (Fig. 1), we assumed either one or both interactions lead to
189 reversal suppression. We hypothesized that adhesion forces that hold agents together will increase reversal
190 suppression by physically increasing the contact duration. To differentiate WT cell-cell contacts at high-
191 cell densities from those that occur between TraAB OE cells, we introduced a time delay between agent

192 adhesion events and reversal suppression signaling. That delay was set at 5 min to ensure no CAs formed
193 in WT agent simulations as explained below.

194 In the presence of the signaling delay, with only weak adhesion (representing the parent strain with low
195 TraAB levels; Fig. 4A), agent interactions were short, and reversals were not substantially inhibited,
196 resulting in normal patterns (compare with Fig. 2A). As shown, less than half of the agent contacts lasted
197 long enough to produce reversal suppression. Next, we performed a simulation of agents with stronger and
198 consequently longer adhesion events and as a result, the frequency of reversal suppression was dramatically
199 increased (Fig. 4B). Under these conditions, CAs readily formed, and in agreement with experiments,
200 showed the unidirectional rotation of agents in a clockwise or counter-clockwise direction (Movie S3). This
201 result supports our hypothesis that reversal suppression was necessary for CA formation. Furthermore, our
202 simulations found that when adhesion strength gradually increases from WT to TraAB OE levels the
203 number of agents participating in contact signaling gradually increased (Fig. 4C). However, the effect of
204 the adhesion strength on the duration of adhesion (time before adhesion bond broke) was more dramatic
205 (Fig. 4D). Therefore, to ensure our simulations were consistent with the lack of CAs in the WT strain and
206 based on our findings, we assumed the transient contacts that were shorter than 5 min in the simulations do
207 not suppress reversals. This threshold was important because CAs would form even with weak adhesion in
208 its absence (Fig. S5).

209 Notably, the behaviors on individual agents in our model match the trends for experimentally tracked cells.
210 With tracking data, we quantified how cell speed and angular speed change as a function of distance to an
211 aggregate center. The experimental results demonstrate that cell speed increases while angular speed
212 decreases as a function of distance from the aggregate center (Fig. S6A, B). Similarly, by quantifying agent
213 speed and angular speed in simulations as a function of distance to the aggregate centers (Fig. S6C, D), we
214 demonstrated that trends were qualitatively consistent with experimental observations (see Fig. S6 legend
215 for details).

216 The contact-mediated reversal suppression model accurately predicts emergent patterns of 217 multi-strain mixtures

218 To further interrogate our model and computationally investigate the interplay between the kin recognition
219 and emergent patterns, we conducted simulations where two types of agents were mixed. In the first
220 simulation, agents overexpressed TraAB receptors of different types (alleles). These receptors do not match
221 and hence the different type agents cannot adhere to each other (16, 17), and reversal suppression only
222 occurs when two agents of the same type engaged in sustained contact. Initially, we hypothesized that
223 differential adhesion would lead to "phase-separation" between agent types, analogous to phase-separation

between oil and water. However, in contrast to this prediction, our simulations found that both agent types were mixed within CAs (Fig. 5A). To explain this result, we suggest that reversal suppression and the ability of agents to follow each other's slime trail overpowered their distinct adhesive forces.

Next, to investigate the impacts of heterogeneous cell-cell adhesion forces across populations, we simulated a TraAB OE (green) mixed with a WT (red) agent. These agents contained different adhesive forces. WT had similar weak adhesions between themselves and with TraAB OE agents (16, 17), and hence they were less susceptible to prolonged cell-cell contacts and reversal suppression. In contrast, TraAB OE agents had strong adhesion among themselves. Interestingly, the simulations showed that the WT agents impeded the formation of CAs by TraAB OE, perhaps by breaking cell-cell adhesions and blocking prolonged contacts that are required for reversal suppression (Fig. 5B, compare to Fig. 4A). Furthermore, to test the role of reversal suppression in CA formation, we conducted a simulation where a TraAB OE agent was mixed with a weakly adhering agent that does not reverse, i.e. a Frz mutant. Strikingly, in this case, the non-reversing and TraAB OE agents formed mixed CAs together (Fig. 5C). This result again demonstrates the key role reversal suppression plays in the emergent CA behavior.

To test our model predictions, we experimentally mixed strains in a manner analogous to simulations. Importantly, for all three strain mixtures, experimental results showed CA patterns or lack thereof, that correlated with all three corresponding simulations (Fig. 5, compare A-C to D-F). Additionally, the degree that strains did or did not mix also correlated well with simulation outcomes, given the latter represents agents in two dimensions while the former shows cells in three dimensions. Specifically, we found that: (i) Introduction of cells with low cell adhesion capabilities (e.g. WT) blocked the emergence of CAs by apparently disrupting prolonged cell-cell adhesions between TraAB OE cells and hence disrupting reversal suppression (Fig. 5E). Moreover, these disruptions were potent because even a minority of such cells, e.g. 7:1 ratio of TraAB OE to WT, reduced CA formation (Fig. S7B). (ii) As found in our simulations and above experiments, reversal suppression played a crucial role, because, in contrast to mixtures with WT cells (Fig. 5E), TraAB OE cells readily formed CAs in 1:1 mixtures with Frz non-reversing mutants, which express TraAB at wild-type levels (Fig. 5F). (iii) When two strains overexpressing incompatible TraA receptors were mixed, they also formed CAs together (Fig. 5D). Therefore, the ability of different strains to strongly adhere to each other was not critical, as long as cell reversals were suppressed, whether by cell-cell adhesion or by *frz* mutations. That is, when divergent populations were mixed, where their cell reversals were suppressed, either by TraAB OE or genetically (*frz*$^{-}$), they readily merged and jointly form CAs by following their reinforced slime trails.

Discussion

Emergent behaviors transcend the properties of individual components and result in complex functions that are often difficult or impossible to understand mechanistically at a systems level. However, here we investigated a tractable emergent behavior, whereby thousand to millions of cells form spinning CAs. By using experimental and biophysical agent-based modeling, we elucidated the underlying mechanism. Strikingly, our models revealed that the formation of CAs only occurs when cellular reversals are suppressed and cells follow their slime trails, as we previously suggested (15). Experiments confirmed that reversal suppression is required, which is triggered by cell-cell adhesion within dense groups. That is, isolated cells that overexpress TraAB have WT reversal frequencies and necessarily are not constituents of CAs. Using these observations, we hypothesized that reversals were suppressed by long-lasting cell contacts that adhesins stabilized. This model is supported by several experimental findings, including that TraAB OE cells do not reverse within CAs and when reversals are induced by IAA addition CAs cannot form. Secondly, CA formation is phenocopied to some extent by mutants (e.g. *frz*) that are blocked in reversals. Third, our model not only explained the differences in patterns between WT and TraAB OE cells but also qualitatively matches how actual cellular linear and angular speeds change within CAs. Finally, our model accurately predicted emergent patterns when strains with distinct behaviors were mixed.

Central to our model, the formation of CAs only requires cells to lay and follow slime trails (29-31), and adhesion to stabilize cell-cell contacts, thereby leading to reversal suppression. Strikingly, however, for non-reversing agents (or strains), the requirement of adhesion forces can largely be bypassed. This conclusion is supported by the observation that Frz non-reversing mutants form detectable amounts of CAs (Fig. S4B and (24, 26)) and that Δ*mglC* mutation that reduces cellular reversal frequencies also induces similar patterns (25). Thus TraAB-driven cell adhesion is primarily required for reversal suppression rather than for the formation of CAs *per se*. In contrast, another theoretical study showed that non-reversing agents could also form CAs by instead invoking a short-range active guiding mechanism (45). In this model, agents do not follow slime trails, but instead, generate active guiding forces that allow the lagging agent to seek and maintain a constant distance from the leading agent. This active guiding force is assumed to arise from physical adhesion and/or attraction between cell poles, which could be generated by polar type IV pili. Importantly, these models make different predictions on CA dynamics. In one case CAs rotate as rigid bodies (45), whereas CAs based on slime trail following (15) showed that despite the increase in speed there is a decrease in angular velocity the farther agents were from the aggregate center. These patterns of cell speed and angular velocity from experiments qualitatively match our model predictions (Fig. S6) and do not match the predictions of (45). However, since simulations were performed in a single agent layer, which contrast with multilayer cell experiments, the size of simulated CAs remains smaller and thus no

288 quantitative agreement between simulations and experiments is expected. In this context, it is foreseeable
289 that cell adhesion between cell layers further stabilizes CAs and allows them to grow much larger. However,
290 conducting such simulations requires alternative modeling formalism and beyond the scope of this work.

291 Laboratory competition experiments and characterization of cells from environmentally-derived fruiting
292 bodies reveal that robust kin discrimination systems lead to near homogenous segregation of kin groups
293 from diverse populations (12, 14, 46). OME, mediated by TraAB, plays a central role in these processes by
294 exchanging large suites of polymorphic toxins (13, 20). This ensures that only close kin survive these social
295 encounters because they contain cognate immunity proteins. Here, we found that overexpression of TraAB
296 from kin cells results in the formation of organized social groups that move in synchrony. However,
297 surprisingly, *in silico* and experimental overexpression of divergent TraA recognition receptors, thus
298 representing distinct kin groups, or genetic suppression of reversals by *frz* mutations, resulted in mixed
299 populations within CAs (Fig. 5). Importantly, however, these mixed laboratory groups were between
300 engineered strains derived from the same parent, and hence they were socially compatible because they
301 contained reciprocal immunity to OME toxins as well as type VI secretion system toxins (14). In other
302 words, consistent with ecological findings from fruiting bodies (12), we do not expect mixed CA formation
303 between divergent *M. xanthus* strains that antagonize one another, and thus serving as a barrier to social
304 cooperation (14).

