

Dynamically induced spatial segregation in multispecies bacterial bioconvection

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Active matter, from motile bacteria to animals, can exhibit striking collective and coherent behavior. Despite significant advances in understanding the behavior of homogeneous systems, little is known about the self-organization and dynamics of heterogeneous active matter, such as complex and diverse bacterial communities. Under oxygen gradients, many bacterial species swim towards air-liquid interfaces in auto-organized, directional bioconvective flows, whose spatial scales exceed the cell size by orders of magnitude. Here we show that multispecies bacterial suspensions undergoing oxytactic-driven bioconvection exhibit dynamically driven spatial segregation, despite the enhanced mixing of bioconvective flows, and the fact that these species coexist in their natural habitat. Segregation is observed as patterns of spatially interlocked domains, with local dominance of one of the constituent species in the suspension. Our findings suggest that segregation mechanisms are driven by species-specific motile behaviors under conditions of hydrodynamic flow, rather than biochemical repulsion. Thus, species with different motile characteristics in the same ecological context can enhance their access to limiting resources. This work provides novel insights on the role of heterogeneity in active matter, as well as on the dynamics of complex microbial communities, their spatial organization and their collective behavior.

Active matter systems often exhibit self-organized, collective motion that can give rise to the emergence of coherent spatial structures¹. Prime examples covering many length scales range from mammal herds, fish schools and bird flocks, to insect, bacterial and robot swarms^{2–7}. In the case of bacteria and other unicellular organisms, the confluence of motility, gravity and abiotic gradients, such as oxygen, may give rise to collective convection patterns dubbed bioconvection^{8–10}. Bioconvection has been observed both in the sea¹¹ and lakes^{12,13}, and the ecological consequences of spatially-localized structures have been demonstrated^{14–17}. Oxytactic-driven bioconvection occurs when up-swimming flagella-propelled bacteria that are denser than the surrounding fluid medium migrate towards an air-liquid interface where oxygen is available, creating a layer that

is denser than the medium below, and an ensuing Rayleigh-Taylor-type instability¹⁸. The gravitational collapse sets up laminar convective currents that auto-organize into patterns (Fig. 1a), whose characteristic spatial scales are orders of magnitude larger than the bacteria themselves and which promote enhanced mixing of oxygen and nutrients^{12,19–23}. Oxytactic bioconvection has been studied experimentally, solely in single bacterial species suspensions, using model organisms such as *B. subtilis* and *E. coli*^{10,17,24}. The study of bioconvective patterns of mixed bacterial species as examples of heterogeneous active matter is scant^{8,9}, despite the fact that bacteria in nature typically live in highly diverse communities, in which different species cooperate and/or compete for resources, including space²⁵.

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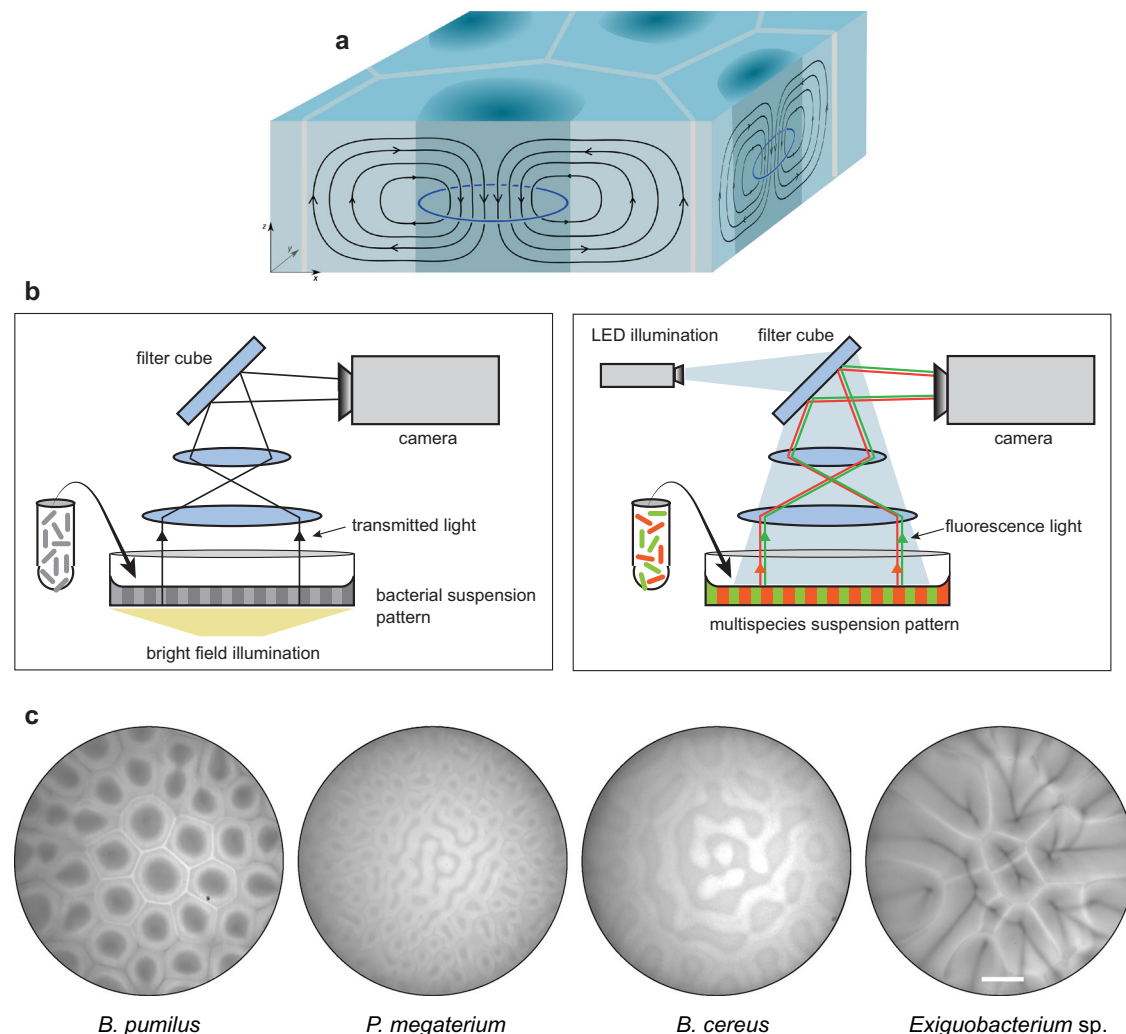


Fig. 1 | Bioconvective patterns of different species. **a** Schematic depiction of streamlines in a typical bioconvective pattern (black lines in cross section) and stagnant regions (blue oval at medium height). Darker regions from the top represent higher bacterial concentration. **b** Experimental setup scheme. Left panel: bioconvective pattern visualization using transmitted bright field light illumination (yellow). Gray ovals depicted in test tubes represent bacteria. Right panel:

epifluorescence elicited by LED illumination (light blue) in bacterial multispecies suspension (ovals represent two fluorescently labeled species). See Methods for experimental details. **c** Typical quasi-stationary bioconvective patterns observed from above by transmitted light through the indicated species suspensions. Quasi-stationarity is attained within ~5 min, well before cell density increments become appreciable. Scale bar = 2 mm.

