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Self-organized patchiness facilitates survival in a cooperatively growing *Bacillus subtilis* population

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Ecosystems are highly structured. Organisms are not randomly distributed but can be found in spatial aggregates at many scales, leading to spatial heterogeneity or even regular patterns[1]. The widespread occurrence of these aggregates in many different ecosystems suggests that generic factors intrinsic to the populations—such as interactions between the organisms—play a major role in their emergence[1,2]. Beyond the emergence of spatial patchiness, its functional consequences remain unclear. Here we show in *Bacillus subtilis* that cooperative interactions in a spatial environment are sufficient to form self-organized patches. These patches allow for survival even when the microbe density is too low to sustain growth in a well-mixed environment. Decreasing cell mobility leads to more compact patches that enhance this survival advantage but also reduce the overall growth. Our results highlight that even populations lacking specific group-forming mechanisms can nonetheless form spatial patterns that allow for group survival in challenging environments.

Heterogeneity and spatial patterns can be found in a large variety of microbial ecosystems[3–5]. Several explanations have been proposed for this heterogeneity, such as selection by locally different environments and colonization, differentiation and extinction dynamics[5]. However, even for rather homogeneous environments, spatial patterns can be found in bacterial and algal mats, culminating in the complex architecture of stromatolites and microbial reefs[6–8]. These patterns are often rather periodic and resemble those well known in plants in arid or peatland environments[1]. For those patterns a major role of intrinsic factors is assumed: they are probably self-organized and cooperative interactions have been suggested to be of importance[1,2,9–13]; e.g. in arid environments grass may increase the local infiltration of rainwater and thus facilitate growth nearby[2]. Many microbes are known to show cooperative interactions as a consequence of 'public good' production[14], raising the question of whether similar mechanisms for pattern formation exist in the microbial realm. Another major question is if there is an ecological benefit from the formation of patterns. Organisms in a group may benefit from each other, but they also experience stronger competition[15].

To experimentally investigate a possible connection between cooperative growth and spatial heterogeneity and what ecological consequences follow, we established a microbial model system giving rise to self-organized patchiness. The Gram-positive motile bacterium *Bacillus subtilis* is able to digest starch by secretion of the enzyme amylase[16] (Fig. 1a, Supplementary Figs 3 and 4). Amylase cleaves the starch and the resulting oligomers can be taken up into the cell, allowing *B. subtilis* to grow on starch as the sole carbon source. Since the amylase enzyme is secreted, the breakdown of starch is cooperative and higher cell densities lead to faster breakdown of starch and thus faster growth. Indeed, in well-mixed culture we find a minimal bacterial density of more than 10^7 c.f.u. ml^{-1} that is necessary to break down enough starch for the population to survive (Fig. 1b and Supplementary Fig. 2). Above this critical cell density all populations reach saturation and below it all populations go extinct; population biologists describe this as a strong Allee effect[17,18].

This system allows us to explore the effect of cooperative growth in a spatial environment by embedding *B. subtilis* in low-percentage agar containing 0.5% starch as the sole carbon source. Agar stays fluid at 37 °C for a few hours, thus allowing us to mix the bacteria uniformly in the agar (Methods and Supplementary Fig. 9). The bacteria–starch–agar mixture was poured into Petri dishes, solidified at room temperature (RT), and incubated at 30 °C (Fig. 1c). Although the bacteria were initially uniformly distributed in the agar (initial bacterial density: 5×10^6 c.f.u. ml^{-1}), growth in the agar–starch mixture occurred in patches (Fig. 1c). It is important to note that these patches are not simple colonies arising from single cells as can be seen by embedding the same (high) number of bacteria in complex media, which leads to homogeneous growth even in the absence of motility (Supplementary Fig. 8B). *B. subtilis* therefore spontaneously forms patches when growing cooperatively on starch.

One way such patches could form is by bacteria sensing each other or the starch breakdown products and swimming towards each other. However, we found that a motile mutant lacking chemotaxis (Δ*cheB*)[19] also formed patches (Fig. 1c). Indeed, even a mutant without flagella and thus completely lacking motility (Δ*hag*)[19] formed small, compact patches (Fig. 1c, details Supplementary Fig. 8). We therefore conclude that patch formation does not require the active mechanisms, such as collective movement and/or chemotaxis, which have been explored in other examples of group formation[20,21].