305 Our findings on CA formation also provide insight into the natural emergent behavior of development. That
306 is, during starvation-induced development cells form spherical fruiting bodies; a process that requires the
307 Frz pathway and reversal suppression (5, 9). Although much is known about development (47), how fruiting
308 bodies emerge remains poorly understood. In light of our findings, we suggest that during development
309 cells increase their adhesiveness, perhaps mediated by C-signaling (48-50), which results in sustained cell-
310 cell contacts, and hence reversal suppression, which similarly is critical for fruiting body formation (9, 44).
311 In a second developmental behavior, cell collision-induced reversals are known to trigger rippling (8, 10).
312 Here we suggest that these collisions could break long-standing cell-cell contacts of aligned groups of cells,
313 thus disrupting their reversal-suppression and triggering reversals. Future studies need to investigate how
314 cell-cell adhesion and sustained cell contacts might change during development and the roles they play
315 during fruiting body morphogenesis and rippling.

316 Central to the sociality of *M. xanthus* is the control of their cellular reversals that coordinates their
317 multicellular behaviors. Although significant progress has been made in understanding the molecular
318 regulation of reversals (5, 37, 51), major knowledge gaps remain. Here we show that engineered sustained
319 cell-cell contacts suppress cellular reversals. Our findings indicate that the methylation state of the FrzCD
320 MCP is not altered, suggesting Frz mediated adaptation is not involved in reversal suppression.

321 Nevertheless, given that the Frz system plays a major role in reversal control and yet has no known ligand
322 binding domain, our findings do not exclude the possibility that a downstream component, such as FrzE or
323 FrzZ, senses and signals sustained cell-cell contacts. Alternatively, reversal suppression could occur
324 independently of Frz. For example, other systems that regulate reversals include the Dif chemosensory
325 pathway as well as the MglC, PlpA and PixA proteins (25, 37, 38, 41, 52, 53). Additionally, there are
326 undiscovered pathways that suppress reversals as exemplified by the EPS (extracellular polysaccharides)
327 signal (54). Finally, in an alternative scenario, TraAB-dependent cell-cell adhesion could mechanically
328 block the A-motility motor from physically switching cell poles and hence suppress cellular reversals.
329 Consistent with this model, TraAB and the A-motility motor reside in the cell envelop and are mobile
330 macromolecular complexes frequently found at the poles (16, 17, 22, 51).

331 In summary, our approach provides a roadmap for how strain engineering and modeling helped to elucidate
332 mechanistic insights into an emergent behavior that arises from cell reversal control. These insights are also
333 likely relevant for the natural emergent behavior of fruiting body development. By extension, in other
334 biological systems and model organisms, seemingly complex emergent behaviors, can be broken down and
335 tackled by using a combination of modeling and simplified experimental manipulations to uncover their
336 mechanisms of action.

337 Materials and methods

338 Bacterial strains and growth conditions

339 All strains used in this study are listed in Table 1. *M. xanthus* cells were routinely grown in CTT medium
340 (1% [w/v] Casitone; 10 mM Tris-HCl, pH 7.6; 1 mM KH_2PO_4; 8 mM $MgSO_4$) in the dark at 33 °C. For a
341 nutrient rich medium CYE was used (1% Casitone, 0.5% yeast extract, 8 mM $MgSO_4$, 10 mM MOPS, pH
342 7.6). 1.5% (w/v) agar was added to the medium to make plates. To prepare agarose pads for microscopy,
343 Casitone was reduced to 0.2% (w/v), and 1% (w/v) agarose was added to the medium. *E. coli* strains were
344 routinely cultured in LB medium at 37 °C. As needed for antibiotic selection or protein induction,
345 50 μg ml^{-1} of kanamycin (Km), 10 μg ml^{-1} oxytetracycline, or 1 or 2 mM IPTG was added to media.
346

347 Plasmid and strain construction

348 All plasmids and primers are listed in Table 1. To maximize the expression of TraAB adhesin, pPC57 was
349 constructed where the native GTG start codon of *traA* was changed to ATG and TraAB expression is driven
350 by a heterologous *pilA* promoter (P_{pilA}). This site-directed mutagenesis was done by using primers
351 containing the desired mutation, and the amplified *traAB* fragments were ligated into pDP22 (linearized
352 with XbaI and HindIII) with T4 DNA ligase. To achieve inducible overexpression of TraAB (pPC58),

353 *traAB* fragments were PCR amplified and then ligated into pMR3487 (linearized with XbaI and KpnI)
354 through Gibson Assembly (New England Biolabs). To create pPC59, primers were designed to amplify
355 fragments of *traAB* and omit the region encoding for OmpA, and the resulting fragments were ligated into
356 XbaI and HindIII digested pDP22 through Gibson Assembly. Plasmid construction was done in *E. coli*
357 TOP10. All plasmids were verified by PCR, restriction enzyme digestion, and if necessary, by DNA
358 sequencing. To construct *M. xanthus* strains, plasmid or chromosomal DNA was electroporated into cells
359 and integrated into the chromosome by site-specific or homologous recombination. For pSWU19 derived
360 plasmids integration occurs at the Mx8 attachment site, while pMR3487 recombines at another site and
361 expression is induced with IPTG (55).
362

363 ## Aggregate formation

364 *M. xanthus* cells were grown to logarithmic growth phase in CTT, washed with TPM buffer (CTT without
365 Casitone), and resuspended to the calculated density of 5×10^8 cells per mL. 5 µL of cell suspension was
366 then spotted onto ½ CTT (CTT medium with 0.5% Casitone) agar plates supplemented with 2 mM $CaCl_2$.
367 In some cases, different strains were mixed at desired ratios before spotting. Spots were air-dried and plates
368 were then incubated at 33 °C overnight before imaging. When necessary IPTG was added during liquid and
369 plate growth. To assess the impacts of cellular reversals on CA formation, isoamyl alcohol (IAA) was
370 supplemented to agar media at indicated concentrations.
371

372 ## Microscopy

373 CA formation on agar plates was imaged using a Nikon E800 phase-contrast microscope (10× phase-
374 contrast objective lens coupled to a Hamamatsu CCD camera and Image-Pro Plus software), or an Olympus
375 IX83 inverted microscope (10× objective lens coupled to a ORCA-Flash 4.0 LT sCMOS camera and
376 cellSens software), or an Olympus SZX10 stereomicroscope (low magnification coupled to a digital
377 imaging system). To track isolated cell reversals, cells were mounted on an agarose pad and imaged with a
378 20× phase-contrast objective lens. Fluorescence microscopy was used to track individual cells within CAs
379 with a 10× lens objective and a Texas Red filter set. Cell-cell adhesion was imaged directly from overnight
380 cultures mounted on glass slides with a 100× oil immersion objective lens.

381 ## Immunoblot

382 To optimize separation of different FrzCD isoforms, SDS-PAGE was done as essentially described by
383 McClearly et al (42). Briefly, equal amounts of cell extract were separated on a 14 cm resolving gel
384 consisting of 11.56% acrylamide, 0.08% bis, 380 mM Tris pH-8.6, 0.1% SDS, 0.1% ammonium per sulfate
385 and 0.04% TEMED. The stacking gel consisted of 3.9% acrylamide, 0.06% bis, 125 mM Tris pH-6.8, 0.1%

386 SDS, 0.1% ammonium per sulfate and 0.01% TEMED. To remove non-specific binding, the rabbit α-FrzCD
387 serum was first pre-absorbed against a blot from a Δ*frzCD* strain and then used at a 1:15,000 dilution on
388 experimental blots. For detection, HRP-conjugate goat-anti-rabbit secondary antibody was used (1:15,000
389 dilution, Pierce) and developed with SuperSignal West Pico Plus chemiluminescent substrate (Thermo
390 Scientific).

391 The Agent-Based-Simulation framework

392 The simulation model framework is adapted from our previous work (15, 28). A brief description of the
393 previous model, simulation framework as well as the new changes introduced in the framework are
394 presented below. All of the parameters are summarized in Table 2. Each agent is represented as a connected
395 string of N (= 7) circular nodes with a total cell length L (= 6 μm) and width w (= 0.5 μm) (see figure S1 in
396 (28) and additional details in (15)). Neighboring circular nodes are kept at a fixed distance apart by (N-1)
397 rectangular spacers. Neighboring circular nodes and rectangular spacers are connected by linear (spring
398 constant, k_l) and angular (spring constant, k_b) springs. Linear springs here resist elongation and
399 compression of cell nodes. The linear spring constant is managed by the model engine to keep the agent
400 length constant. Angular springs resist bending from straight-line configuration to simulate elastic bending
401 behavior of *M. xanthus* cells.