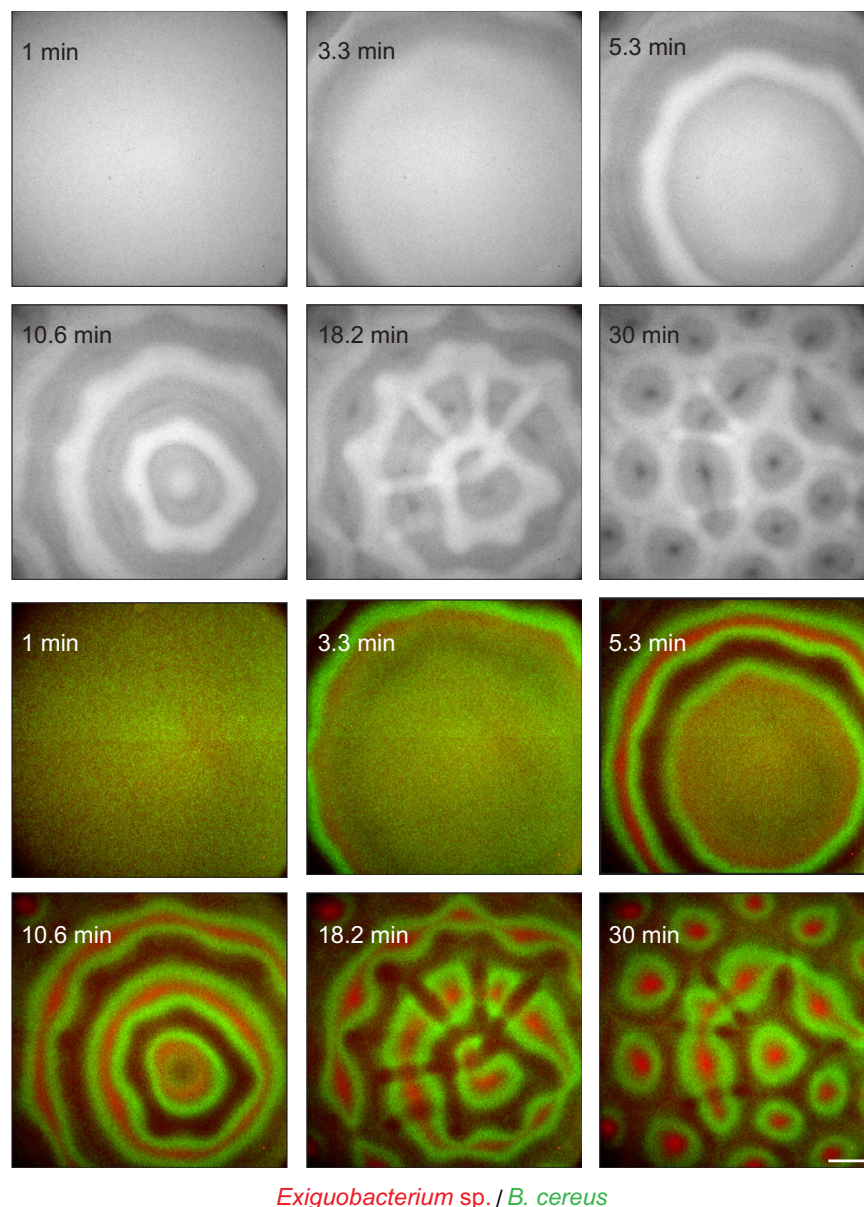
Here we report observations of bioconvection in multispecies suspensions of bacteria from the Cuatro Ciénegas Basin (CCB), Mexico, a unique ecosystem in which basic questions on early life on Earth, its evolution and ecology can be addressed^{26–29}. CCB communities exhibit unique and endemic hyper-diversity under extreme conditions, remnant from pre-Cambrian oceans (circa 700 million years ago)³⁰. These conditions, which represent a barrier to colonization/invasion by outside microorganisms, have kept CCB in a largely pristine state. The shallow pond ecosystem lifestyle of CCB supports a rich microbial diversity, and the extremely low yearly precipitation makes conditions for bioconvection favorable.

Results

Pattern diversity across species and bacterial density distribution in convection cells

Cooperative and/or competitive interactions between species in a community can take place under different lifestyles, such as in planktonic and sedimentary environments. We selected a small consortium of bacteria collected from shallow sediments of the Churince lagoon in CCB (Table S1), and tested for their bioconvection characteristics in a stereomicroscope setup (Fig. 1b). Bacteria in sediments can display

significant collective motion³¹. In spite of the generic nature of the instability that triggered the initial stages of bioconvection, we found marked differences between the bioconvective patterns across species. Typical well-formed, quasi-stationary patterns observed under our experimental conditions are shown in Fig. 1c. Quasi-stationarity was attained within ~5 min, before increases in bacterial density due to cell division became appreciable. Patterns with finer scales formed at longer times (Movie S1), consistent with previous observations¹⁷. Local differences in intensity correspond to differences in bacterial concentrations, with dark regions within convective cells corresponding to denser, down-flowing streams of bacteria (Fig. 1c). Whereas *Bacillus pumilus* exhibited well-delineated polygonal, convex convective cells with nearly-uniform down-flowing currents, *Priestia megaterium* and *Bacillus cereus* convective cells were serpentine, and those of *Exiguobacterium* sp. exhibited marked auto-focusing, observed as dark elongated stripes. To estimate bacterial density differences between light and dark regions, we calibrated the local transmitted light intensity within the observed patterns, using as reference the intensity of well-mixed suspensions of known bacterial concentration. Density maps for selected patterns obtained with different species show that local bacterial densities may differ by about five-fold between the



Exiguobacterium sp. / *B. cereus*

Fig. 2 | Spatial segregation and locking in bioconvective patterns of a binary suspension. Top panels: Transmitted light images of the dynamics of pattern formation in a 1:1 volume ratio mixed suspension of *B. cereus* and *Exiguobacterium* sp., starting from a well-mixed homogeneous state. Bottom panels: Same images as

above observed through fluorescence channels with *B. cereus* and *Exiguobacterium* sp. fluorescently labeled in green and red, respectively (see Methods). The time following mixing of the suspension is indicated in each panel. See also Movie S3. Scale bar = 2 mm.

central and outer regions of convection cells (Fig. S1). A high resolution microscopic observation of a convection cell in an *Exiguobacterium* sp. suspension from the bottom surface shows that cells are tightly packed together in the region of the plume, and pushed away from it by a steady directional flow, until reaching the edge of the convection cell (Movie S2).

Bioconvection in binary suspensions of motile/motile species

We studied bioconvection in a mixed suspension of *Exiguobacterium* sp. and *B. cereus*, both species being motile. We show in the upper half of Fig. 2 transmitted light snapshots of the observed dynamics. A few minutes after the well-mixed suspension was deposited in a well, convection started near the rim, and gradually moved inward towards the well center. Bioconvective patterns then evolved into spatially interlocked, nested domains, with features characteristic of each of the constituent species being predominant in different

regions, suggesting spatial segregation of the species. To support the notion of segregation, we fluorescently stained *Exiguobacterium* sp. in red and *B. cereus* in green prior to mixing. Overlays of red and green fluorescence channels are shown in the bottom half of Fig. 2, corresponding to the frames shown in the upper part. Intense, self-focused, down-flowing plumes of *Exiguobacterium* sp. (red) were observed to be consistently localized within down-flowing plumes of *B. cereus* (green) (Movie S3). Darker brown areas correspond to up-flowing currents where no segregation is observed. After the transient stages during which the patterns were formed, the relative areas occupied by colored down-flowing currents achieved a nearly steady-state value (Fig. S2). Similar behavior was observed when cells were labeled using green fluorescent protein (GFP) expressed constitutively from plasmids (Fig. S3). This striking behavior strongly supports the notion of spatial segregation between species, despite enhanced mixing in bacterial suspensions undergoing large-scale