Another important factor in this system is cooperative growth on starch. To check whether this causes these patches, we supplemented the starch with a small amount of glucose. Glucose can be taken up directly, meaning that cells no longer benefit as much from the presence of other cells (Supplementary Fig. 7). We found that adding glucose makes the growth increasingly homogeneous (Fig. 1e). Cooperative growth is therefore essential for the formation of patterns in this system.

Bacteria with and without mobility formed strikingly different patches (Fig. 1c), raising the question of how mobility and patch formation are linked. Bacterial mobility can be controlled by the agar concentration, as high agar concentrations slow down bacterial swimming[22] (Supplementary Figs 6, 8 and Supplementary Movie 1) without affecting the diffusion of proteins and small molecules (Supplementary Information and Supplementary Fig. 12)[23]. At high mobilities the patches are large and diffuse, whereas at lower mobilities the patches appear much more compact and in higher numbers (Fig. 1d and Supplementary Fig. 8). Patch formation occurs even at agar concentrations sufficiently high (1%) to stall the bacteria completely (Supplementary Fig. 6 and Supplementary Movie 2), resulting in patches that look similar to those of the

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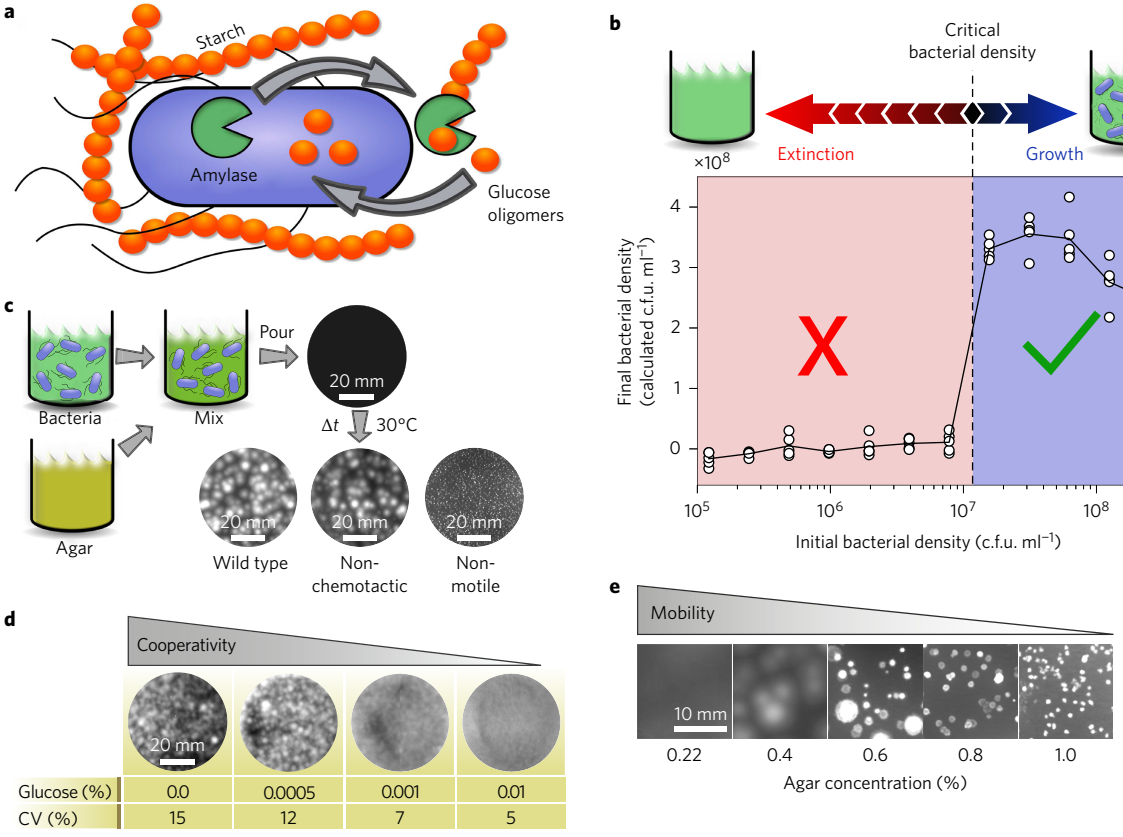

