**Materials and Methods**

**Strains and Growth Conditions.** Chemicals used were of analytical grade and obtained from Merck. To construct plasmid pGFP-acoA, carrying the *Bacillus subtilis* acoA-L promoter region followed by a perfect ribosomal binding site fused with the *gfpmut1* gene, a PCR with the primers acoA-F (5'-CCCAAGCTTGCGCCTATGAGCATGACGATTCAG-3') and acoA-R (5'-CGGAATTCCATTTCCTCCTCCTCTATTAGGGTTGACCATTATAATGC-3') was performed, using chromosomal DNA of *B. subtilis* 168 as template. The amplified fragment was subsequently cleaved with HindIII and EcoRI and ligated into the corresponding sites of pSG1151 (1). *B. subtilis* strain acoA-gfp was obtained by a Campbell-type integration of plasmid pGFP-acoA into the chromosomal *acoA-L* promoter region of *B. subtilis* 168. Transformants were selected on TY agar plates containing chloramphenicol (Cm, 5 µg/ml). Correct integration was verified by PCR (data not shown). To obtain strain IIA/skf/sdp, strain IIA-gfp (2) was transformed with chromosomal DNA of strains EG168 and EG321 (3), respectively. Transformants were selected on TY agar plates containing spectinomycin (Sp), tetracycline (Tc), and Cm, after overnight incubation at 37°C. Correct mutation of the *skf* and *sdp* genes was verified by PCR (not shown). To obtain *B. subtilis* strain Sik243::Tc, in which the Cm resistance marker of strain Sik243 is switched to a tetracycline (Tc) resistance marker, strain Sik243 was transformed with linearized plasmid pCm::Tc (4). Correct transformants were scored on Cm sensitivity and Tc resistance. To obtain *B. subtilis* strain IIA/0A, strain IIA-gfp (2) was transformed with chromosomal DNA of strain Sik243::Tc. Transformants were selected on TY agar plates containing Tc and Cm, after overnight incubation at 37°C. Finally, *B. subtilis* strain IIA/Δ 0A/spo0A (PspooA-gfp,Δspo0A amyE::Pspac-spo0A) was obtained by transforming strain IIA/0A with chromosomal DNA of strain SWV215 (spo0A::Km) (5). Transformants were selected on TY agar plates containing Tc, Cm, and kanamycin (Km), after overnight incubation at 37°C. All strains used are derivatives of the fully sequenced *B. subtilis* 168 laboratory strain (6).

**Recombinant DNA Techniques and Oligonucleotides.** Procedures for DNA purification, restriction, and ligation, and agarose gel electrophoresis and transformation of *E. coli* were carried out as described in ref. 7. Enzymes were obtained from Roche. Oligonucleotides were purchased from Biolegio BV (Malden). *B. subtilis* was transformed as described before (8).

**Time-Lapse Microscopy.** We found that the growth medium used (both in overnight liquid cultures and within the semisolid matrix) is crucial for the generation of monolayered sporulating microcolonies of large enough size to generate analyzable lineages. Cells were grown overnight in liquid minimal medium (MM) at 30°C and continuously shaken at 200 rpm. MM contained 62 mM K2HPO4, 44 mM KH2PO4, 15 mM (NH4)2SO4, 6.5 mM sodium citrate, 0.8 mM MgSO4, 0.02% casamino acids, 27.8 mM glucose, and 0.1 mM L-tryptophan. The pH was set to 7 using a KOH solution. The next morning, cells were diluted 25× into a liquid chemically defined medium (CDM). CDM is a MM solution, but without casamino acids, containing 2.2 mM glucose, 2.1 mM L-glutamic acid, 6 µM L-tryptophan, 7.5 µM MnCl2, and 0.15× metal (MT) mix. This CDM was then diluted to 15% before use. The 50× MT mix was prepared as described in ref. 9. Exponentially growing cells were inoculated onto a thin semisolid matrix of CDM–agarose attached to a microscope slide. These slides were prepared as follows. A 125 ml Gene
Frame (AB-0578; ABgene House) was attached to a standard microscope slide (CML). The resulting cavity was filled with heated CDM supplemented with 1.5% low-melting-point agarose (A4718; Sigma–Aldrich) and covered with a standard microscope slide. After cooling and removal of the cover slide, strips of CDM–agarose were removed with the use of a surgical scalpel blade (Lance Blades Ltd.), resulting in a small strip of CDM–agarose (~1.5 mm wide) in the center of the Gene Frame. This provides air cavities that are essential for efficient growth and spore formation. Cells were spotted onto the strip, and the Gene Frame was sealed with a coverslip (24 × 60 mm; Menzel GmbH).