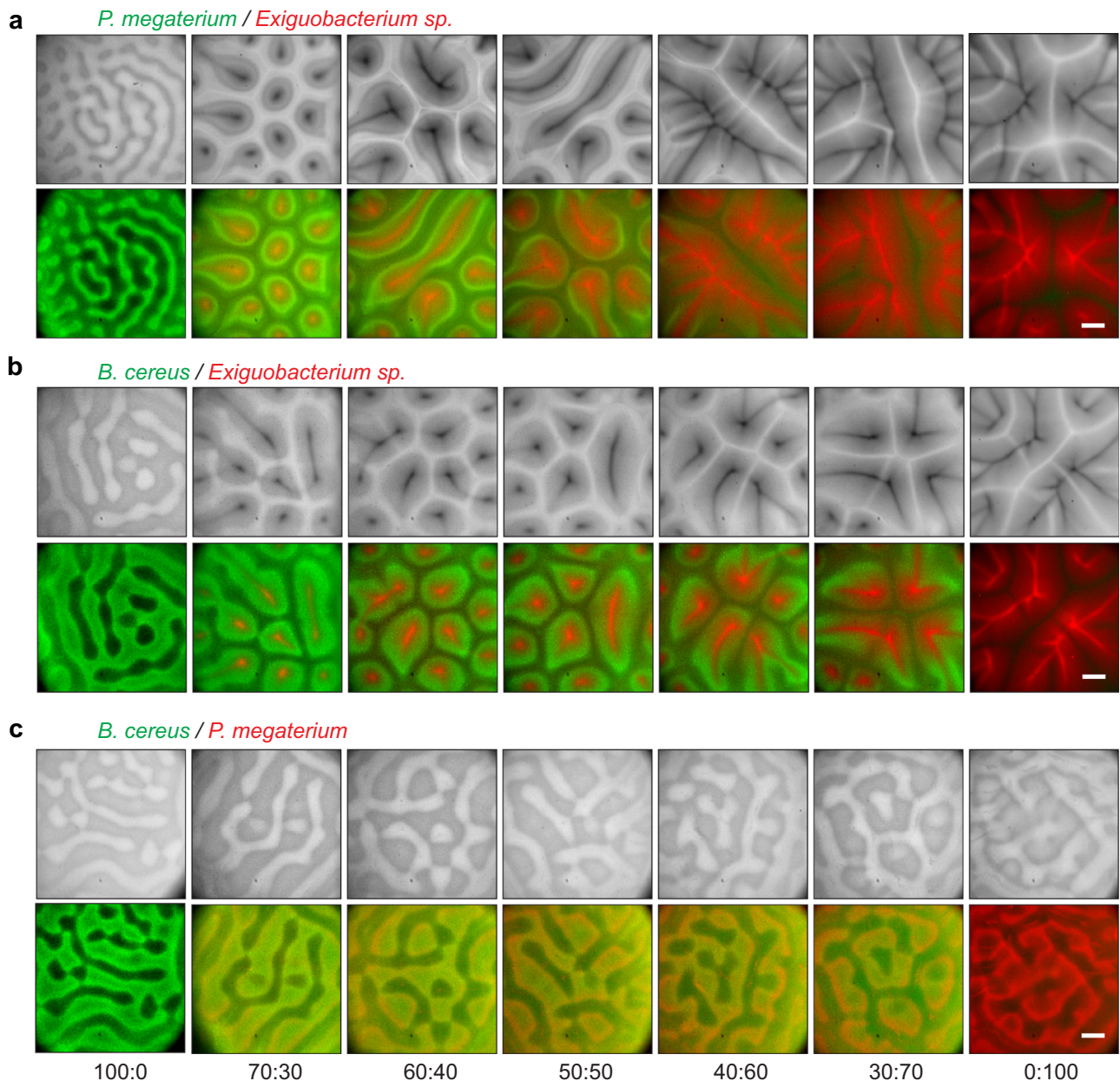


Fig. 3 | Variation in DISS bioconvective patterns as a function of the relative concentration of constituent species in binary suspensions. Bioconvective patterns in binary suspensions of bacterial species at the indicated relative volume ratios of the two constituent species. For each species pair, bottom panels show overlays of red and green fluorescence channels (see Methods), whereas top panels

show the same patterns recorded with transmitted light. **a** *P. megaterium*/*Exiguobacterium* sp. green and red respectively, 30 min following mixing (see also Movie S4); **b** *B. cereus*/*Exiguobacterium* sp. green and red respectively, 60 min following mixing; **c** *B. cereus*/*P. megaterium* green and red respectively, 60 min following mixing. Scale bar = 1 mm.

convective flows²⁰. Segregation was also observed in binary suspensions of other motile species, and was robust against variation in the ratio of both constituent species, resulting in a smooth interpolation in pattern characteristics of the individual constituents (Figs. 3 and Fig. S4). It is noteworthy that no segregation was observed in the absence of bioconvection, and that the onset of bioconvection in a binary suspension was determined by the total bacterial concentration of both species. Therefore, bioconvection in the binary suspension was attained at a lower concentration of each of the constituents, compared to when each constituent underwent bioconvection alone (Fig. S5). Furthermore, once patterns attained a steady state, they recovered rapidly (~2 min) following thorough mixing (Fig. S2). De novo segregation and recovery characteristic timescales are significantly smaller than bacterial cell division times

under our experimental conditions (Fig. S6). Moreover, while patterns varied, segregation was robust against changes in suspension height h (Fig. S7), once the critical value of an appropriate Rayleigh number Ra was exceeded (of note, $Ra \sim n_c((\rho_c - \rho_w)/\rho_w)h^3$, where n_c is the bacterial concentration, ρ_c the density of the bacterial interior, and ρ_w the density of the medium. See Hillesdon and Pedley²²). We term the phenomenon observed in the above suspensions dynamically induced spatial segregation (DISS).

Bioconvection in binary suspensions of motile/sessile species

The motility of both bacterial species as a necessary condition for DISS is supported by bioconvection experiments in binary suspensions in which one of the constituent species was non-motile. To illustrate this, we studied binary suspensions consisting of the sessile

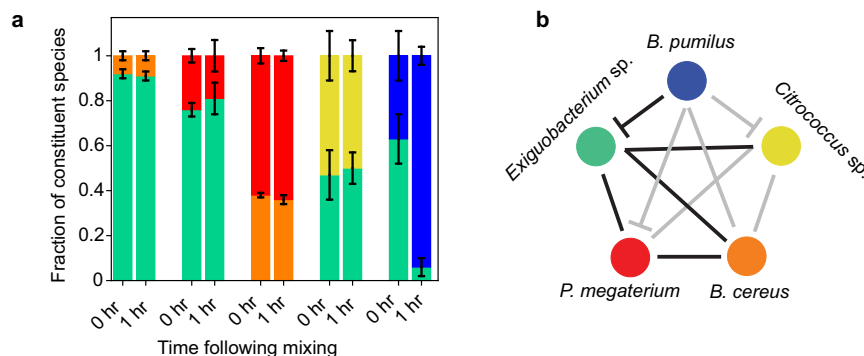


Fig. 4 | Characterization of interspecies interactions. **a** Mean colony-forming units' fraction of constituent species sampled before mixing (0 hr) and from binary suspensions after one hour (1 hr). Error bars denote standard errors of the mean over three independent experiments. **b** Schematic interaction network between different species, as determined from spot-lawn assays (Fig. S9). Black lines

represent interactions between pairs also shown in the left panel. Gray lines represent interactions determined by spot-lawn assays. Blunt lines denote antagonist interactions, whereas simple lines represent neutral interactions. The color code representing the different bacterial species is the same for panels a and b.

actinobacterium *Citroccoccus* sp. with the motile *Exiguobacterium* sp. in different ratios (Fig. S8a). Patterns obtained with mixed suspensions show that for small *Citroccoccus*/*Exiguobacterium* ratios, *Citroccoccus* sp. co-localizes spatially with *Exiguobacterium* sp., and is thus passively advected by the latter, effectively serving as a scalar tracer. However, as the ratio was increased, we observed an increase in the segregation of *Citroccoccus* sp. towards the rim, forming an outer annulus where no bioconvective currents were observed. This annulus enclosed a central bioconvective region rich in *Exiguobacterium* sp., with *Citroccoccus* sp. present in a smaller concentration as a flow marker (from ratios 60:40 and above). In the central bioconvective region, dual fluorescence labeling indicates that the spatial distribution of *Citroccoccus* sp. cells is nearly homogeneous, with small modulations locked to the bioconvective pattern set by *Exiguobacterium* sp. (Figs. S8b and Movie S5). Note that *Citroccoccus* sp. alone did not elicit bioconvection (see Fig. S8a, 0:100).