Figure 1 | Cooperative growth in a spatial environment leads to formation of patches. a, Upon starvation *B. subtilis* secretes the enzyme amylase, which breaks down starch. The resulting glucose oligomers can then be imported into the cell and utilized. **b**, In well-mixed conditions with 0.5% starch there is a minimal starting cell density required for survival. Below the critical cell concentration the bacteria die; above it the cells survive and the population reaches saturation (strong Allee effect). Final cell concentration is measured after 7 days at 30 °C ($n = 5$). Optical density (OD) is measured and transformed to c.f.u. ml^{-1} (see Methods). This transformation leads to negative values in some cases; direct plating confirmed that they are exactly zero (Supplementary Fig. 2). **c**, To study patchy growth in spatial environments bacteria are mixed with liquid agar with starch and poured into petri dishes (initial bacterial density: 5×10^6 c.f.u. ml^{-1}, 0.4% agar, 0.5% starch). Incubation at 30 °C leads to patch formation in the agar ($\Delta t = 150$ h) also in the absence of chemotaxis or movement. White equals high fluorescence signal (cell density). Images are representatives of three replicates. **d**, Addition of glucose weakens the cooperative nature of growth and leads to homogeneous growth (0.4% agar, 0.5% starch). Coefficient of variation (CV) of the fluorescence intensity decreases as the glucose concentration increases. Images were chosen to have similar mean intensities (initial bacterial density: 5×10^7 c.f.u. ml^{-1}) and are representative out of two repetitions. **e**, Increase in agar concentration leads to smaller but more patches (initial bacterial density: 5×10^6 c.f.u. ml^{-1}). Images were obtained after 300 h incubation at 30 °C. They are representatives of three replicates. Images in **c** have different colour-mapping. Colour-mapping is the same for images within **d** and **e** but different between **d** and **e**.

non-motile mutant (compare Fig. 1c right and Fig. 1e right, details in Supplementary Fig. 8). Thus mobility in this system is undirected and 'blurs' out the growing patches.

We demonstrated in well-mixed conditions that sufficiently high cell densities are required for population survival (Fig. 1b and Supplementary Fig. 2). Local bacterial density is higher in patches than in the surrounding volume, raising the question of whether patch formation influences population survival. To explore this, we measured the minimal starting cell density that allows for survival at different mobilities (agar concentrations). We found that the bacteria can survive best at low mobility where smaller and more compact patches can be found (Fig. 2). Populations initiated in 1% agar can survive starting cell densities that are more than 1,000 times lower than required in well-mixed conditions (Fig. 2, Supplementary Figs 5,6 and 19). As the agar concentration decreases, the bacteria become more motile and the environment approaches the well-mixed condition where the (enforced) motility is highest. Accordingly, the minimal cell density required for survival converges to that measured under well-mixed conditions (Fig. 2). Spatial structure and patch formation therefore allow the bacterial population to survive conditions that would be lethal in a well-mixed environment: the patterns increase the resistance against adverse conditions.

How can we explain the formation of these patterns and their positive effect on survival? In a well-mixed solution no stable spatial heterogeneities can form, and the local density of cells is equal to the mean concentration. If this mean concentration is above the critical concentration, the bacteria live; otherwise, they all die (Figs 3a,1b, and Supplementary Fig. 2). In a spatial system, the mobility is limited and local heterogeneities can exist over a certain time depending on the organism's mobility. Moreover, the bacteria themselves are expected to show some phenotypic heterogeneity, e.g. amylase synthesis per cell[24], which increases this spatial noise. Given that these sources of variation are multiplicative, a reasonable hypothesis is that the net variation is log-normal (see Supplementary discussion, Supplementary Figs 9 and 10), which in a spatial environment causes some local concentrations to be above the critical concentration even when the mean density is below it (Fig. 3a). Based on this idea we built a reaction–diffusion model that captures the basic properties of the bacterial system: randomly distributed bacteria grow cooperatively and show undirected mobility. The bacterial motility is described as diffusion, with a larger