For SI Movies 1, 2, 3, and 5, microscope slides were incubated in a temperature-controlled (Cube and Box incubation system; Life Imaging Services) automated microscope (Zeiss 200M) at 30°C for up to 44 h. Images were obtained with a CoolSNAP HQ (Princeton Instruments) at a magnification of either ×63 or ×100. Up to 12 fields, each containing one or two cells, were manually identified and their XYZ positions stored in the Metamorph microscope control software (Universal Imaging). Phase-contrast and fluorescent images were recorded at each field every 5 min for the first 122 frames and every 12 min for the remainder of the time lapse. To prevent phototoxicity, the excitation light (480–520 nm for 2 s) was limited to 5% of the output of a 100-W Hg-vapor lamp by neutral density filters. Emission wavelengths were 505–565 nm (filters from Chroma). For SI Movies 6, 7, 8, and 10, CDM–agarose contained 0.4 μg/ml of the membrane dye FM5-95 (Invitrogen), and microscope slides were incubated in a temperature-controlled room (30°C) and mounted on a Deltavision RT automated microscope (Applied Precision). Images were obtained up to 44 h with a CoolSNAP HQ at a magnification of ×60, ×63, or ×100. Fluorescent images were recorded at each field every 15 min. To prevent phototoxicity, the excitation light (480–500 nm for 0.1 s for GFP, and 541–569 nm for 0.3 s for FM5-95) was limited to 32% of the output of a 100-W Hg-vapor lamp by neutral density filters. Emission wavelengths were 509–547 nm (GFP) and 580–653 nm (FM5-95) (filters from Chroma). For SI Movies 4 and 9, FM5-95 was omitted, and images were captured every 8 min. Because cells tend to form multiple layers in the presence of xylose, a final concentration of agarose of 1.2% was used for SI Movie 9. Furthermore, the CDM was further diluted by adding 500 μl of water to 5,000 μl of 15% CDM and complemented with 0.125% xylose.

**Data Analyses.** To test the null hypothesis that the pole age of a cell is not a factor in the decision to form a spore or lyse, we performed random simulations generating control datasets with a normal binomial distribution and compared them with the observed results (t test), as described previously (10). χ² tests were performed to determine whether cells were biased to form a spore at the old pole.

To test whether spore formers are distributed randomly over the microcolony, we used a sampling approach. We randomly sampled a subset of all cells (sporulating and nonsporulating) and calculated the mean nearest neighbor distances (MNND) as described in ref. 11 to arrive at a sampling distribution (the size of the subset was thereby equal to the number of sporulating cells). The MNND of the spore formers can then be tested against this sampling distribution. In two of five cases, the observed MNND of spore formers differed significantly from the sampling distribution (p values = 0.002, 0.003, 0.107, 0.135, 0.303), indicating that spore formers show a propensity toward spatial spore grouping.
Phylogenetic Analyses. The true bacterial lineage of the microcolony after 20 h, containing 476 cells, including earlier spore formation and lysis, was plotted in a radial fashion by the use of Mathworks Matlab Bioinformatics toolbox (SI Fig. 14A) or was visualized as a dendrogram lineage tree by using custom BHV software (10) (SI Fig. 14B). Every terminal branch was color coded according to the fate of each bacterial cell.

To generate a parsimony map of fluorescence distribution within the lineage (Fig. 4A), fluorescence information of 356 cells after 669 min (~11 h) of growth was made binary ("on" or "off") on the basis of the cutoff value of 40 arbitrary fluorescence units above background. This time point was chosen because it coincides with the general growth arrest point (~669 min), and all cells express GFP above background. The cutoff fluorescence value was chosen by plotting the distribution of fluorescence values. This clearly shows two distinct subpopulations of GFP expressing cells (Fig. S19).


18. Fujita M, Losick R (2005) Evidence that entry into sporulation in *Bacillus subtilis* is governed by a gradual increase in the level and activity of the master regulator Spo0A. *Genes Dev* 19:2236-2244.
Fig. 6. *B. subtilis* utilizes nutrients released from its dead relatives. Images were recorded after cells went through the diauxic shift. Expression of GFP is indicative for growing cells (strain *PabrB-gfp*) (12). After the occurrence of lysis, some cells continue growth, and others complete spore formation (indicated with arrows).
SI Movie 1

**Movie 1.** Growing and differentiating *B. subtilis* microcolony. This film shows 35 h (247 frames) of the growth of a *B. subtilis* microcolony harboring the PspoIIA-gfp construct condensed to 23 s. For the first 122 frames, images were taken every 5 min; for the remaining frames, images were taken every 12 min. The complete lineage history of the entire microcolony from the single initial cell to all descendents has been tracked and recorded, allowing pole ages and cell fates to be assigned to every cell. The data derived from this microcolony were used for Figs. 1, 3, and 4.