Biological interactions in binary suspensions during bioconvection

Both biological and physical interactions between species in a suspension may give rise to spatial segregation. The former include for instance the secretion of chemo-repellents, whereas the latter, short-range cell-cell collisions and longer-scale hydrodynamic interactions. Spatial patterns in bacteria can also be formed by toxins, but primarily on surfaces³². To shed light on the importance of biological interactions beyond competition for resources and metabolites³³ we first tested for antagonistic effects that may alter the relative bacterial concentrations in a suspension. To this end, the relative concentrations of bacteria in a binary suspension were measured by colony counting right near the onset of bioconvection and one hour later. As shown in Fig. 4a, there were no detectable reductions in the number of cells of either of the paired species, except for a pair involving *B. pumilus*, which is antagonistic to *Exiguobacterium* sp. and *P. megaterium*²⁹, but not to *B. cereus*^{29,34} (Fig. 4b). The interactions between different species pairs, measured by spot-lawn assays (Fig. S9), are consistent with the results observed in the bioconvective state (Fig. 4a). To rule out biological interactions that could potentially induce segregation, e.g., by the secretion of a repelling factor, we tested for segregation in the absence of bioconvection, while preserving the species' motile state. Bioconvection was prevented by either reducing the suspension's height within the well, reducing cell density, or by increasing the viscosity of the extracellular medium by the addition of agar to a final concentration of 0.3%, which allows cell motility but impedes bioconvection. As Fig. S1,0 shows, no spatial segregation was observed under these conditions, confirming that

spatial segregation is a dynamically-induced process, rather than a biological response.

Cell morphology and swimming behavior

We studied the extent to which morphological factors contribute to bioconvective pattern formation and DISS. Phase contrast images of representative bacterial cells sampled under conditions of bioconvection are shown in Fig. 5a. Despite the similarity in size between *Exiguobacterium* sp. and *P. megaterium* cells, a glance at Fig. 1c shows that their bioconvective patterns are different. In addition, images of the flagella of different bacterial species cells using transmission electron microscopy (TEM) and atomic force microscopy (AFM) showed that all species are peritrichous (Fig. S11), except for *Citroccoccus* sp., which lacks flagella. Being peritrichous, they have multiple, long flagella arranged along the length of the rod-shaped cell body. This indicates that they are low-Reynolds number pushers³⁵.

Theoretical considerations have suggested that self-propelled pushers interact primarily by long-range, hydrodynamic dipolar interactions³⁶. However, more recent studies indicate that these dipolar interactions are weakened beyond length scales of a few microns, by noise stemming from orientational Brownian motion and intrinsic swimming stochasticity³⁷. To evaluate orientational memory and the role of long-range dipolar interactions, we aimed to quantify the tendency of different species to change orientation by evaluating the persistence length of individual cell trajectories. A persistence length quantifies the tendency to move straight and avoid collisions with neighbor swimmers. It is an important parameter for understanding correlations in active matter, particularly in the low density regime³⁸. Note that the persistence length might be affected by bacterial concentrations, e.g., by the number of collisions. To this end, we first visualized the motion of cells of different species, in suspensions sampled from a bioconvecting state, by phase contrast microscopy (Movie S6). While *B. pumilus* displayed a propensity to swim in nearly straight trajectories, *Exiguobacterium* sp. and *P. megaterium* cells carried out significant rotations as they swam. Cells of all species exhibited considerable orientational noise and in addition, a significant proportion displayed incomplete cell division or a filamentary structure, deviating from the paradigmatic run-and-tumble behavior of model bacteria such as *E. coli*^{39,40}. Next, we calculated the average value of the persistence lengths (ξ_p) of two-dimensional trajectories as a function of cell concentration for the different species, by diluting suspensions from OD₆₀₀ = 1 (Fig. 5b). These dilutions cover the range of cell concentrations relevant for bioconvection and show that the hierarchy of ξ_p values is maintained between species, irrespective of bacterial density. Note that species differed in the behavior of their

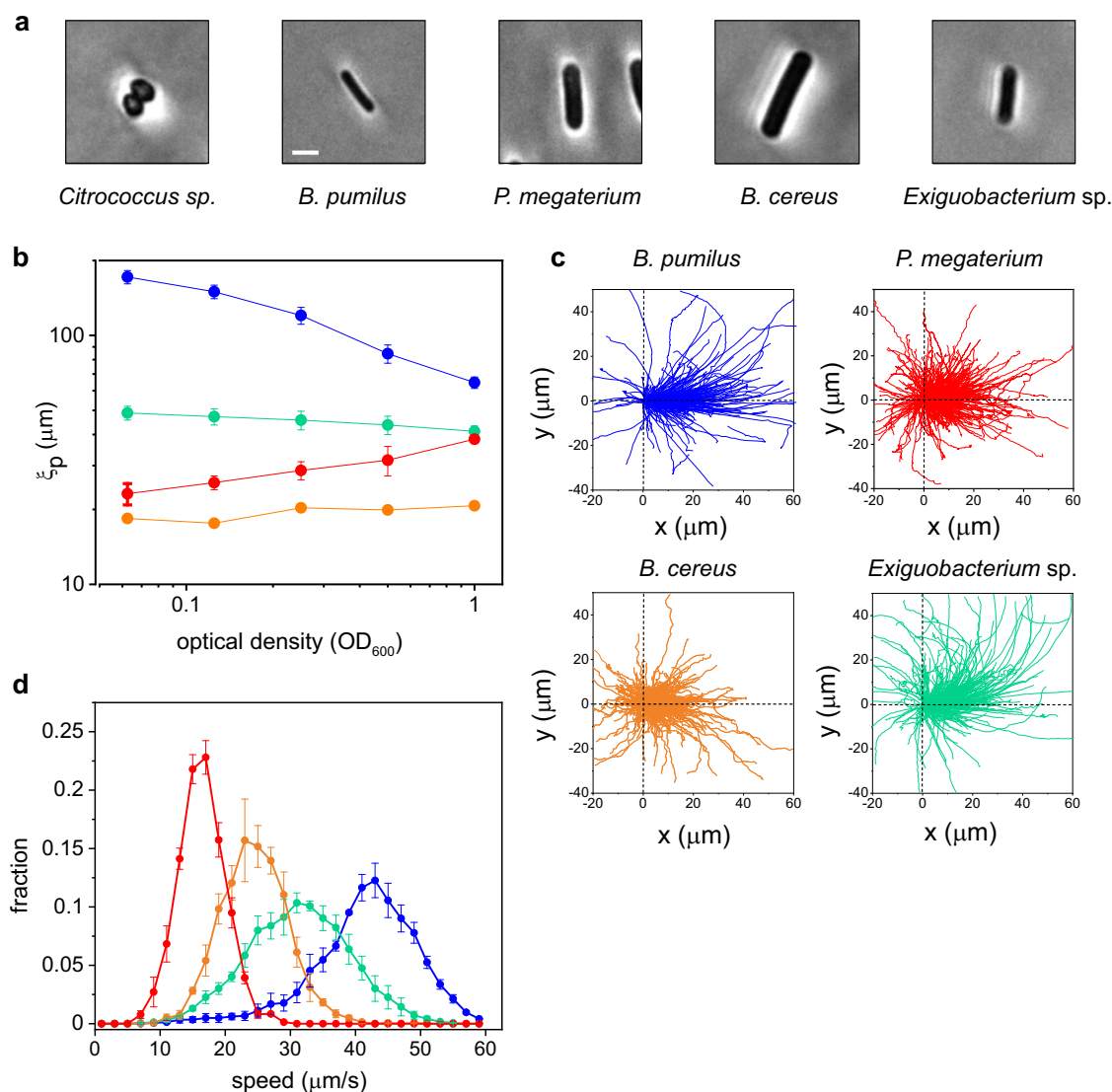


Fig. 5 | Characterization of bacterial cells and their motility in a bioconvective state. **a** Phase contrast images of the indicated species cells. Scale bar = 2 μm for all species. The images are representative results from at least three independent experiments. **b** Mean persistence length ξ_p as a function of bacterial concentration for four bacterial species (*B. pumilus*, blue; *P. megaterium*, red; *B. cereus*, orange; and *Exiguobacterium* sp., green), whose cultures were adjusted to OD₆₀₀ = 1. The mean values of ξ_p for each value of OD₆₀₀ were obtained by averaging over all trajectories from two independent experiments except for *P. megaterium* (three repeats), and error bars representing standard error of the mean were estimated from 10000 bootstrap samples. The total number of trajectories, n , for OD₆₀₀ values ranging from 0.06 to 1 were correspondingly: *B. pumilus* (7712–12745); *P. megaterium* (1263–10869); *B. cereus* (514–4048) and