402 Each agent moves forward by the propulsive forces. Since the experiments are performed with the cells
403 lacking S-motility, we only implement gliding (A) motility of *M. xanthus* cells based on distributed force
404 generation model (22, 56-58). At each node i a propulsion force ($\boldsymbol{F}_{p,i} = F_T/(N-1), F_T$ is the total
405 propulsive force) is applied in the current travel direction towards the neighboring node. Viscous drag
406 forces ($\boldsymbol{F}_d$) arising from the surrounding fluid/slime act on nodes opposing their movement with the force
407 proportional to the velocity or each node with proportionality coefficient c (drag coefficient).

408 Agent movement is affected by collisions, periodic reversals, random turns, and slime-trail-following by
409 agents. Collisions in our model are resolved by applying repulsion forces on nodes that keep agents from
410 overlapping. Further, adhesive attachments between the agent and the underlying cell substrate (based on
411 focal adhesion model of gliding motility in *M. xanthus* (57)) at each node resist lateral displacement of the
412 nodes during collisions with other cells. These attachments are modeled as linear springs (spring constant,
413 k_a) and are detached at a threshold distance $d_{a,max}$. For each agent, the first and last nodes in the current
414 cell travel direction are designated as head and tail nodes respectively. Periodic reversals in our model are
415 introduced by switching the roles of head and tail nodes and reversing the propulsive force direction at the
416 inner nodes. Reversals in agents are triggered asynchronously by an internal timer expiring at the end of
417 the reversal period (τ_r) after which the timer is reset to zero. *M. xanthus* cells exhibit random turns during

movement on solid surfaces (28). These random turns are added to the model by changing the direction of the propulsive force on the head node of the agent by 90° (either clockwise or anti-clockwise chosen randomly) for a fixed amount of time (1 min) at regular time intervals (τ_t) triggered another internal timer. Slime-trail-following by *M. xanthus* cells is a known phenomenon (29), in which cells leave a slime trail on the substrate and other cells crossing these trails later start following them. We added slime-trail-following of agents in our model using a phenomenological approach where we gradually change the direction of propulsive force ($\boldsymbol{F}_p$) on the head node of the agent parallel to the direction of slime trail ($\hat{e}_s$) it is currently crossing. (See (15) for implementation details of slime-trail-following mechanism in our model).

Cell adhesion

To simulate adhesive interactions between agents, we apply lateral adhesive forces (F_{adh}) on nodes of neighboring agents if the two nodes are closer than a specific threshold distance. In the simulation, we include end-end adhesion where one agent's head node is attached to another agent's tail node and lateral adhesion where an agent is attached to a nearby agent side by side. The threshold distance for lateral adhesion $d_{thr} = 0.9$ µm and for end-end adhesion $d_{thr} = 1.5$µm. This is because we assume the cell wall/membrane can be stretched more along the long axis.

We use the following equation to calculate cell adhesion force:

$$F_{adh} = \begin{cases} 0 & d > d_{thr} \\ k_{adh} \dfrac{d - w}{w} F_T & d_{thr} > d > w \end{cases}$$

Here, d is the distance between neighboring nodes, w is the width of cells, k_{adh} is the adhesion force factor describing the ratio of the maximal adhesive force to the total propulsive force of the agent F_T. For OE cells, $k_{adh} = 0.1$ for WT cells $k_{adh} = 0.01$. These adhesive forces are applied on each node in the direction towards the neighbor node center.

Reversal suppression induced by cell contacts

In the model, cell reversal is controlled by a reversal clock in the agent. If the reversal clock records a time longer than the chosen reversal period, the reversing happens and the reversal clock is reset to 0. In this work, we assume if the adhesion lasts longer than a threshold time (τ_{thr}, set to be 5 min unless indicated otherwise), agents suppress their reversals. We set the threshold to be 5 minutes. When suppression of reversal happens, the reversal clock is slowed down or even turned back for every time step that agents

remain in contact past the threshold. For each agent, we calculate the total suppression from end-end pairs and lateral suppression contacts, i.e.:

$$r_{t+1} = \begin{cases} \max(r_t + dt(1 - \sum_{\substack{\text{end-end} \\ \text{pairs}}} \delta R_e - \sum_{\substack{\text{lateral} \\ \text{pairs}}} \delta R_l), \quad 0) & if\ r_t < \tau_r \\ 0 & if\ r_t \geq \tau_r \end{cases}$$

Here, r_t is reversal clock at time step t, r_{t+1} is reversal clock at time step $t+1$, τ_r is reversal period, δR_e is end-end reversal suppression factor, δR_l is lateral reversal suppression factor and dt is the time step.

Simulation of the mixed agent population

To simulate mixed populations of two types of agents, we assign each agent a label that corresponds to the strain it represents. We use WT label for parent strain, OE for TraAB-expression, and NR for non-reversing. Adhesion interactions are assumed to be 10x stronger ($k_{adh} = 0.1$) if both agents have OE labels as compared to all other pairs ($k_{adh} = 0.01$). For simulations of mixture OE agents of different TraAB alleles, no adhesion between agents with different alleles is occurring ($k_{adh} = 0$), thus the reversal suppression also will not occur. Since in our model adhesion is required for reversal suppression, these interactions do not affect reversals.

Simulation procedure

The simulation procedure here is similar to (15). We study collective behaviors of cells by simulating mechanical interactions among a large number (M) of agents on a 2D simulation region with periodic boundary conditions in an agent-based framework.

We initialize agents one by one on a square simulation region (dimension L_{sim}) over a few initial time steps until the desired cell density (η) is reached. Agents are initialized in random positions over the simulation region with their orientations (θ) chosen randomly in the range $[0, 2\pi]$. Agent nodes are initialized in the straight-line configuration. During initialization, agent configurations that overlap with existing agents are rejected. After initialization, the head node for each agent is chosen between its two end-nodes with 50% probability.

At each time step of the simulation, agents move according to the various forces acting on their nodes. Changes in node positions and velocities are obtained by integrating the equations of motion based on Newton's laws. We use the Box2D physics library (59, 60) for solving the equations of motion and for effective collision resolution. Snapshots of the simulation region, the orientation of each agent, and its node positions are recorded every minute for later analysis.

Simulations are implemented in Java programming language with a Java port of Box2D library (http://www.jbox2d.org/). The parameters of the simulation are shown in Table 2. Other parameters of the model are the same as in (15, 28). Each simulation is run for 250 min. The codes and datasets are available in the https://github.com/Igoshin-Group/CircularAggregatesPaper repository.

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485 ## Figure Legends

486 **Fig. 1**. Emergent behavior triggered by TraAB overexpression (OE). Cells adhere from shaker flask growth
487 (left panel), while on agar surfaces motile populations form circular aggregates (CA) when grown on rich
488 media (middle/right panels, 12 h growth). For simplicity, cells only contain one functional gliding motility
489 system.

490 **Fig. 2**. Biophysical model predicts non-reversing and slime-following agents required for CA formation.
491 (A) Reversing agents do not form CAs in the presence of adhesion or (B) with adhesion forces stronger
492 then motor forces. (C) Non-reversing agents form CAs even in the absence and (D) in the presence of
493 adhesion. (E) Reversing agents with long reversing periods (70 min) initiate CA formation. (F) Non-
494 reversing agents without slime-following do not form CAs.

495 **Fig. 3**. TraAB OE suppresses cell reversals via the Frz chemosensory pathway. (A) Digitally labeled
496 trajectories of marked cells from Movie S2 . Each colored curve represents a trajectory of one cell; 443
497 cells were tracked for the duration of the whole movie (60 min, 1 min intervals). (B) Run duration
498 distribution of the tracked cells: vast majority of cells did not reverse during the observation. (C) CA
499 formation occurs independently of FrzCD methylation changes. A negative control (Δf*rzCD*), parent and
500 TraAB OE strains were harvested from indicated agar plates grown for 24 hr. Only the TraAB OE strain
501 with 2 mM IPTG formed CAs. Representative immunoblot probed with α-FrzCD serum shown. Left,
502 molecular weight standards; *non-specific loading control band.

503 **Fig. 4**. Strong adhesion and cell contact-dependent reversal suppression results in CAs in simulations. (A)
504 Agents with weak adhesion (WT phenotype) show only a small number engaging in reversal suppression
505 (blue) with no emergent behavior. Agents with no reversal suppression are red. (B) Stronger adhesion leads
506 to prevalent reversal suppression (blue) and formation of CAs. (C) Fraction of agents with suppressed
507 reversals as a function of adhesion strength at end of the simulation. (D) Average adhesion bond time at the
508 final 10 min of simulation. The adhesion factor k_{adh} is defined in SI. WT, k_{adh} = 0.01; OE, k_{adh} = 0.1.

509 **Fig. 5**. Correlations between simulations and experiments when different combinations of agents or cells
510 were mixed 1:1. (A) Simulation of two different agents (red and green) that adhere to themselves but not
511 each other. (B) Simulation of adhesive agents (TraAB OE, green) mixed with weakly-adhesive agents (WT,
512 red). (C) Simulation of adhesive agents (green) mixed with weakly-adhesive non-reversing agents (red).
513 (D) Experimental mixture of two strains that overexpress different TraA receptors (red and green) that
514 adhere to themselves but not each other. (E) Mixture of TraAB OE strain (green) mixed with a strain that
515 does not adhere (WT, red). (F) Mixture of TraAB OE strain (green) mixed with a non-adhesive non-
516 reversing mutant (red). D-F merged images; see Fig. S7A for single-channel images.