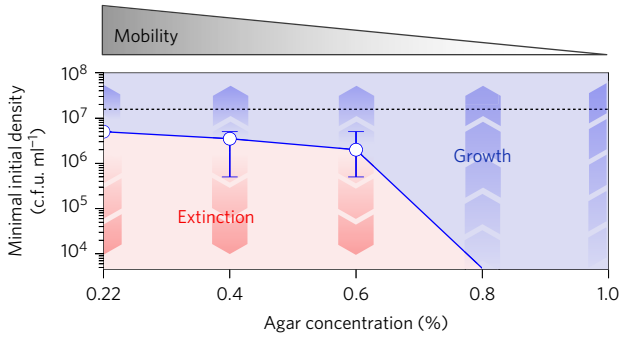

Figure 2 | Self-organized patches allow bacteria to survive lower starting cell densities. Similarly to well-mixed conditions (Fig. 1b), there is a minimal initial cell density required for survival in spatial environments. The dashed line represents the minimal bacterial density necessary for growth in well-mixed solution (see also Fig. 1b), whereas the blue line shows the minimal density in spatial environment (error bars are minimum and maximum of three replicates). Increasing agar concentrations causes more compact patches (Fig. 1e) and allows the bacterial population to survive lower starting cell densities; at 1% agar even lower densities than depicted here can survive (Supplementary Fig. 6). Growth was assayed after a 320 h incubation at 30 °C ($n = 3$ for every condition and initial bacterial density). See supplementary material for description of measurement and analysis.

diffusion constant corresponding to increased mobility[25]. In this model, we find that bacterial populations can survive and grow in distinct patches, even if the mean bacteria density is below the critical cell density (Fig. 3b, upper left). However, too high mobility causes those initial patches to diffuse apart and lose coherence, thus leading to population extinction (Fig. 3b, upper right). Accordingly, the likelihood of the population surviving decreases with increasing mobility (Fig. 3b, lower) and implies that low mobility is, in principle, advantageous.

On the other hand, at sufficiently high initial cell densities the bacteria can grow regardless of mobility, and high mobility allows the patches to expand faster, resulting in larger patches (Fig. 3c upper left and right and Supplementary Figs. 13,15, and 16). This faster expansion also increases the maximal rate of population growth across the plate (Fig. 3c, lower and Supplementary Fig. 14). Thus this model suggests that low mobility allows for survival at initially lower cell densities but the price for this is slower spatial expansion of the population. The optimal level of mobility therefore depends on the starting cell density: at high initial densities fast mobility is preferred, whereas at low density slow mobility is preferred. The same result is obtained with a cellular automaton model (Supplementary Information, Supplementary Figs 17 and 18).

To explore the predicted trade-off between survival and expansion, we took fluorescence images of the pattern formation over time at different agar concentrations. As predicted by the model, we found a survival–growth trade-off: at high mobility (0.22% agar) only the two initially highest cell densities were able to survive, but were able to expand and grow rapidly (Fig. 4a,b). At low mobility (1% agar), all initial densities survive but grow more slowly (Fig. 4a,c). Thus, for starting cell densities below the critical density it is better to be in the low-mobility regime, to at least survive in some localized patches (Fig. 4d, violet line). However, for starting cell densities above the critical density it is better to be in the high-mobility regime, where bacteria are able to colonize the entire plate (Fig. 4d, orange line; Supplementary Fig. 20).

We have demonstrated that cooperative growth in a spatial environment can cause microbes to form self-organized patches, even without complex signaling or collective movement to coordinate the actions of the many cells involved. The most important factor of this process – cooperative growth – is a direct consequence of 'public goods' production which is very common in microbes[14], but in macroorganisms cooperation also exists as a consequence of collective behavior or sexual reproduction[17].

With the generic appearance of cooperativity in nature, the basic ingredient for pattern formation, heterogeneity and patterns are expected as the rule rather than the exception. Many patches found in natural ecosystems have been described as Turing patterns[26], with cooperative local interactions facilitating local growth, whereas resource depletion inhibits growth over some distance. The combination of local activation and distant inhibition can also be found in the *B. subtilis* starch system: whereas cooperative growth is sufficient to cause the initial formation of patterns, resource depletion guarantees their long-term stability and prevents neighboring patches from combining into larger patches (Supplementary Information, Supplementary Figs 23 and 24). This combination of local cooperation and global competition makes the system similar to an activation–depletion Turing pattern (see Supplementary Information for further discussion).