Exiguobacterium sp. (1544–9790). The value of n for each OD₆₀₀ is given in Fig. S12. **c** Two-dimensional trajectories of bacterial cells near the bottom surface under conditions of bioconvection for a given concentration (Methods), at least 100 trajectories were randomly chosen from three independent experiments. The initial tangents in the trajectory plots were aligned to the x-axis to facilitate visualization. **d** Mean speed distributions of bacterial species in diluted suspensions of a given concentration (Methods) obtained from three independent experiments. Error bars denote standard errors of the mean and the color code for the different species is the same as in **b** and **c**. The number of trajectories n obtained from three biological replicates were *B. pumilus* (5509); *P. megaterium* (1441); *B. cereus* (1038) and *Exiguobacterium* sp. (4122).

persistence length: while *B. pumilus* and *Exiguobacterium* sp. displayed a significant negative correlation with increasing cell concentration as previously observed in other bacteria⁴¹, *B. cereus* and *P. megaterium* behaved differently. Examples of two-dimensional trajectories of individual cells, with their first segments aligned parallel to each other in the horizontal direction are shown in Fig. 5c. Notably, the values of ξ_p for *B. cereus* and *P. megaterium* were small, and therefore these cells changed their orientation after coasting distances a few times the bacterial size. In contrast, the persistence length for *Exiguobacterium* trajectories was about threefold larger, despite the fact that many *Exiguobacterium* cells carried out significant rotations as they swam (Movie S6). The persistence length for *B. pumilus* was significantly

larger than for all other species. Well defined DISS patterns were observed when the contrast in ξ_p values were significant (Fig. 5b), as between *Exiguobacterium* mixed with either *B. cereus* or *P. megaterium* (Figs. 2 and 3a, b). DISS was however weaker when ξ_p values were similar, as for *B. cereus* and *P. megaterium* suspensions (Fig. 3c). These results indicate that for the latter two species, orientational noise is substantial and that long-range dipolar interactions play a minor role. Therefore, we hypothesize that their bioconvective collective motion is dominated by short-range interactions such as collisions, as well as cell-cell scattering e.g., by lubrication forces^{37,42,43}.

Short range interactions may facilitate orientational order in the suspension⁴⁴, and this in turn reduces collisions, suggesting their

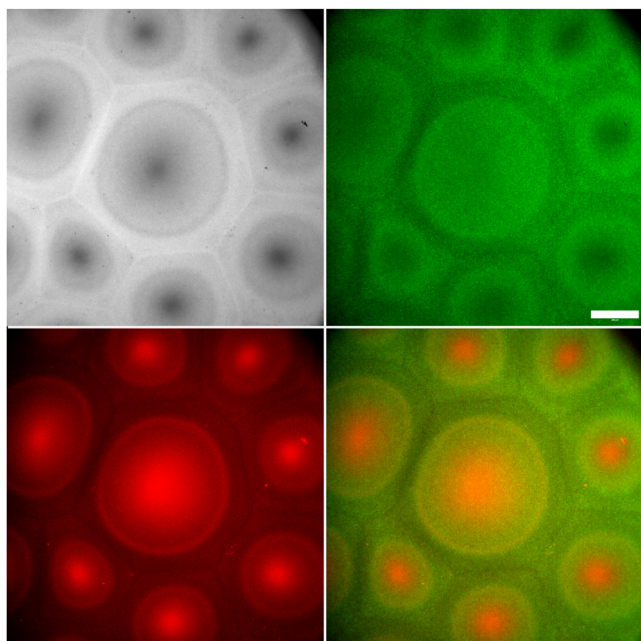


Fig. 6 | Bioconvection in a complex five-species suspension. Bioconvective pattern in a suspension consisting of five species: *Bacillus infantis*, *Rosellomorea aquimaris*, *P. megaterium*, *B. cereus* (labeled in green), and *Exiguobacterium* sp. (labeled in red), all of which are motile, and none of which interact by strong antagonistic biotic interactions. All species were initially present in the same volume proportion, diluted from individual suspensions with $OD_{600} = 0.7$. *B. cereus* and *Exiguobacterium* sp., are labeled in green and red, respectively. The images were taken 80 min after mixing. See also Movie S7. Scale bar = 1 mm.

possible role in DISS. To substantiate the extent of collisions between species in a bioconvective multispecies suspension, we measured the velocity distributions of the different species when bioconvecting alone at a given concentration (Fig. 5d). Notably, *B. cereus* and *P. megaterium* were the slowest, and when either one of them was combined with *Exiguobacterium* sp., the corresponding bioconvective patterns were qualitatively similar (see patterns in Fig. 3a, b). In contrast, a *B. cereus*/*P. megaterium* suspension displayed weak segregation (Fig. 3c), suggesting that both a large speed contrast and magnitude are necessary for marked DISS. Note that cell size does not correlate with speed in a straightforward fashion (Fig. 5a); whereas *B. cereus* and *P. megaterium* cells are the largest, their mean speeds are smaller than those of *Exiguobacterium* and *B. pumilus*. Similarly to the behavior of ξ_p , the order of speed values is maintained between species, irrespective of bacterial density (Fig. S12). Note also that the faster species was typically located in the center of nested domains, when differences in speed were significant as for *B. cereus*/*Exiguobacterium* sp., *P. megaterium*/*Exiguobacterium* sp. (Figs. 3a and b, Fig. S3) and *B. cereus*/*B. pumilus* binary suspensions (Fig. S4).

Oxygen consumption

Given that oxytaxis is the driving force behind bioconvection, we tested the hypothesis that differential oxygen consumption between species may play a role in DISS. To this end, the oxygen consumption rate was measured for each bacterial species separately, in the same setup used for bioconvection experiments (Methods). The measurements show that the oxygen consumption per cell varies significantly between species (Fig. S13a). While the consumption rates of *Exiguobacterium* sp. and *B. pumilus* are similar and the lowest, those of *B. cereus* and *P. megaterium* are ~ 1 – 2 orders of magnitude higher. The sessile *Citrococcus* sp. oxygen consumption rate lies between those of the other species. Furthermore, normalization of these results by the

mean cellular volumes preserved this trend, with smaller differences between *B. cereus* and *P. megaterium* (Fig. S13b).

Bioconvection in five-species suspensions

Lastly, we asked whether bioconvection and spatial segregation occur in complex suspensions of more than two species. To address this question, we followed a suspension consisting of five species, *Bacillus infantis*, *Rosellomorea aquimaris*, *B. cereus*, *P. megaterium* and *Exiguobacterium* sp., all of which are motile and elicit bioconvection alone. Both bioconvection and spatial segregation indeed take place, as shown in Fig. 6, where a convective pattern formed in such a suspension is shown, and in which only *B. cereus* and *Exiguobacterium* sp. were fluorescently labeled (Movie S7). Note that the fastest species, in this case *Exiguobacterium* sp., was found to be at the center of convection cells. Weak antagonism is detected between *R. aquimaris* and either *B. infantis* or *P. megaterium* by spot-lawn assays (Fig. S14). Note that the halos are smaller than those elicited by *B. pumilus* in Fig. S9.

Simulation of segregation in a binary mixture of particles moving with different speeds in the same direction

A possible mechanism that may account for segregation between species characterized by different speeds is the minimization of cell-cell collisions under conditions of a prominent flow direction. To test this hypothesis, we carried out computer simulations of a three-dimensional toy model of two particle types that can collide while moving in the same direction, and whose characteristic speeds could be varied (Fig. S15a). The results show that when the particles move with the same speed, no segregation is observed (Fig. S15b). However, when speeds differ and the speed contrast between particle types increases (Figs. S15 c–e respectively), segregation is more marked.