517

518

519 ## Supplemental Materials

520 **Fig. S1.** Induction of CAs**.** (A) Time course after plating of constitutive P_{pilA}-*traAB* OE strain and its parent
521 on agar spotted at a cell densities of 3×10^9 cfu/ml. (B) Strain with an inducible promoter (P_{IPTG}-*traAB* OE)
522 plated in the presence and absence of inducer and grown for 36 h.

523 **Fig. S2**. Cell tracking and reversal tracking. (A) Fluorescent micrograph of CAs. Labeled and non-labeled
524 cells mixed 1:250. White arrows, counter-clockwise rotations; yellow arrows clockwise rotation. Time-
525 lapse series shown in Movie S2. (B) TraAB OE does not suppress cell reversals of isolated cells at low cell
526 density. Cell movements of isolated cells were tracked by time-lapse microscopy in three independent
527 experiments and compared between TraAB OE to WT cells.

528 **Fig. S3**. Outer membrane exchange (OME) is not required for CA formation. (A) Cell-cell adhesion absent
529 in Δ*traAB* strain and present in two TraAB OE strains (red arrows). (B) Stimulation assays assess function
530 of *traAB* alleles in OME. Here, two nonmotile strains were mixed 1:1 and when OME occurs the recipient
531 cells receive missing motility lipoproteins (CglC and Tgl) and swarm out from the colony edge (middle
532 panel arrow) (34). When OME is defective the recipient cells remain nonmotile and produce sharp colony
533 edges (left, right panels). (C) Ability of *traAB* alleles to promote CAs (middle, right panels) or not (left
534 panel). See Table 1 for strain details.

535 **Fig. S4**. Role of Frz in CA formation. (A) Chemical induction of cellular reversals blocks CA formation.
536 Isoamyl alcohol (IAA) stimulates cell reversals by activating the Frz pathway. IAA was added at indicated
537 concentrations to agar media and micrographs taken at 24 h. The two left panels with no IAA are also
538 shown in Fig. 1. (B) Frz non-reversing mutant forms CAs, though not as distinctive as the TraAB OE strain.

539 **Fig. S5**. WT agents without time threshold for reversal suppression form CAs. Black agents not reversal-
540 suppressed; blue agents are reversal-suppressed.

541 **Fig. S6**. Linear speed and angular speed as measured experimentally and in simulations. Experimentally
542 determined cell linear speed (A) and angular speed (B) measured as a function of distance to the center
543 from Figure 3A and Movie S2. Each data point represents the average of cells within a 5 μm window. Agent
544 linear (C) and angular speed (D) measured as a function of distance to center from simulations. Each data
545 point represents the average of cells within a 2 μm ring. The data and simulations indicate that the CAs do
546 not rotate as a rigid body, because that is defined as cells having the same angular speed throughout CAs.
547 Note the quantitative agreement between the speed values is not possible because the CAs in the experiment
548 were larger (radius 120 μm) than in the simulation (radius < 20 μm). This discrepancy was mainly due to
549 computational limitations as our simulation can only simulate a limited number of agents in a small
550 simulation domain (200 μm× 200 μm).

551 **Fig. S7**. Impact of strain mixing on CA formation of TraAB OE strain. (A) CA phenotypes of strain
552 mixtures (1:1 ratios) expressing different *traAB* alleles. Panels show single channels and merged images
553 used to create Figure 6D-F and strains used. Media contained 1 mM IPTG to allow $TraAB^{Mx}$ overexpression
554 (DW2540). See Table 1 for additional strain details. (B) WT cells inhibit CA formation by TraAB OE. The
555 ratios of TraAB OE to WT are indicated. Micrographs taken at 24 h.

556 **Movie S1** Time-lapse series of strains from Figure S1 at indicated times after cell plating. Time intervals
557 between frames 30 sec; each series for 15 min. See Table 1 for strain details.

558 **Movie S2** Time-lapse movie that is represented in Figures 3A and S2. Time intervals between frames 1
559 min. See Table 1 for strain details.

Movie S3 Time-lapse movie from Figure 4B. The movie records the last 60 mins of a 180 min simulation with a different color scheme for better contrast. In each frame, white cells represent reversal-suppressed cells and red cells are not reversal-suppressed.

563 **Table 1** Plasmids, strains and primers used in this study

Plasmids	Relevant features	Figure(s) used	Source
pCV10	pSWU19-P_{R3+4}-eGFP, Km^r		(46)
pDP21	P_{pilA}-*traAB* in pSWU19 (Mx8 *attP*), Km^r		(18)
pDP22	P_{pilA} in pSWU19, Km^r		(61)
pMR3487	IPTG-inducible promoter, Tc^r		(55)
ptdTomato	pMR3487-*tdTomato*, Tc^r		Larry Shimkets
pPC4	P_{pilA}-$traAB^{Mf}$ in pSWU19, Km^r		(16)
pPC57	P_{pilA}-(ATG)-*traAB* in pSWU19, Km^r		This study
pPC58	pMR3487-(ATG)-*traAB*, Tc^r		This study
pPC59	P_{pilA}-(ATG)-*traAB*(ΔOmpA) in pSWU19, Km^r		This study
Strains			
TOP10	*E. coli* cloning strain		Invitrogen
DK1622	Wild-type *M. xanthus*; all strains derived		(62)
DK8601	DK1622 *aglB1* (*aglQ1*) Δ*pilA*::tc, nonmotile, Tc^r	1 top/left panel	(62, 63)
DW1463	DK8601 (pDP21), Km^r, Tc^r	1 bottom left panel	(18, 63)
DK10410	DK1622 Δ*pilA* (markerless)	1 top middle/right panels, 3C, S1A (top), S4, S5, S7B, Movie S1	(18, 64)
DW2292	DK10410 (pPC57), Km^r	1 bottom middle/right panels, 3A, 3B, S1A (bottom), S2, S3, S4, S5, S6, S6A, S6B, S7B, Movies S1-S2	This study
DW2293	DW2292 (ptdTomato), Km^r, Tc^r	3A, 3B, S2, S6A, S6B, Movie S2	This study
DW2294	DK10410 (pPC58), Tc^r	3C, S1B	This study
DW712	DK1622 Δ*frzA-E*::km Δ*pilA*::tc, Km^r, Tc^r	S4B	This study
DW1466	DK1622 Δ*cglC* Δ*tgl* (markerless)	S3B	(18)
DW1483	DK8601 Δ*traAB* (markerless)	S3B	(16, 18)
DW2296	DW1463 (pPC57), Km^r, Tc^r	S3B	This study
DW2297	DW1483 (pPC59), Km^r, Tc^r	S3B	This study
DW2298	DW2270 (pPC59), Km^r	S3A, S3C	This study
DW2270	DK10410 Δ*traAB* (markerless)	S3A, S3C	(17)
DW2295	DK10410 (ptdTomato), Tc^r	5E, S7	This study
DW2539	DW2294 Δ*frzA-E*::km, Km^r, Tc^r	3C	This study
DW2538	DW2295 Δ*frzA-E*::km, Km^r, Tc^r	5F, S7	This study
DW2300	DW2270 (pPC4, ptdTomato), Km^r, Tc^r	5D, S7	This study
DW2540	DW2294 (pCV10), Km^r, Tc^r	5D, 5E, 5F, S7	This study

Primer name	Sequence (5'→3', Restriction sites are underlined)
(ATG)-TraA-RBS-XbaI-F	GACGACTCTAGAGGAAACCAAGAATAGAAATAGAAAGGAGAATTAATGGGAGATATCCCTCATTG
TraB-HindIII-R	GACGACAAGCTTGGAGTTCTTCACCTCGGACTC
XbaI-(ATG)-TraA-F	GGATAACAATTAAGGAGGCTCTAGAATGGGAGATATCCCTCATTG
TraB-KpnI-R	TGATTACGAAGGCGAGCTCGGTACCGGAGTTCTTCACCTCGGACT
pSWU19-EcoRI-P_{pilA}-F	AGGAAACAGCTATGACCATGATTACGAATTCCGTCATGTTGGACGAGGT
TraB-ΔOmpA-R	GAAGTCGACGCGGTTGAGCTGCTCCAGGATGACAAT
TraB-ΔOmpA-F	GGAGCAGCTCAACCGCGTCGACTTCACCATCC
pSWU19-XbaI-HindIII-TraB-R	TGCATGCCTGCAGGTCGACTCTAGAAAGCTTGGAGTTCTTCACCTCGGACTC

564

565 **Table 2** Simulation parameters

Description	**Symbol**	**Value**
Agent length	L	6 μm (10, 65)
Agent width	W	0.5 μm (10, 65)
Agent mass	m	$1.2 \times 10^{-15}\ kg$
Number of nodes per agent	N	7
Linear spring constant	k_l	Managed by Box2D (59)
Angular spring constant	k_b	$10pN \cdot \mu m/\mathrm{rad}$ (65-67)
Total propulsive force per agent	F_T	55 pN (28)
Drag coefficient	c	22 $pN \cdot min/\mu m$
Substrate adhesions spring constant	k_a	100 $pN/\mu m$ (28)
Substrate adhesion break distance	$d_{a,max}$	0.5 μm (28)
Reversal period	τ_r	8 min (6)
Direction change period	τ_t	5 min
Simulation region dimension	L_{sim}	200 μm
Agent density	η	0.074 $cell/\mu m^2$
Time-step	dt	0.0067 min
Adhesion force factor	k_{adh}	WT: 0.01, OE: 0.1
End-end suppression factor	δR_e	1
Lateral suppression factor	δR_l	0.04
The minimum time for suppression activation	τ_{thr}	5 min
Maximal adhesion length	d_{thr}	1.5 μm for end-end adhesion 0.9 μm for lateral adhesion