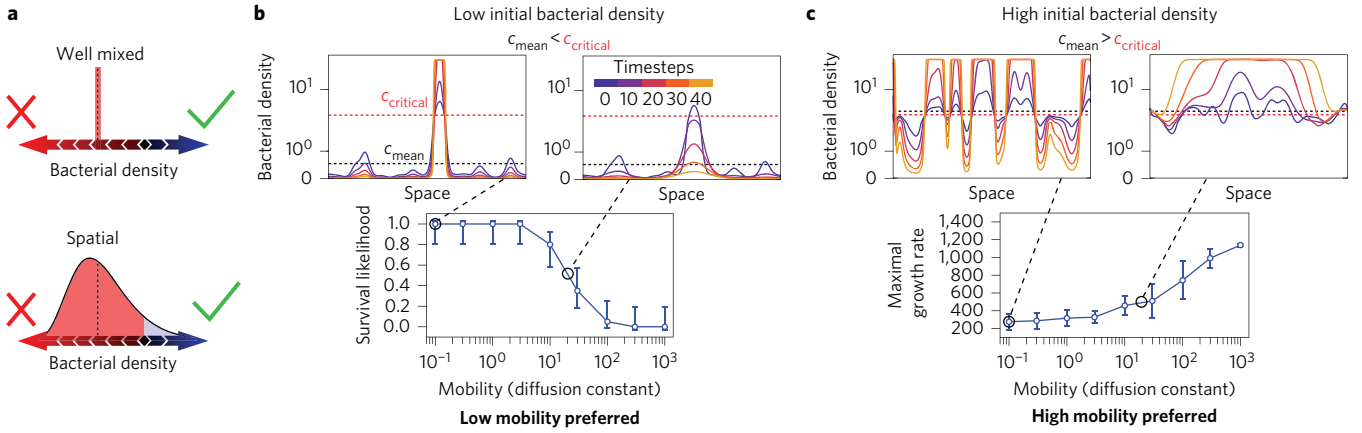

Figure 3 | Reaction-diffusion model describes pattern formation. a, In a well-mixed situation (top) the local concentration (shading) always equals the mean concentration (dashed line). In contrast in a spatial system (bottom) the local bacterial concentration may differ from the mean and be locally above the critical concentration (blue area) even when the majority is below it (red area). **b**,**c**, Growth and movement of bacteria in 1D space. Dimensionless time evolves from blue to yellow. The red dashed line is the critical cell concentration required for growth, the black dashed line is mean concentration. At low initial bacterial densities low mobility ensures survival (**b**, error is 95% Agresti–Coull confidence interval from $n = 20$ simulations runs), whereas at high initial densities high mobility allows fast spreading and growth (**c**, error bars are s.e.m. from $n = 20$ simulation runs). For description see main text and Supplementary Information.