Discussion

The picture that emerges from our study is that despite enhanced mixing by bioconvective flows, physical interactions together with sufficiently large differences in motility characteristics can give rise to marked spatial segregation between different species undergoing oxytactic bioconvection. The motility of both species in a binary suspension is necessary to observe segregation into locked nested domains. Importantly, segregation is contingent upon bioconvection as no segregation was observed below the onset, suggesting that repulsive biological interactions do not play a major role. In addition, the combinations of species were such that inter-species interactions did not inhibit mutual growth, and therefore were not antagonistic. Segregation is robust against changes in the relative proportion of the constituent species and with respect to the height of the suspension. Furthermore, segregation was also observed in a multispecies suspension of motile bacteria collected from the same pristine ecosystem²⁹. Our experiments support the notion that when present as part of a planktonic community, species can reap the benefits of bioconvection – e.g., enhanced access to oxygen or other resources¹⁷ – at a much lower concentration of each of the constituents, than the one needed to elicit bioconvection alone: it is the total bacterial concentration that sets the onset for bioconvection. Similarly, it was observed that the heterogeneity in mixed swarms of bacteria of different sizes in agar plates can lead to cell alignment by means of a purely physical mechanism and, thus, to an advantageous increase in swimming speed of individual cells.^{45,46}

Spatial segregation in multispecies suspensions undergoing bioconvection displayed patterns of spatially-interlocked domains in which down flowing currents of one species were nested within down-flowing currents of the second species. Within these domains, each bacterial species largely preserved their pattern characteristics. These findings suggest that DISS allows different bacterial species to auto-organize and maintain their resource intake vis-à-vis their metabolic

needs¹⁷, as nearly as possible as when each species undergoes bioconvection separately.

A possible mechanism that may allow different bacteria to segregate during bioconvection is the auto-organization of cells of different species in flows that minimize collisions and cell-cell scattering. These short range interactions are determined in large part by differences in the motility characteristics of the species involved. Differences in motility may in turn account for the qualitatively different bioconvective patterns we observed among species, as well as for variations in cellular concentrations that individual species exhibit across patterns. Therefore, we studied in detail both the speed and the orientational noise as measured by the persistence length ξ_p under different concentrations. Our results indicate that segregation in multispecies bioconvection is more marked when contrasts in ξ_p and speed are large, and that the dependence of these quantities on bacterial concentration varies considerably between species. This is illustrated by the negative correlation of ξ_p and speed of *B. pumilus* and *Exiguobacterium* sp. with increasing cell concentration—as previously reported for other bacteria⁴¹—and by the positive correlation of these quantities for *P. megaterium* and *B. cereus*. This positive correlation may be due to a possible progressive alignment induced by scattering⁴⁷. We surmise that the negative correlation of ξ_p and speed with concentration in the case of *B. pumilus* and *Exiguobacterium* sp. may be due to the relatively larger values of ξ_p and speeds, making these quantities more sensitive to (extrinsic) concentration effects by allowing cells of these species to interact more significantly by short range interactions, e.g., by collisions. Simulations of a toy model of two particle types moving in the same direction exhibit segregation when the speeds are different, supporting the notion of collision minimization as a mechanism behind DISS. We note that one cannot rule out that other mechanisms such as shear-induced depletion could also play a role in segregation⁴⁸.

Interestingly, segregation was also observed in a binary suspension in which one of the species was non-motile, but the patterns differed: the non-motile species were largely excluded out into a macroscopic domain surrounding a bioconvective region consisting mainly of the motile species, instead of the formation of nested spatially locked domains. This behavior suggests that the mechanism of segregation is different from DISS. Furthermore, since the motile and non-motile species were different in our experiments, the mechanism behind *Motility-Induced Phase Separation* (MIPS)⁴⁹ may not account for the observed segregation.

In an attempt to characterize better the differences in the motility between the bacterial species in this study, we carried out high resolution morphological characterizations of their flagella distributions. The SEM and AFM images revealed that the motile species are peritrichous. While peritrichous bacteria typically display run and tumble motility⁴⁰, our experiments revealed that there are considerable differences in their swimming behavior. It is remarkable that differences in swimming at cellular scales appear to manifest themselves at scales three orders of magnitude larger as differences in bioconvective patterns. Predicting patterns in bioconvection from the motility characteristics of individual bacteria remains a formidable problem, in the same way as determining the shapes and dynamics of flocks from the flight of individual birds⁶ and other animal aggregations⁵.

The driving force behind bioconvection in this study is oxytaxis. This led us to characterize the oxygen consumption rates of the different species under the same physiological conditions as the bioconvection experiments. Their oxygen consumption rates per cell were found to be different between species, ranging from -0.5×10^6 to 10^7 O₂ molecules per second. These values are comparable to those measured for other bacterial species^{50,51}. Even when taking into account the large variation in the mean volumes of cells between species, the differences persisted, suggesting different oxygen metabolisms between species. Bacteria in a bioconvective pattern navigate

in a complex local oxygen landscape, involving oxygen input from the external environment, hydrodynamic mixing and bacterial consumption with different physiologies. It has been previously shown that oxygen gradient sensing is logarithmic in the case of *B. subtilis* and in other bacterial species^{47,52}. Furthermore, the bacterial swimming response to changes in oxygen concentration is immediate in the case of *B. subtilis*⁴⁷. We speculate that log-sensing also characterizes the response of the species in our study, especially for *B. pumilus*, which is genetically closely related to *B. subtilis*²⁹, and may provide high sensitivity in a bioconvective setting. We cannot rule out that differences in oxygen consumption rates may play a role in DISS, given that marked segregation was observed between species whose oxygen consumption rates differed significantly. In *B. subtilis* the nature of the motion including speed and orientational noise is strongly dependent on oxygen concentration⁴⁷, suggesting that ξ_p and speed in the species in our study may also be affected similarly.

DISS bears features in common with the kinetic segregation observed in multilane traffic flows, in which vehicles segregate into lanes according to their speed⁵³, as well as with lane nucleation instabilities in complex active flows exemplified by pedestrian traffic⁵⁴. Other mechanisms of purely physical segregation based on differences in motility characteristics have previously been discussed in the literature, including both differential activity and substrate geometry⁵⁵, differential activity alone⁵⁶, as well as differential diffusivity⁵⁷. Segregation according to motility differences has been observed in sessile colonies of the peritrichous *P. mirabilis*⁵⁸. Collision minimization also plays pivotal roles in segregation of heterogeneous vertebrate collectives⁵⁹, and in heterogeneous robotic swarms⁶⁰. Segregation in multispecies systems of bacterial cells driven by a synthetic, density-dependent mutual regulation of motility between species reported previously⁶¹ differs sharply from the phenomenon we report here. DISS makes it necessary to rethink how bacteria interact in planktonic communities and paves the way for the study of emergent behavior of heterogeneous active matter.

Methods

Strains, species and culture conditions

The bacterial strains and species used in this work are listed in Table S1. These strains were isolated from superficial sediment at the margins of the oligotrophic Churince lagoon in the Chihuahuan desert, Cuatro Ciénegas Coahuila, Mexico²⁹. *B. cereus* was transformed with the plasmid pNW-sfGFP (SPS6) encoding the super folder green fluorescence protein (sfGFP). Transformation was carried out as described by Yi et al., with modifications⁶². Briefly, 1 ml of an overnight culture was diluted into 100 ml of LB medium with 2% (wt/wt) glycine and incubated at 30 °C and 180 rpm, until the OD₆₀₀ reached 0.4–0.7. Bacterial cells were harvested, washed sequentially with ice-cold glycerol (2.5%, 5%, and 10%), resuspended in precooled 10% glycerol, and shock-frozen in liquid nitrogen. The electro-competent suspension and 5 µg plasmid DNA were transferred into 2-mm precooled electroporation cuvettes. Electroporation was carried out using the ECM630 electroporation system (BTX Harvard Bioscience Inc) at 2.0 kV, 25 µF, and 200 Ω in the cuvettes. After pulsing, 1 ml of LB medium was added and bacterial cells were recovered at 30 °C and incubated at 150 rpm for 2 h, selected with 25 µg/mL chloramphenicol on LB agar plates and incubated overnight at 30 °C. Bacterial stocks were stored in marine medium⁶³ with 15% glycerol at -80 °C. For the experiments, samples from the stocks were streaked on marine media plates with 1.6% agar and incubated overnight at 28 °C, except for *Citrococcus* sp., which was incubated for two days.