566

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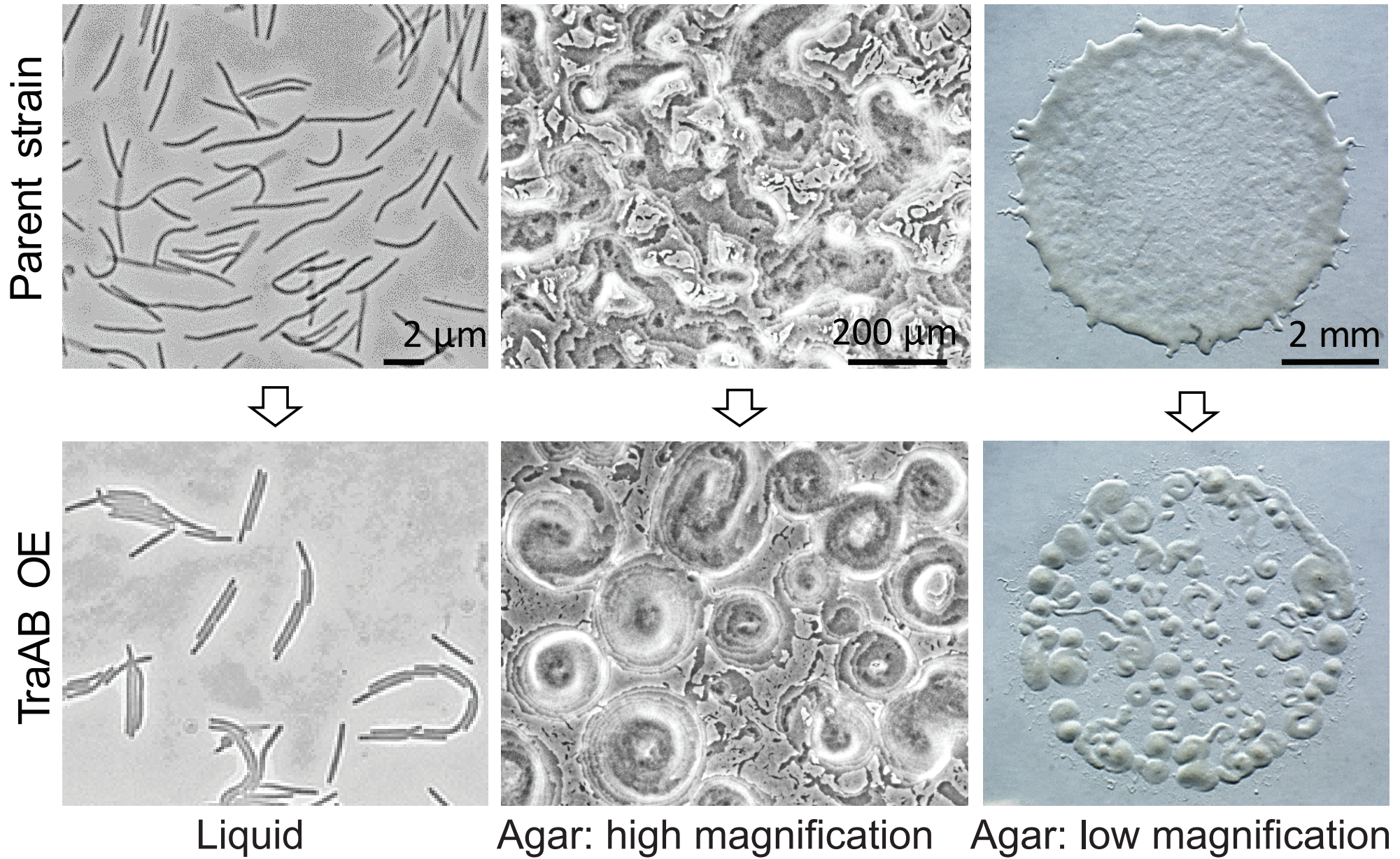
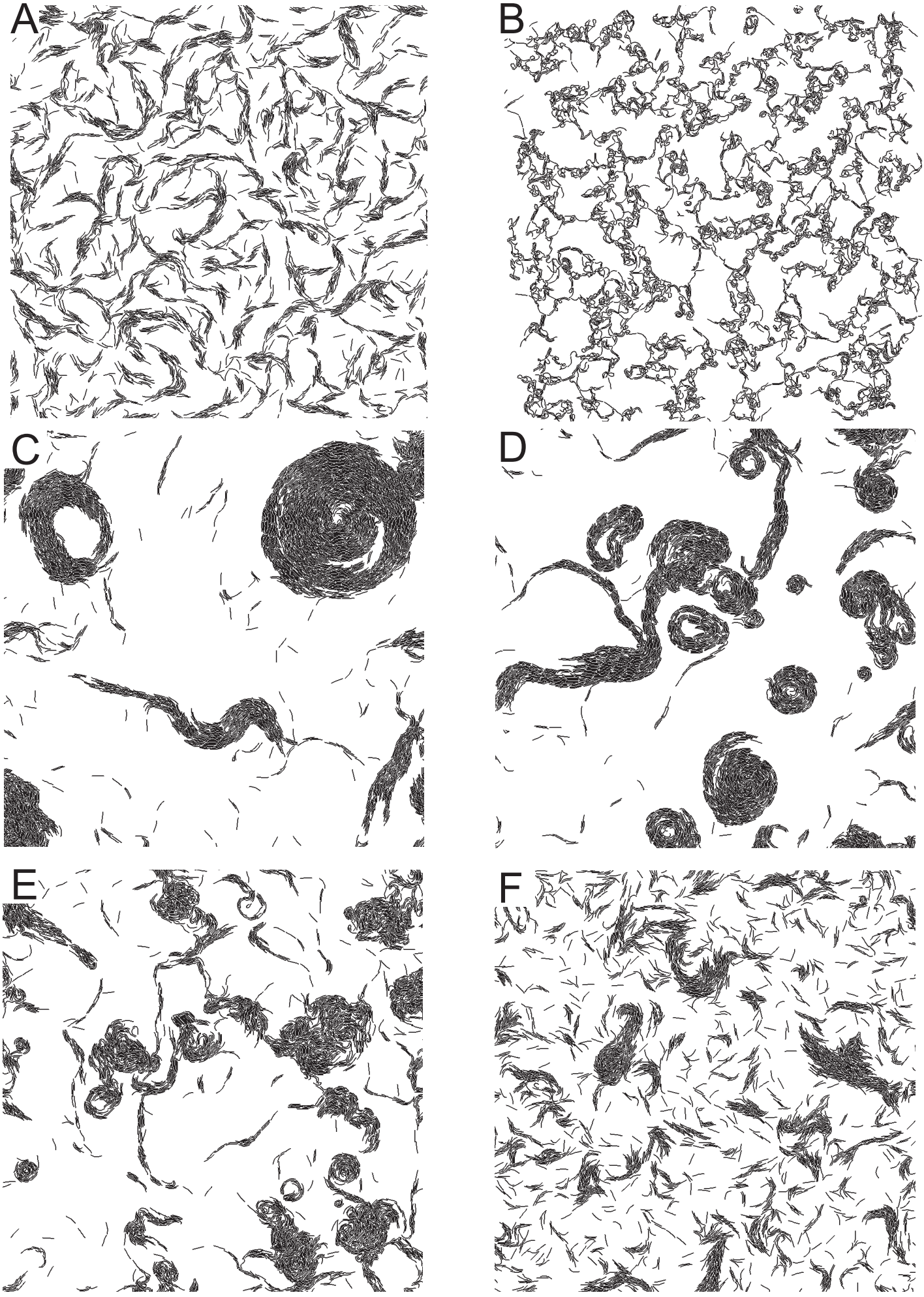
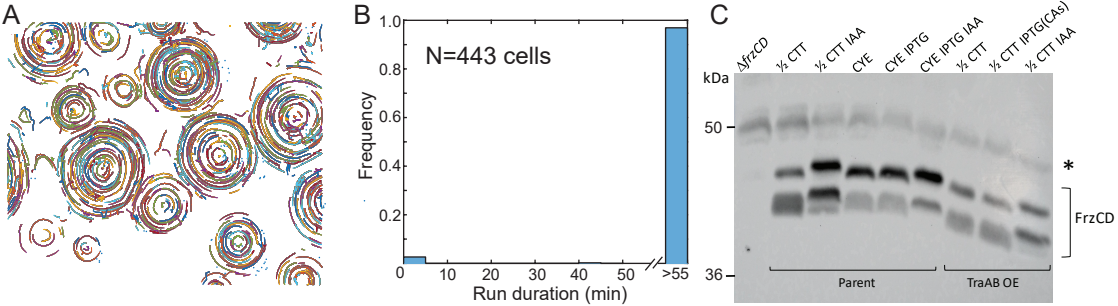
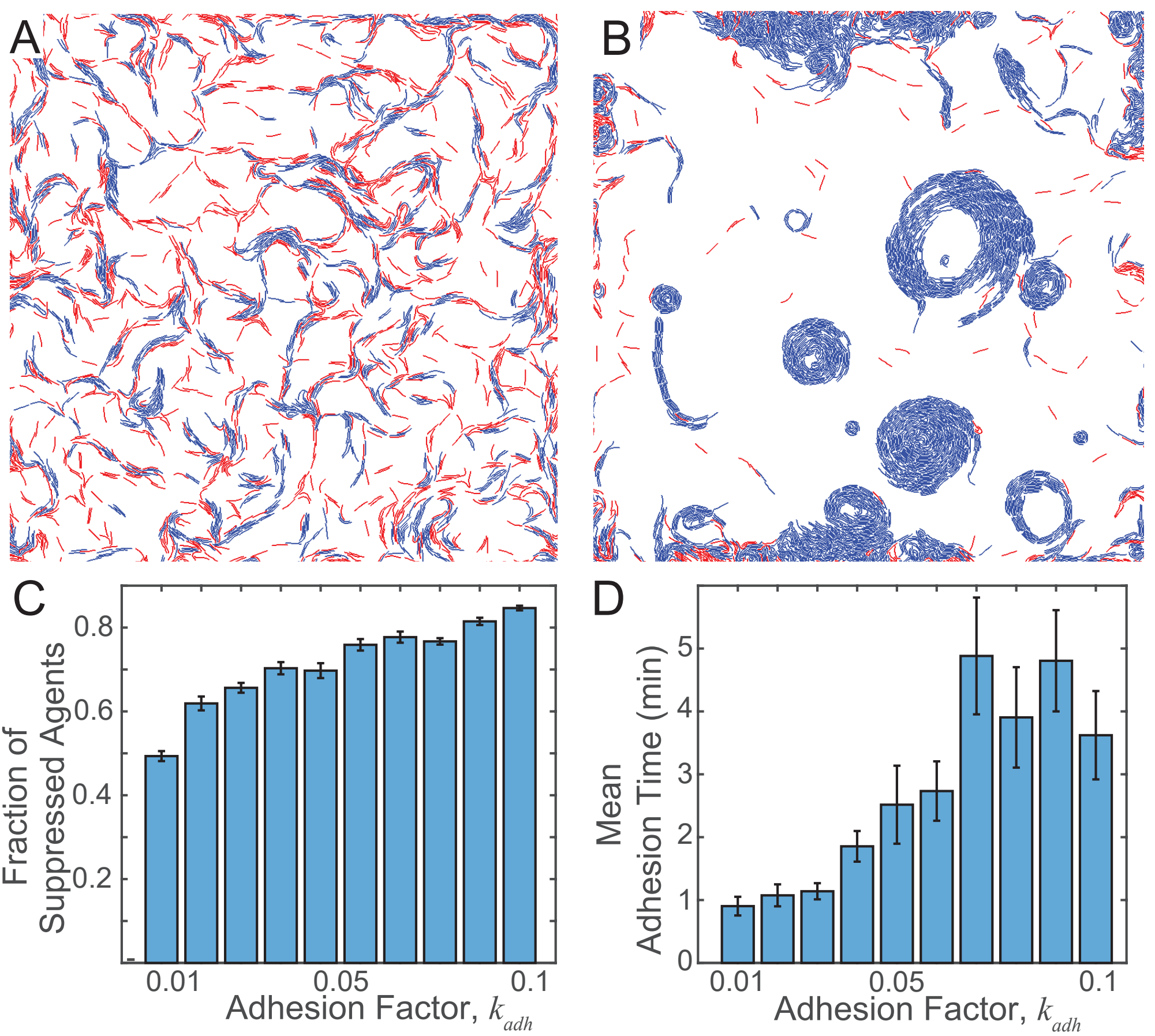
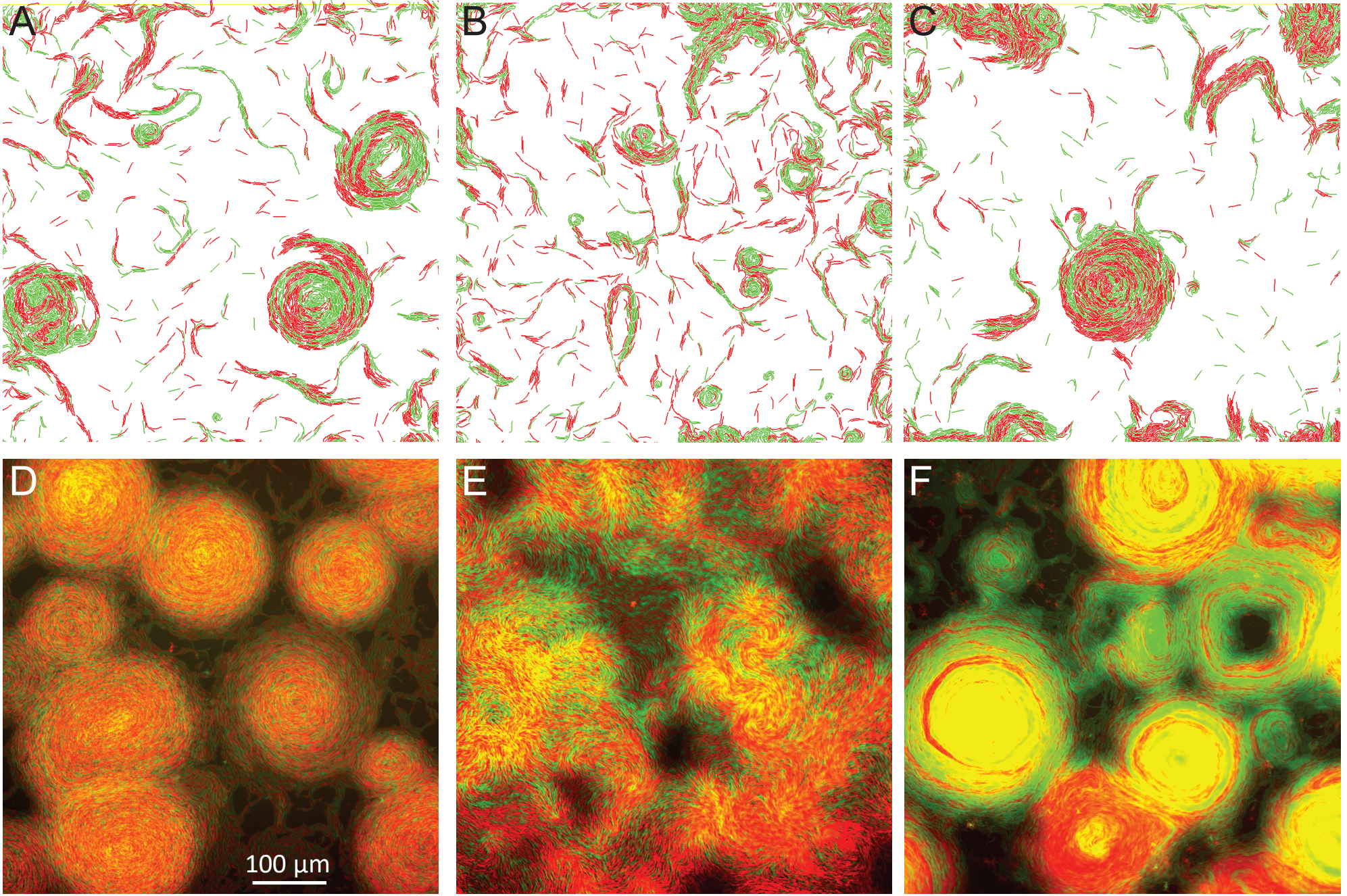
718