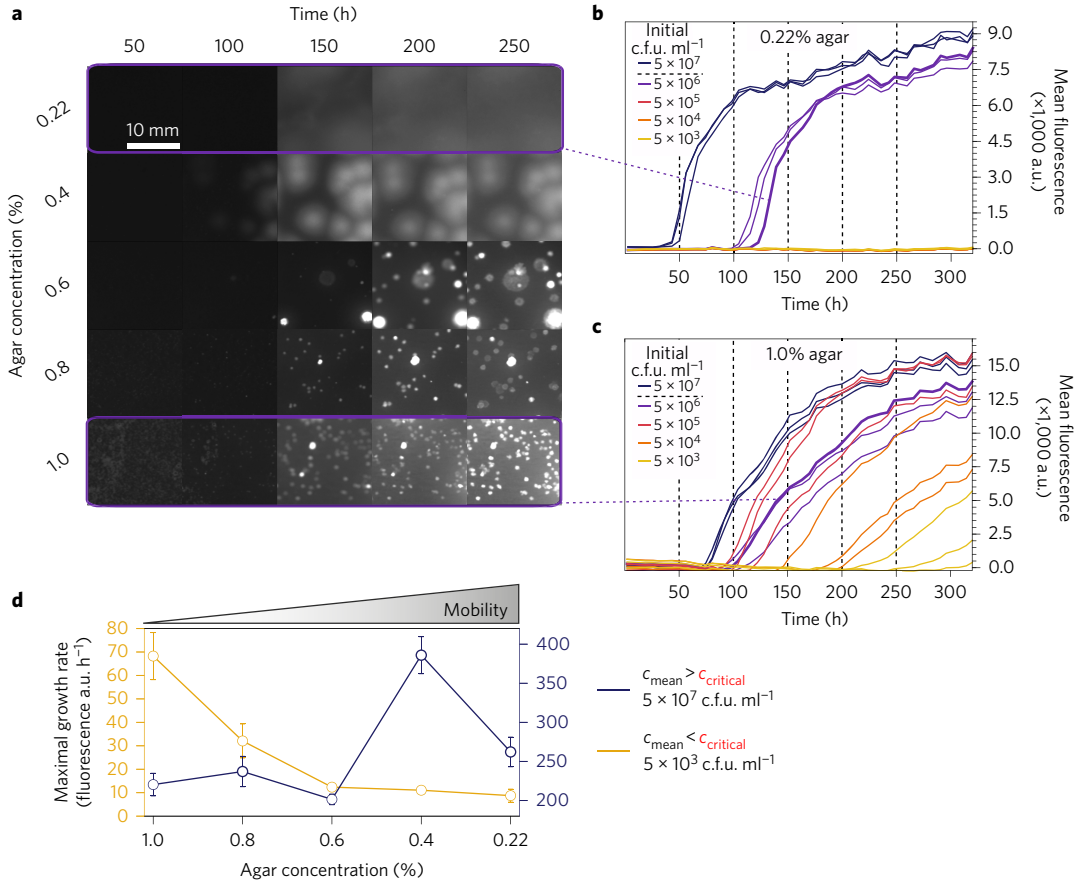

Figure 4 | Trade-off between survival and growth. a–c, Patch formation observed over time at different agar concentrations (**a**) gives growth curves (**b**,**c**) of the bacteria ($n = 3$ for every condition and initial bacterial density, all growth curves shown in Supplementary Fig. 11). Thicker purple line corresponds to images shown in **a**. We found that the fluorescence signal has an approximately linear relation to the bacterial density (Supplementary Fig. 21). In 1.0% agar there are no bacteria between the patches (the background signal is probably caused by light scattering, see Supplementary Fig. 19). Images are representatives of three replicates for each condition. **d**, Trade-off of the maximal growth rate (maximal slopes in growth curves (**b**,**c**) and Supplementary Fig. 11, $n = 3$ for every condition) can be found as predicted by the model (Fig. 3).

Although the emergence of heterogeneities and patterns in ecosystems is well documented, their functional consequences are often unclear. In our bacterial population we find that the formation of patches increases the ability of the group to survive low starting cell densities in environments that require collective action. Local group formation allows the cells to survive otherwise lethal conditions. Although the group formation studied here is essentially caused by growth, it is also likely that in other contexts group formation by active movement can increase survival[15]. Consistent with this expectation, group formation is found in many microbes either by active gathering[20], collective movement[21] or by passive grouping, like in biofilm formation[27]. Often this group formation is accompanied by cooperative activities such as cell differentiation[20] or sharing of 'public goods'[28]. Thus those behaviors might be adaptations to benefit from group formation.

Offspring spreading into new territory have to face the risk of failing to establish, but on the other hand, staying in an already occupied territory causes strong intra-species competition[29]. In the presence of cooperative effects, local higher densities increase fitness and higher mobility becomes disadvantageous at low densities[30]. If the local growth is high enough then expansion is beneficial since it allows spreading of the organism but also lowers local competition. Reducing dispersal at low densities, as has been observed in *Vibrio cholerae*[31], might therefore be a strategy in stressful environments.

In summary, we described here a very simple microbial system that shows self-organized formation of patches that allow for survival in otherwise lethal conditions. The simplicity and generality of the mechanisms suggests that similar phenomena may underlie the formation of patterns in a wide variety of other ecological systems.

Methods

Molecular biology. All experiments were done with *Bacillus subtilis* 3610 (ref. 19). To allow fluorescence imaging of the cells a yPET (ref. 32) expression cassette was used with a yPET sequence optimized for the expression in *B. subtilis*. For constitutive expression PlepA was used as the promotor[33] with the BioBricks Bba_B0014 Terminator. The cassette was fused into a pDR183 integration vector[34] (a kind gift of David Rudner) and integrated into *B. subtilis* 3610 strain. Correct integration was checked with a galactosidase assay[35] (Supplementary Fig. 1). The *B. subtilis* wt and Δ*hag* mutant were a kind gift of Daniel Kearns.