Growth curves and doubling times

Growth curves were measured for all species in marine medium to confirm ability of all to grow similarly in the chosen medium (Fig. S6). Overnight cultures of each bacterial species were diluted to an

OD_{600nm} of 0.01, and four biological replicates of 200 µL were inoculated in a 96-well plate with 50 µL of mineral oil on top to prevent evaporation. Measurements of optical density (OD_{600nm}) were taken every 6 min for 24 h at 28°C, with agitation of 200 rpms, using a CLARIOstar Plus (BMG Labtech, Germany) plate reader. The mean growth rate μ and standard deviation of the four replicates for each species (in the late-exponential/early-stationary phase, where bioconvection occurs) were calculated, and the mean as a function of time t was fitted with the function $\mu = A \exp(-\frac{t}{\tau}) + B$. The doubling time $t_d = \tau / \ln 2$ was then calculated from the value of τ obtained as fitting parameter for each species.

Quantification of bacterial density

In all experiments, bacterial density was estimated by measurements of colony-forming units (CFUs). A bacterial suspension sample (350 µL) was serially diluted 10-fold in marine medium without peptone and yeast extract. Serial dilutions of the samples from 10⁻⁴ to 10⁻⁶ were plated (100 µL) on marine medium agar plates at least in duplicates. Plates were incubated overnight at 28 °C, then colony counting was carried out with a ProtoCOL Scan3 scanner (Synbiosis, UK). The mean and standard deviation of the replicate plates and their concentration in CFUs•mL⁻¹ were calculated.

Bioconvection sample preparation

A single colony was picked from a plate and incubated overnight in liquid marine medium at 28 °C and 250 rpm. The next day, the overnight culture was diluted to an OD₆₀₀ of 0.7 (NanoDrop One C, Thermo Fisher Scientific) with fresh marine medium. For *B. cereus* CH_111, 2 % of the overnight culture was inoculated in fresh marine medium and incubated until an OD₆₀₀ of 0.7 was reached. This was done because this *B. cereus* strain tends to aggregate and form large clumps, and this impeded a clear visualization of bioconvection patterns. The approach to the end of the exponential growth phase enabled the increased motility necessary for bacterial cells to elicit bioconvection^{64,65}. Neither re-growth in fresh marine medium nor the dilution method had an impact on the bioconvective behavior of any of the species. A volume of 400 µL of bacterial suspension was added to a flat-bottomed circular well (diameter=15.6 mm, resulting in a 1.3 mm depth in the center and 2.3 mm at the borders due to the meniscus). We used 24-well plates (Nunc™ Cell-Culture Treated Multidishes, Thermo Fisher Scientific). The plates were closed with their lid and either placed on the stereo-scope stage at room temperature (25–27 °C), or at 26 °C within a stage incubator (Okolab, Italy). For this range of temperatures, no difference was observed in the bioconvection dynamics and patterns. Pattern visualization was performed with a SMZ-25 stereomicroscope controlled by the NIS-Elements software (Nikon, Japan) equipped with an Andor Zyla 4.2 Plus USB3 camera (Oxford Instruments, United Kingdom) and a SOLA Light Engine (Lumencor, USA).

Mixed species bioconvection

For mixed species bioconvection experiments, once each species developed their characteristic patterns in separate wells, appropriate volume ratios of each culture were mixed and 400 µL of the mixture was placed in a well for observation. At least three biological replicates of each species mixture were done. To observe spatial segregation between species, we used two fluorescent cell dyes. FM™ 4–64 is a membrane-impermeable red fluorescent dye. Stocks of the dye were prepared by making 1 µL aliquots of 20 mM FM™ 4–64 in DMSO and stored at -20 °C. PBS buffer was added to one aliquot to have a 1.6 mM FM™ 4–64 working solution. Final concentration of the dye in the bioconvection samples was 1.6 µM for *Exiguobacterium* sp. and 4.8 µM for the other species. MitoTracker™ Green FM is a membrane-permeable green fluorescent dye. Stocks (1 mM) of the dye were prepared in DMSO and stored at -20 °C. The final concentration of the dye in the bioconvection samples was 0.5 µM for all species. We made sure

that the dyes did not interfere with the bioconvection. An isogenic *B. cereus* strain carrying a plasmid that expressed GFP (CH_111 [*pNW-sfGFP(SPS6)*]) was used in a bioconvection experiment with *Exiguobacterium* sp. DISS was observed in this paired experiment, as observed using the dyes (Fig. S3). In addition, the same *B. cereus* strain was used in an experiment with *B. pumilus* testing the robustness of DISS to changes in the relative composition of a binary suspension (Fig. S4). For image acquisition, 16-bit black and white images and fluorescence images were captured using the SMZ-25 stereomicroscope setup. MitoTracker™ Green was visualized using a 49002 – ET – EGFP filter set (Chroma Technology Corporation, USA), with an exposure of 400 ms and 50% light intensity. FM™ 4–64 dye was visualized using a 49008 – ET – mCherry, Texas Red® filter set (Chroma Technology Corporation, USA), with an exposure of 600 ms and 50% light intensity.

Image analysis of patterns of fluorescently labeled species

For analysis of fluorescence images of patterns in binary mixtures of different relative concentrations, the background mean intensity was subtracted for each fluorescence channel. The percentages of area occupied by the down-flowing currents (red and green regions representing *Exiguobacterium* sp. and *B. cereus* respectively, in Fig. 2 (bottom panels)) were calculated using ImageJ. For each fluorescence channel, the first frame (with no bioconvective pattern) was subtracted as background from each of the panels at later times. Then a threshold was chosen to select the appropriate regions and the percentage of total area was determined. The error bars in Fig. S2 correspond to an estimate of the error due to the choice of the threshold.

Biotic interactions during mixed species bioconvection

Samples of two different species undergoing bioconvection were mixed in 1:1 volume ratios in 24-well plates (Nunc™ Cell-Culture Treated Multidishes, Thermo Fisher Scientific). Cell concentration (CFUs•mL⁻¹) of each species was measured prior mixing and after one hour. The concentrations of single species cultures before mixing were used to calculate concentrations in the binary mixture at time zero. As controls, single-species bioconvection samples were measured in parallel. The CFUs•mL⁻¹ measurements were used to calculate the relative cell abundances of the constituent species before and after mixed bioconvection. The distinctive colony morphology of each species allows CFUs counting in the mixed-species sample plates. At least three independent experiments were performed for each pairwise interaction.

Motility characterization

For swimming speed characterization and track aligning of species under bioconvective conditions, the samples were diluted to an OD₆₀₀ of ~0.175 for *B. cereus* and *P. megaterium*, and ~0.125 for *B. pumilus* and *Exiguobacterium* sp., so that the mean distance between cells was about tenfold larger than the cell size, to enable the recording of separate trajectories. A 100 µL aliquot of each species was placed in a µ-Slide VI 0.4 Bioinert device (Ibidi, Germany). At least three independent experiments were performed.

For experiments of motility characterization at different bacterial concentrations, bioconvection samples were grown until they reached OD₆₀₀=1 (the concentration values corresponding to OD₆₀₀=1 in CFUs•mL⁻¹ correspond to: *B. cereus* (5.1 ± 2.6) × 10⁷; *P. megaterium* (12.0 ± 4.8) × 10⁷; *B. pumilus* (6.8 ± 1.7) × 10⁸; *Exiguobacterium* sp. (9.7 ± 5.8) × 10⁸ and *Citrococcus* sp. (6.6 ± 1.4) × 10⁸). Next, the suspensions were serially diluted in two-fold steps and a 100 µL aliquot of each dilution was placed in a µ-Slide VI 0.4 Bioinert device (Ibidi, Germany). At least two independent experiments were performed.

Bright-field phase contrast videos with an interval of 0.03 sec between frames were recorded using an Eclipse Ti2 inverted microscope (Nikon, Japan) controlled by the NIS-Elements software using a

60 N.A. 1.40 oil immersion phase contrast objective lens (Nikon plan-apochromat 60 1.40), equipped with an Andor DU-897 X-4889 camera (Oxford Instruments, UK). The videos were processed using the Fiji distribution of ImageJ-win64⁶⁶. The videos were pseudo flat-field corrected to account for heterogeneous illumination. They were then binarized and scaled. Tracking and mean speeds of individual trajectories were computed using the Trackmate plugin⁶⁷. A custom MATLAB code was used to calculate the persistence length from the two-dimensional tracks obtained for the different bacterial species at different concentration measurements, as well as to align the trajectories to the x-axis for visualization, as described previously^{48,68}.