SI Movie 2

**Movie 2.** Growing and differentiating *B. subtilis* microcolony. This film shows 44.5 h (293 frames) of the growth of a *B. subtilis* microcolony harboring the PspoIIAgfp construct condensed to 29 s. For the first 122 frames, images were taken every 5 min; for the remaining frames, images were taken every 12 min. The complete lineage history of the entire microcolony from the single initial cell to all descendents has been tracked and recorded, allowing pole ages and cell fates to be assigned to every cell.

SI Movie 3

**Movie 3.** Time-lapse analysis of strain abrB-gfp (*PabrB-gfp*) after the diauxic shift. This movie shows growth (indicated by increased GFP expression from the *abrB* promoter) and sporulation by utilization of the nutrients released from dead cells.
Fig. 7. Mother cells of lysing cells can be found in both pathways: diauxy growers and sporulators. Every birth point shown indicates the birth of a cell for which it is certain that either this cell or all of its descendants will follow a specific fate. The average growth rate of this cell during its life is represented on the y axis (AU). Diamonds show the growth rates of all birth points, blue circles show cells committed to spore formation, open upward triangles show diauxic growth fate cells, orange circles show mothers of lysing cells, and red triangles indicate lysing cells.
### Table 1. Diauxic growth cells contribute the most in terms of total offspring

<table>
<thead>
<tr>
<th>Cell fate</th>
<th>Average no. events*</th>
<th>Contribution to total offspring</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spores formed during glucose-based growth</td>
<td>49 +/- 23</td>
<td>16 +/- 8.2 %</td>
</tr>
<tr>
<td>(before lysis)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spores formed by diauxic growth cells</td>
<td>55 +/- 13</td>
<td>19 +/- 6.8 %</td>
</tr>
<tr>
<td>(after lysis)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total spore contribution</td>
<td>105 +/- 33</td>
<td>35 +/- 13 %</td>
</tr>
<tr>
<td>Vegetative cells formed from diauxic</td>
<td>211 +/- 99</td>
<td>65 +/- 14 %</td>
</tr>
<tr>
<td>growth cells (after lysis)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vegetative cells and spores formed from</td>
<td>266 +/- 98</td>
<td>84 +/- 8.2 %</td>
</tr>
<tr>
<td>diauxic growth cells (after lysis)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Based on five microcolonies, after 44 h of growth. The range (+/-) indicates the standard deviation.

**SI Movie 4**

**Movie 4.** Comparative time-lapse analysis of strains IIA-gfp (wild type, *Left*) and IIA/skf/sdp (Δskf/Δsdp, *Right*). Strains were grown next to each other within the same slide (thus identical conditions), and phase-contrast images were recorded every 8 min. Two typical microcolonies of these strains are shown.
**Fig. 8.** Average lineage tree showing old pole effect on growth rate. The lengths of the lines connecting the cells to their progeny are proportional to the average growth rate of that cell; a longer line represents a higher growth rate for that cell. At each division, the cell inheriting the old pole is placed on the right side of the division pair and shown in red, whereas new poles are placed on the left side of each pair and shown in blue. Because the position of the start of the growth line for each new generation depends on the generations that preceded it, the difference in growth rates is cumulative. Green lines indicate the point at which the first cell divides in each generation. Seven generations from 10 films encompassing 1,080 cells are averaged in this tree.
Fig. 9. Lengths, at the time of birth, of cells that will form a spore or grow during the diauxic shift. The time frame shown is between 380 and 500 min, around the period of the diauxic shift. We assume that cell length is an approximate indicator for cell cycle state. This graph shows that there are no apparent differences between the two categories of cells with different fates, indicating that cell length is not of critical importance in the decision to sporulate or not to sporulate.
Fig. 10. First spore formers are early deciders. Cells that form spores after the first round of exponential growth activate the *spoIIA* operon early during colony development. It should be noted that not all cells that have activated *spoIIA* at an early stage will form spores (see Fig. 3A).
**Fig. 11.** Individual fluorescence levels based on net change (net GFP production derived from the *spoIIA* promoter). Birth points are shown. Blue circles show net fluorescence change of cells committed to spore formation, open upward triangles show diauxic growth fate cells, orange circles show mothers of lysing cells, and red triangles indicate lysing cells. This graph shows that part of the lysing cells activated Spo0A and attempted to sporulate.