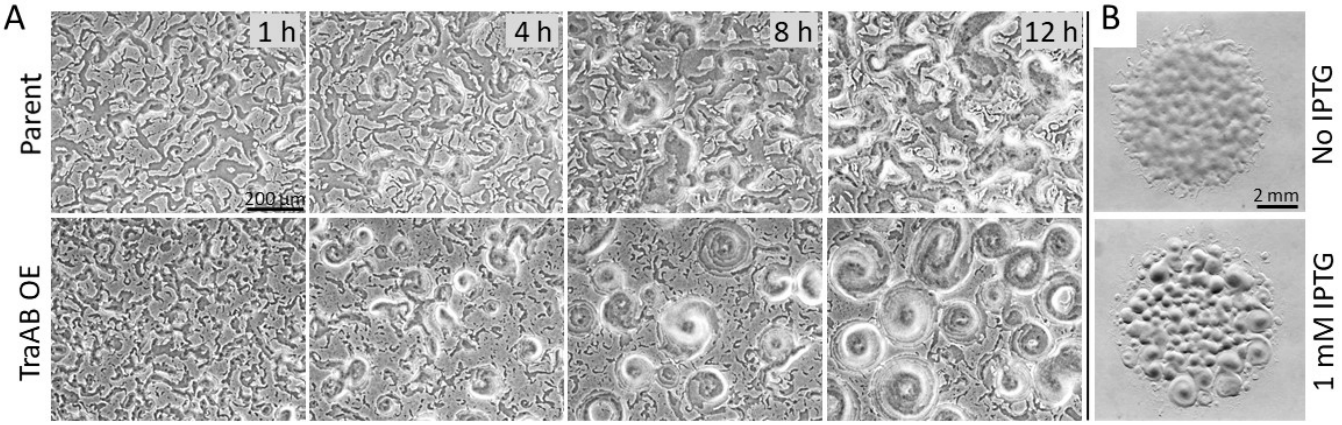

Parent strain
TraAB OE
2 µm
200 µm
2 mm
Liquid
Agar: high magnification
Agar: low magnification