Spot-lawn assays for pairwise antagonism evaluation

Spot-lawn assays⁶⁹ were performed to reveal antagonistic interactions between species. Melted marine media with 0.45 % agar was submerged in a water bath to lower the temperature to 45 °C, and then inoculated with 5 % of an overnight liquid culture of the species to be tested as lawn and mixed to get a thin layer. An appropriate volume of the mix was poured on top of a marine media plate with 1.6 % agar. The plates were left at room temperature for ~1 h for the agar to solidify, and 5 µl drops of the tester species were spotted over the lawn. Plates (10 cm) were incubated at 28 °C for the appropriate time depending on the species and photographed. The development of an inhibition halo around the spot species means that the spot species is antagonizing the lawn species.

Measurement of oxygen consumption

The specific oxygen uptake rate q_{O_2} for each species was measured as previously described⁵¹. Briefly, the oxygen concentration X in the suspension's medium was measured as a function of time using a setup consisting of a computer-controlled NTH-PSt7 optical syringe micro-sensor (Presens, Germany) connected to an OXY-1 ST transmitter (Presens, Germany), at a rate of one measurement per second. Measurements were performed on a volume of 700 µl of bacterial suspension in 24-well plates that underwent bioconvection at an OD_{600} ~0.8 (except for *Citrococcus* that does not perform bioconvection, but was prepared following the same procedures (see Bioconvection sample preparation)), and then were subjected to orbital stirring at 300 rpm at 24 °C. The rate of change of oxygen is described by:

$$\frac{dX}{dt} = k(X^* - X) - q_{O_2}B \quad (1)$$

Where k represents the input rate of oxygen from the atmosphere into the suspension due to diffusion and stirring, X^* is the saturation concentration in the medium without bacteria, and B is the bacterial concentration. For the determination of k , marine medium was bubbled with nitrogen to gas-out dissolved oxygen. The rate k was obtained by fitting the measured oxygen curve with the solution to Eq. 1, without including the second term on the right hand side:

$$X = X^*(1 - e^{-kt}) \quad (2)$$

Oxygen curves were then measured in suspensions of the different species until a quasi-steady oxygen concentration X^{**} was reached. The bacterial concentration was determined at the end of the oxygen measurements (see Quantification of bacterial density). Assuming a steady-state between oxygen input and consumption, the consumption rate per cell is given by:

$$q_{O_2} = \frac{k(X^* - X^{**})}{B} \quad (3)$$

Units were adjusted to $mg \cdot mL^{-1} \cdot min^{-1}$, and then divided by the corresponding $CFUs \cdot mL^{-1}$ to obtain the estimate of consumption rate per bacterial cell. Next, the consumption per physiological volume was obtained by dividing by the cellular volume for each species.

Measurement of local bacterial concentration across bioconvective patterns

An overnight culture of *Exiguobacterium* sp. was serially diluted 2-fold four times, and also concentrated 2-fold by centrifugation (5000 g) for 5 min, to have a total range of six concentrations. The samples were placed in a 24-well plate and images were taken using the SMZ-25 stereomicroscope setup. Images from the different wells were then converted to a 16-bit TIFF format, and the mean intensity pixel value of a circular region-of-interest of the well center excluding the well edge was calculated for each well. The sample with the highest concentration was measured and the concentration in $CFUs \cdot mL^{-1}$ was calculated. For *B. cereus*, the same procedure was applied, except that a culture from the same day was used to avoid aggregation, instead of an overnight culture (Fig. S1a).

To determine the bacterial concentration in the different regions of the bioconvection pattern images in Fig. S1b, the pixel intensities (65,536 values) as a function of bacterial concentration were fitted with the Rodbard function⁶⁶:

$$f(x) = c \left(\frac{x - a}{d - x} \right)^{1/b} \quad (4)$$

Heat maps of transmitted light intensities calibrated in terms of concentration were then generated.

Flagella visualization by AFM and TEM

Atomic Force Microscope imaging of dried samples was carried out with a JPK Nanowizard III AFM microscope (Bruker Nano GmbH, Berlin, Germany) in AC mode, at ambient temperature. Measurements were conducted with an AC240 probe, (Olympus). Different methods were used on each species for sample fixation, and the fixation procedure was carried out after confirmation of a bioconvecting state. A *B. cereus* overnight culture was diluted to an OD_{600} of 0.7 and directly placed on a positively charged glass slide for 1 h, rinsed three times with HPLC water and let to air-dry at room temperature. A *P. megaterium* overnight culture was diluted to an OD_{600} of 1.5, and placed on poly-L-lysine treated slides (P0425-72EA, Sigma) that were previously rinsed with HPLC water to remove dust. The samples were incubated at room temperature in an orbital shaker at 100 rpm for 2 h. Washing of the samples was done by flooding with HPLC water and removing most of the water to avoid drying and crystallization. Washing was repeated three times and in the final wash, all water was removed and the slide was left to air-dry. *B. pumilus*, *Exiguobacterium* sp. and *Citrococcus* cultures were grown overnight, diluted to an OD_{600} of 0.7, and washed 3 times with PBS buffer. The samples were placed on a Super frost PLUS slide (Bar Naor Lab Supplies, Israel), and incubated for 1 h at room temperature. The sample was rinsed by dipping the slide in HPLC water three times and fast dried with nitrogen. For transmission electron microscopy (TEM) imaging, 10 µL of bacterial suspension at OD_{600} of 1.0 were deposited onto a freshly glow-discharged copper TEM grid coated with Formvar and carbon, and stained with 2 % uranyl acetate. Images were collected at a Tecnai T12 transmission electron microscope (Thermo Fisher Scientific), using a TemCam-XF416 CMOS camera (TVIPS).

Statistics and reproducibility

Statistical analyses were performed using OriginPro 2023b and MATLAB R2022b. The images of bioconvective patterns shown in Figs. 1c and 2 are representative results from at least ten independent experiments. Figures 3a–c and 6 are representative results from at

least three independent experiments, which were carried out in separate days. For other figures, reproducibility is indicated in their respective legends.

Simulation of a binary mixture of particles moving with different speeds in the same direction

To test for segregation of particles having different speeds, a toy model was designed to simulate the motion of particles moving in the same direction ($-z$) in three-dimensional space and the effects of momentum non-conserving particle collisions. The model included two types of particles having different speeds u_f and u_s (fast and slow respectively) with arbitrary units. For the simulation, the particles were initially randomly distributed and moved in space in the same direction: $-u_f\hat{n}$ and $-u_s\hat{n}$. At each iteration, a collision event occurred if two particles were closer to each other than a specific distance. If a collision took place, a velocity vector of small magnitude ($\hat{\delta}$) was added to each particle's velocity, where the direction of $\hat{\delta}$ is the vector joining the two particles: $u_f(-\hat{n} + \hat{\delta})$ and $u_s(-\hat{n} - \hat{\delta})$. Throughout the simulation, $|\hat{\delta}| \ll 1$ was assumed constant. The simulation also included random motion of particles by adding a random velocity vector of small magnitude ($\hat{r}$), whose components were drawn from a normal distribution: $u_f(-\hat{n} + \hat{\delta} + \hat{r})$ and $u_s(-\hat{n} - \hat{\delta} + \hat{r})$.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

Source data generated in this study and data supporting the figures in the main text are available at: <https://datadryad.org/stash/share/y-BeGU9IzE9NZbVOX30Z6IDVa2v0D9IbMNDKVUK22yQ> with DOI⁷⁰.

Code availability

Open source software for persistence length analysis was deposited in Zenodo and available at: <https://datadryad.org/stash/share/y-BeGU9IzE9NZbVOX30Z6IDVa2v0D9IbMNDKVUK22yQ> with DOI⁷¹.

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Author contributions

J.S. conceptualized and supervised the study; O.G.N., R.A.G., and J.S. developed the methodology; O.G.N., R.A.G., and J.S. carried out the investigation, visualization and validation; O.G.N. carried out the experiments; G.O.-A. provided bacterial species and strains; O.G.N., R.A.G., E.A. and J.S. carried out analysis; J.S. and R.A.G. wrote the original draft while O.G.N., R.A.G., G.O.-A, E.A. J.S. reviewed and edited until the final version.

Competing interests

Authors declare that they have no competing interests.

Additional information

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