**SI Movie 5**

**Movie 5.** Time-lapse analysis of strain abrB-gfp (*PabrB-gfp*). The *abrB* promoter is directly repressed by Spo0A–P (16), thus expression of GFP is indicative for nonsporulating cells.
Fig. 12. Parsimony reconstructions of the wild-type strain (Ps spoIIA-gfp). Lines that are black and white have no unique parsimonious state. The lineage history of every cell is shown at the tips (O, old pole; N, new pole). After the first division of the primordial cell, one of the offspring cells dies. For clarification of the figure (and that of Fig. 4), this event is not shown. (A) Parsimony reconstruction of the lineage and GFP fluorescence information of 356 cells after ≈11 h of growth. Black tips represent cells with an arbitrary fluorescence value above 40 units. (B) Parsimony reconstruction of randomized data used for A. (C) Parsimony reconstruction of the lineage and fate information of 531 cells (including those that had lysed or sporulated previously) after 25 h of growth. Every end point in the tree represents one offspring cell; black tips are spore-forming cells.
Fig. 13B
Fig. 13. Lineage history and single-cell fluorescence trajectories. Green lines depict the total measured fluorescence in single cells in arbitrary units (AU) above background. This measure differs from the average (per pixel) fluorescence value used in both Fig. 3 and to determine the cutoff value for parsimony reconstruction. By not averaging over the cell area, it is possible to calculate the net change in GFP levels by subtracting the total fluorescence measured at time point \(t\) by the total fluorescence measured at time point \(t - 1\). Assuming that GFP degradation rates are constant, this formula yields an accurate approximation of actual promoter activity. Blue shadings indicate positive promoter activity, and red shadings indicate a decrease in GFP. Note that the scale of the \(y\) axis (fluorescence) is identical in all graphs, but the \(x\) axis (time) differs among graphs. (A) The entire lineage of a subfamily of highly spoI1A-gfp-expressing cells including the time point used for the parsimony reconstruction (669 min). At 669 min, this subfamily consisted of 11 cells, all of which show a total fluorescence higher than 40 AU (green cells). Induction of GFP begins in all eight cells of generation 8 in this lineage approximately concurrently, indicating that the observed correlation in GFP expression among closely related cells is not simply caused by dilution of GFP through division, but rather by the inheritance of a physiological "on" state. This finding is also illustrated in the next generation, where in many cases both siblings inherit extant GFP, but also continue to express GFP, further indicating the inheritance of an "on" state. This subfamily (derived from cell 1_1-NNON) produced 14 cells and successfully formed six spores. (B) The entire lineage of a subfamily in which all cells did not make the fluorescence cutoff and have therefore been categorized as "off" in the parsimony reconstruction. This subfamily, contemporary with the one above, all descended from cell 1_1-NOOO and consisted of 15 cells at 669 min. In contrast to the "on" family, this lineage eventually produced 28 cells, of which none formed spores.
Fig. 14. Lineage fate tree of a *B. subtilis* microcolony. Data from the microcolony used in Fig. 1 was used to generate this dendrogram (SI Movie 1). (A) Radial bacterial lineage of the colony represented in Fig. 1 showing the lineage and cell fates of 476 cells derived from a single ancestor after 20 h. The primordial cell is depicted with an arrow. Green end points represent sporulating cells, red end points represent cells that lysed, and black end points represent diauxy growth cells. (B) The primordial cell is depicted on the Left. After the first division of the primordial cell, one of the offspring cells dies. For clarification of the figure, this event is not shown. Blue end points represent sporulating cells, red end points represent cells that lysed, and black end points represent cells that did not sporulate and used overflow metabolites to continue growth.
Fig. 15. Phylogenetic signal tests on fluorescence data from 356 cells after 669 min of growth (Left) and on cell fate data (spore formers vs. non-spore formers) after 25 h of growth (Right).
Fig. 16

Percentage of cells

Mean fluorescence intensity (AU)

P_{spolA}^{gfp}

Δsp00A

17.5h

P_{spac}^{sad67}, 40 μM IPTG

P_{spac}^{spo0A}, 100 μM IPTG

P_{spac}^{spo0A}, 40 μM IPTG
**Fig. 16.** Fluorescence distribution of cells from Fig. 5. Images after 17.5 h of growth of strain IIA/Δ0A/spo0A grown on either 40 μM (405 cells) or 100 μM IPTG (332 cells) and strain IIA/spo0A/sad67 grown on 40 μM IPTG (149 cells) were analyzed. The frequency distributions of mean fluorescence intensities in arbitrary units (AU) above background level of individual cells are plotted.