A
B
C
D
E
F

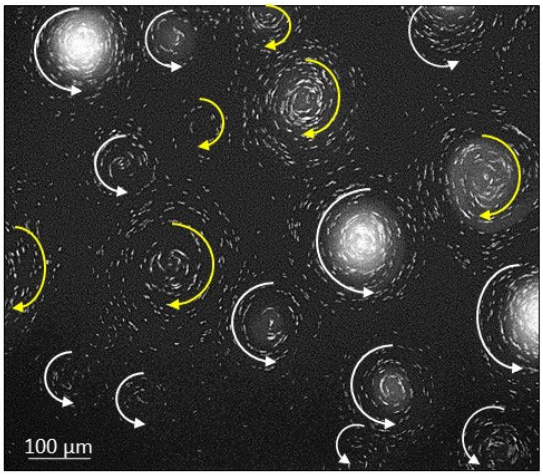

A
B
N=443 cells
Frequency
1.0
0.8
0.6
0.4
0.2
0
10
20
30
40
50
>55
Run duration (min)
C
ΔfrzCD
½ CTT
½ CTT IAA
CYE
CYE IPTG
CYE IPTG IAA
½ CTT
½ CTT IPTG(CAs)
½ CTT IAA
kDa
50
36
*
FrzCD
Parent
TraAB OE


A
B
C
Fraction of Suppressed Agents
0.2
0.4
0.6
0.8
0.01
0.05
0.1
Adhesion Factor, k_{adh}
D
Mean Adhesion Time (min)
0
1
2
3
4
5
0.01
0.05
0.1
Adhesion Factor, k_{adh}