***B. subtilis* on starch-agar plates.** All chemicals were purchased from Sigma Aldrich, if not stated otherwise. 2×M9 broth was prepared and autoclaved. After cooling down to RT 0.2 mM $CaCl_2$, 4 mM $MgCl_2$ and 2× trace elements (Teknova) were added. *B. subtilis* 3610 with genomically inserted yPET was grown in 5 ml TSB (Teknova) at 37 °C (starting with a single colony picked form agar plate streak-out). After 15 h the bacteria were diluted 1/100 into 5 ml TSB and grown to OD=2, followed by another 1/10 dilution (at this point cultures were split into three replicates and treated separately henceforth). After reaching again OD=2 the cells were spun down (2,500 r.p.m., 2 min) in an Eppendorf 5810 tabletop centrifuge with rotor A-4-81 (Eppendorf). The cells were washed three times with 37 °C prewarmed M9 medium without starch (2,500 r.p.m., 2 min). During the process the cells were kept on prewarmed heat-blocks. After the last wash cells were resuspended in 2.5 ml prewarmed M9. The OD was adjusted to 1. In parallel the agar was prepared by autoclaving 2 × 0.5% starch with 2 × 0.22–1.0% agar (Bacto Agar, Beckton Dickinson). After autoclaving the agar was mixed 1/1 vol/vol with the M9 and

22.5 ml were transferred into 50 ml tubes and kept shaking at a platform shaker (300 r.p.m., 37 °C). After cooling down to around 37 °C the bacteria were added and a dilution row in liquid agar with five × 1/10 dilutions was made. The bacteria agar mixture was mixed by massive shaking by hand and by storing prior to pouring on a platform shaker at 300 r.p.m., 37 °C. The bacteria agar was poured into Petri dishes and allowed to cool down to solidify. Afterwards plates were incubated at 30 °C with around 70–80% humidity.

Starch bulk measurements. Bacteria were prepared as for the starch plates, but after the third wash resuspended in M9 with 0.5% starch. A dilution row with dilution factor 1/2 was made in the same medium. The final volumes of 120 µl per well in 96 deep-well plates covered with sterile sealing (AearaSeal, Excel Scientific) were incubated with shaking at 925 r.p.m., 30 °C at around 90% humidity. After 7 days 50 µl were transferred into 96-well flat bottom plates (Corning) and OD was measured with a Varioskan Flash plate reader (Thermo Scientific). To get cell densities from OD the conversion factor (5×10^8 c.f.u. ml^{-1}/(OD cm^{-1}), mean of five technical replicas) was estimated by measuring the c.f.u. ml^{-1} of a 1 OD cm^{-1} bacterial solution (measured with a UV/Vis Spectrometer; Jenway 6320D). To convert the OD measured with the plate reader to OD cm^{-1} another conversion factor was estimated by measuring a 1 OD cm^{-1} bacterial solution in both devices. Direct plating is in good agreement with this conversion (compare Fig. 1 and red axis of Supplementary Fig. 2).

Fluorescence measurements and analysis. To quantify the cells grown on the plate, fluorescence images were taken with a Biospectrum imager 810 and an external Biolite halogen light source (UVP). Epi illumination was used. The excitation wavelength was 502–547 nm and the emission wavelength was 565–625 nm. A scan of every plate was done three times a day (usually 9:00, 16:00 and 23:00). On the obtained images the edge of the Petri dishes were detected with a circular Hough transform and the inner part of the Petri dish was cut out, to avoid reflections from the edge of the plates in the further analysis.

Survival was estimated from the mean fluorescence signal on those plates after 320 h. If the signal was at least 120 fluorescence a.u. above the background it was counted as survival.

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Author contributions

C.R and J.G. designed the research. C.R. performed the research. C.R. and J.G. wrote the manuscript.

Additional information

Supplementary information is available online. Reprints and permissions information is available online at www.nature.com/reprints. Correspondence and requests for materials should be addressed to C.R. and J.G.

Competing interests

The authors declare no competing financial interests.