**SI Movie 6**

**Movie 6.** Time-lapse analysis of strain IIA/Δ0A/spo0A (PspoIIA-gfp, Δspo0A, amyE::Pspac-spo0A). To induce spo0A transcription, CDM–agarose was supplemented with 40 μM IPTG.

**SI Movie 7**

**Movie 7.** Time-lapse analysis of strain IIA/Δ0A/spo0A (PspoIIA-gfp, Δspo0A, amyE::Pspac-spo0A) grown on either 40 (Left) or 100 (Right) μM IPTG.

**SI Movie 8**

**Movie 8.** Time-lapse analysis of strain IIA/spo0A/sad67 (PspoIIA-gfp, Δspo0A, amyE::Pspac-sad67). Sad67 is a mutant form of Spo0A that contains a deletion in the conserved N-terminal region, renders the protein constitutively active, and bypasses the need for activation by the phosphorelay (17). It should be noted that induction of Sad67 does not support actual spore formation. This is presumably a consequence of a rather abrupt activation of the Spo0A-regulon, because it has been shown that for successful spore formation, a gradual activation of first the low-threshold regulon followed by the high-threshold regulon is required (18). To induce sad67 transcription, CDM–agarose was supplemented with 40 μM IPTG.
**Fig. 17.** Phosphorelay activity determines the size of subfamilies of sporulating cells. Parsimony reconstruction of strain IIa-gfp/ΔrapA (PspoIIA-gfp, ΔrapA) (A) and strain IIAgfp, Xrap (PspoIIA-gfp, amyE::PxyI-rap60) (B) grown as microcolonies in the presence of 0.125% xylose (SI Movie 9). The Rap60 phosphatase from *Bacillus amyloliquefaciens* is highly homologous to RapA (13) and can dephosphorylate a component of the phosphorelay of *B. subtilis* (2, 14). Lines that are black and white have no unique parsimonious state. The lineage history of every cell is shown at the tips (O, old pole; N, new pole). Black tips represent cells with an arbitrary fluorescence value above the cutoff value and highly express GFP.

SI Movie 9

**Movie 9.** Comparative time-lapse analysis of strains IIa-gfp/ΔrapA (PspoIIA-gfp, ΔrapA) and IIAgfp/Xrap (PspoIIA-gfp, amyE::PxyI-rap60) grown in the presence of 0.125% xylose. Strains were grown next to each other within the same slide (thus identical conditions), and phase-contrast and fluorescence images were recorded every 8 min. Two typical microcolonies of these strains are shown.
Fig. 18. Sporulating cells express the acetoin catabolism operon. Time-lapse analysis of strain acoA-gfp (PacoA-gfp) shows that expression starts at the end of exponential growth and production of GFP is present in every cell, including sporulating cells (see inset and Movie S10). In microcolonies, cells show a drop in growth rate after a period of rapid growth because of depletion of glucose from the medium. This drop in growth is quickly followed by recovery of growth at a reduced rate, probably caused by utilization of overflow metabolites. Cells may feed on carbon products such as lactate, butanediol, and acetoin, which are excreted during earlier growth under the conditions of excess of glucose (15). As shown, the operon responsible for acetoin catabolism (acoA-L) is activated after exponential growth ceases. Under the conditions tested (also in liquid cultures, data not shown), all cells activate the acoA-L operon, indicating that also sporulating cells use overflow metabolites. This indicates that spore formers invest this energy in spore formation rather than in growth.

SI Movie 10

Movie 10. Time-lapse analysis of strain acoA-gfp (PacoA-gfp). See legend of SI Fig. 18.
Fig. 19. Determining the fluorescence cut-off value used to generate Fig. 4A. To generate a parsimony map of fluorescence distribution within the lineage, fluorescence information of 356 cells after 669 min (~11 h) of growth was made binary ("on" or "off") on the basis of the cutoff value of 40 arbitrary fluorescence units above background. This time point was chosen because it coincides with the general growth arrest point (~669 min; Left, red dotted line), and all cells express GFP above background. The cutoff fluorescence value was chosen by plotting the distribution of fluorescence values (Right). This clearly shows two distinct subpopulations of GFP-expressing cells (dotted red line).