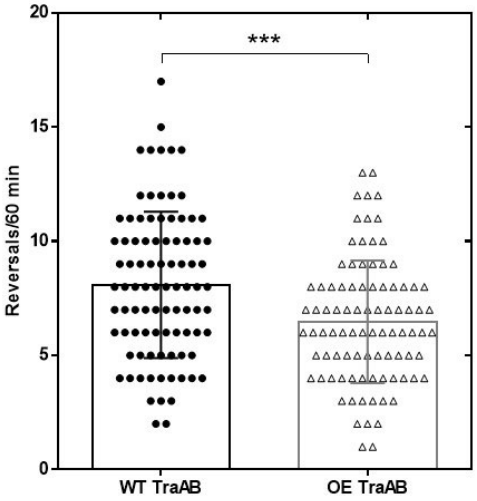

A
B
C
D
E
F
100 µm


A
1 h
4 h
8 h
12 h
Parent
TraAB OE
200 µm
B
No IPTG
1 mM IPTG
2 mm

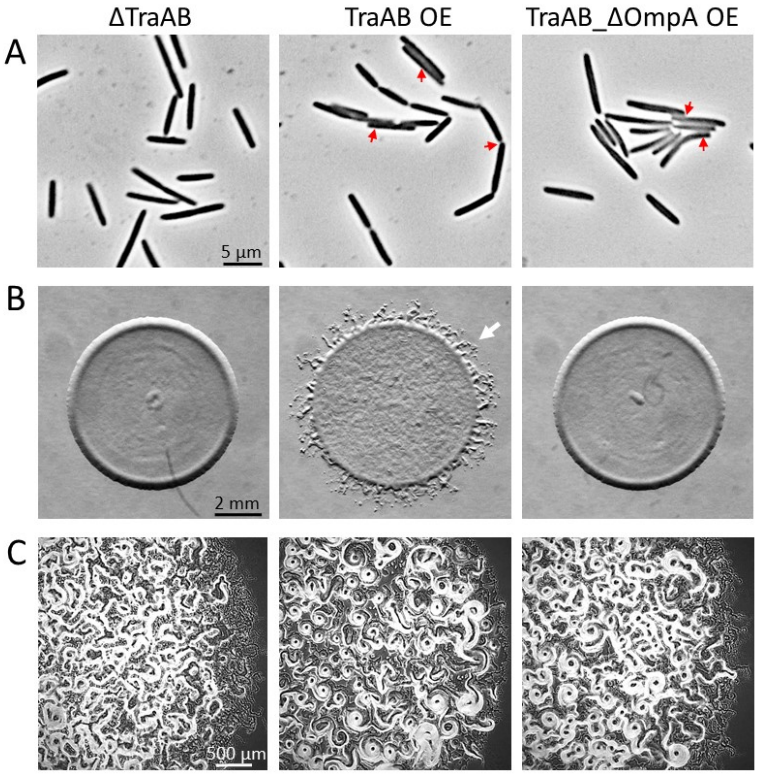
A

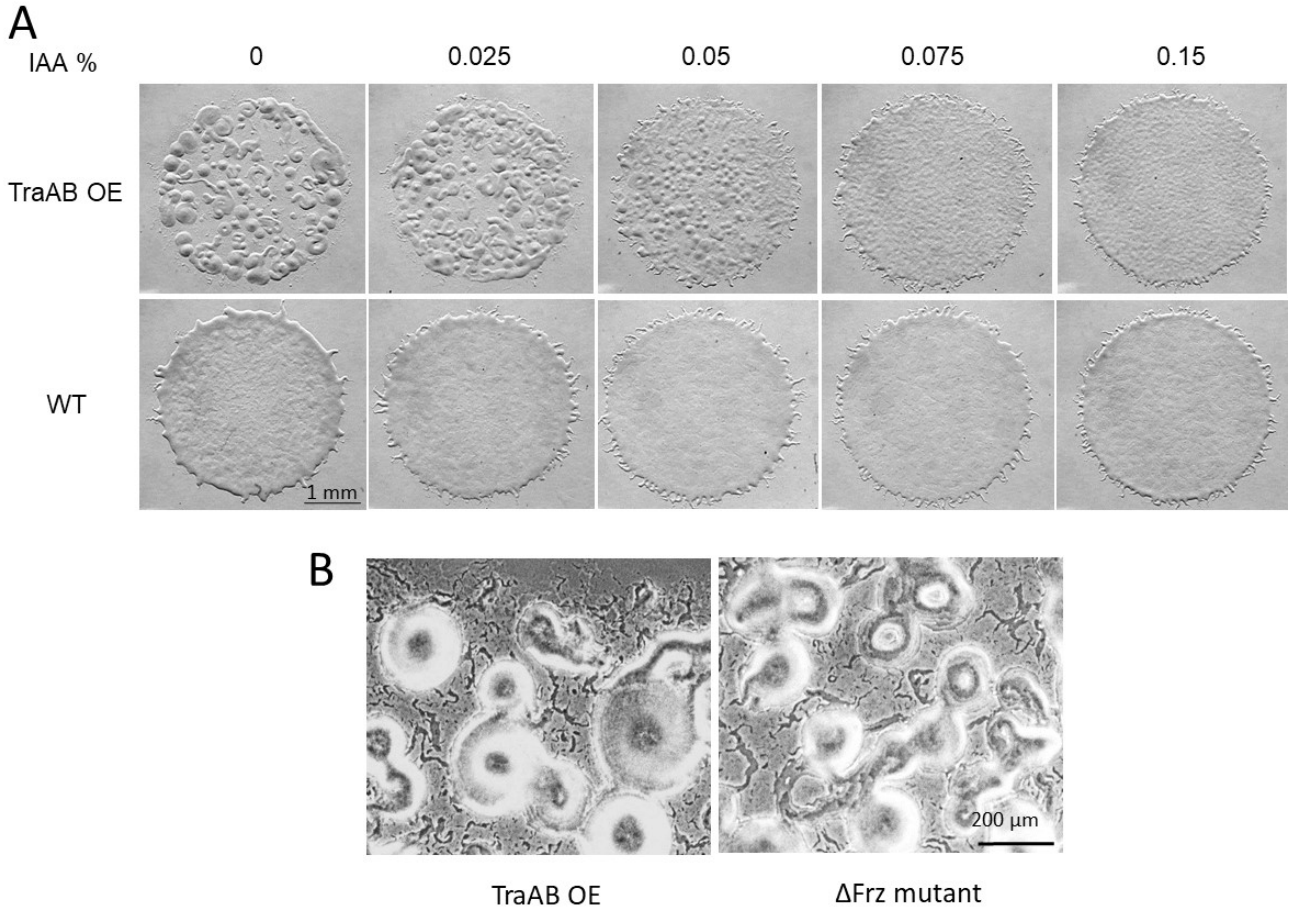


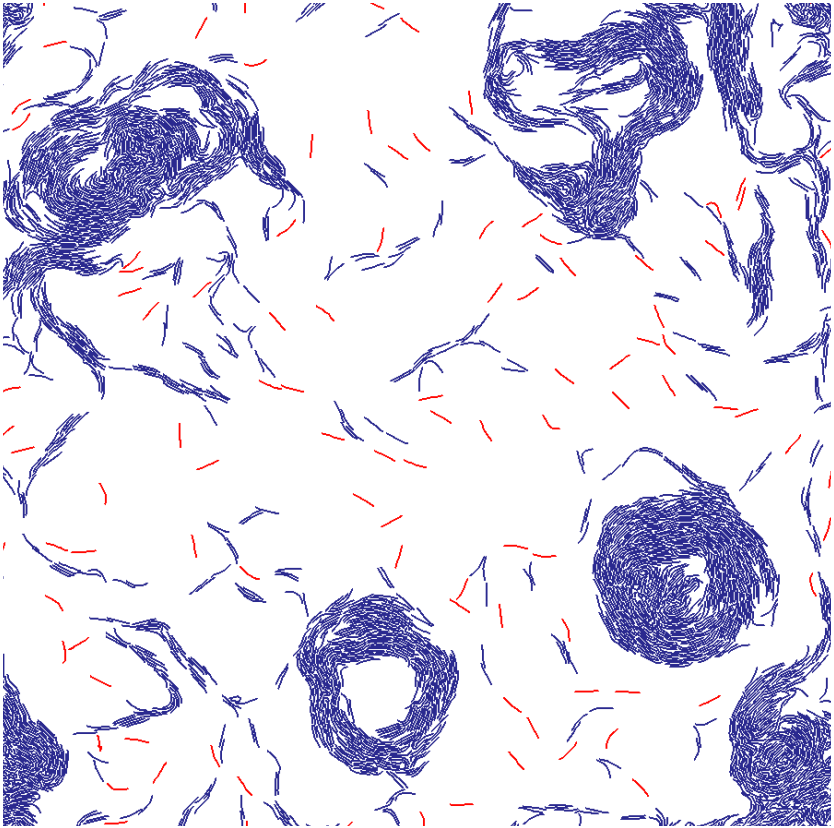
B

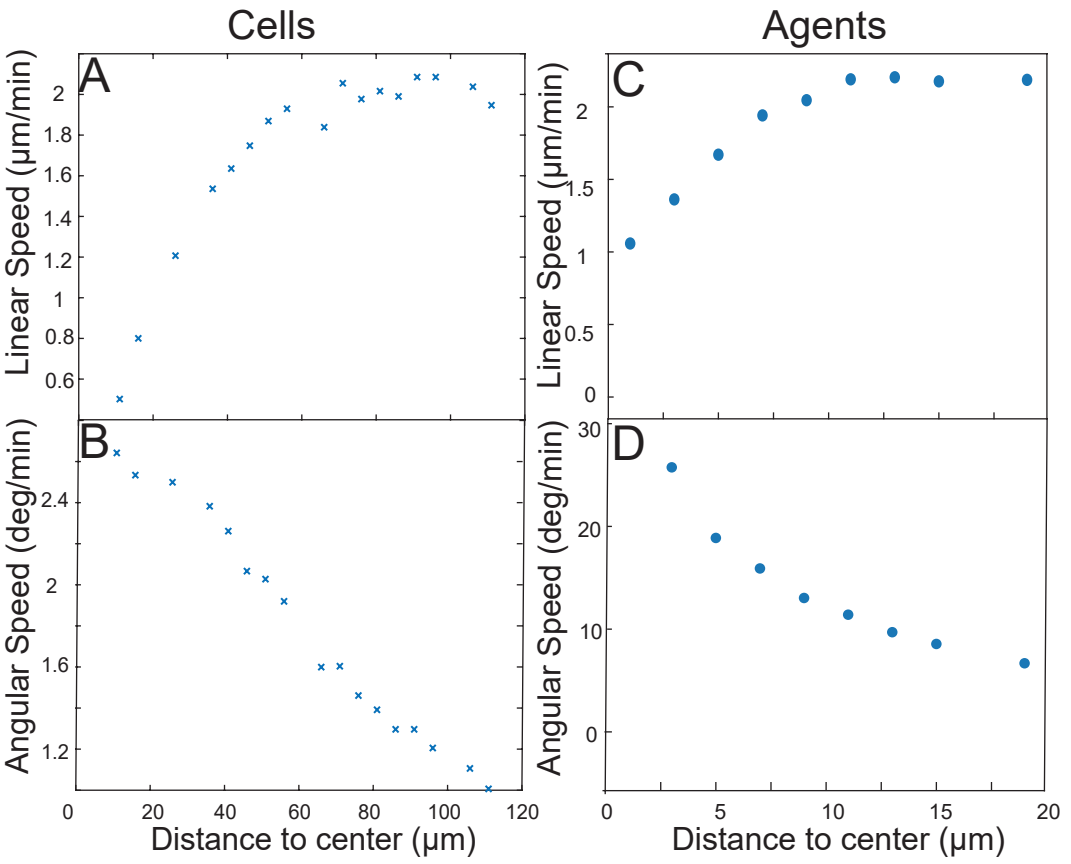


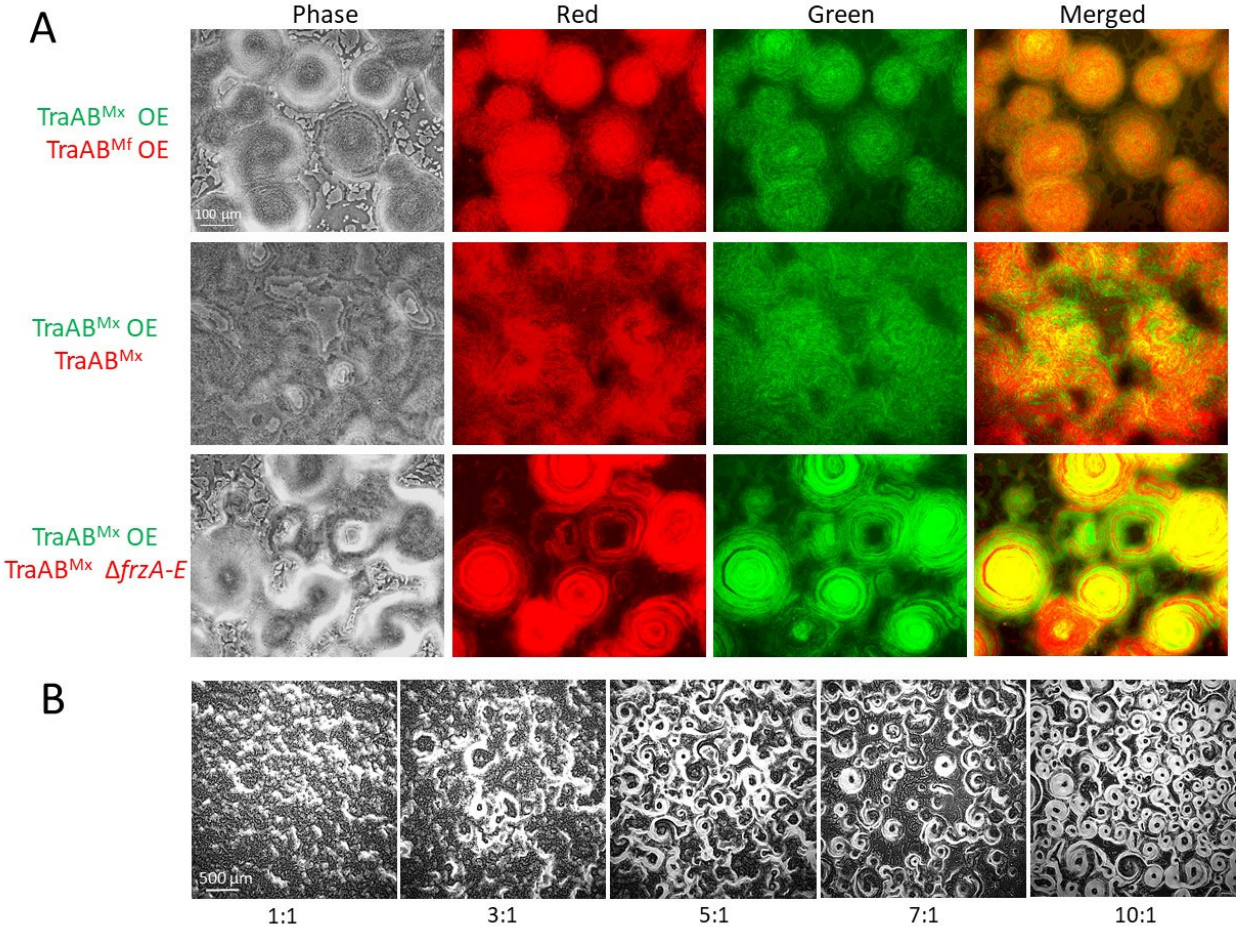
	WT	TraAB OE
Number of cells	91	91
Average reversals/60 min	8.088	6.473
Std. Deviation	3.203	2.689
P value	0.0003	


ΔTraAB
TraAB OE
TraAB_ΔOmpA OE
A
5 µm
B
2 mm
C
500 µm


A
IAA %
0
0.025
0.05
0.075
0.15
TraAB OE
WT
1 mm
B
200 µm
TraAB OE
ΔFrz mutant




Cells
Agents
A
B
C
D
Linear Speed (μm/min)
Angular Speed (deg/min)
Distance to center (μm)
0.6
0.8
1
1.2
1.4
1.6
1.8
2
1.2
1.6
2
2.4
0
0.5
1.5
10
20
30
0
20
40
60
80
100
120
5
15


A
Phase
Red
Green
Merged
TraAB^Mx OE
TraAB^Mf OE
100 µm
TraAB^Mx OE
TraAB^Mx
TraAB^Mx OE
TraAB^Mx ΔfrzA-E
B
500 µm
1:1
3:1
5:1
7:1
10:1