Memory and modularity in cell–fate decision making

Thomas M. Norman1*, Nathan D. Lord1*, Johan Paulsson1 & Richard Losick2

Genetically identical cells sharing an environment can display markedly different phenotypes. It is often unclear how much of this variation derives from chance, external signals, or attempts by individual cells to exert autonomous phenotypic programs. By observing thousands of cells for hundreds of consecutive generations under constant conditions, we dissect the stochastic decision between a solitary, motile state and a chained, sessile state in Bacillus subtilis. We show that the motile state is ‘memoryless’, exhibiting no autonomous control over the time spent in the state. In contrast, the time spent as connected chains of cells is tightly controlled, enforcing coordination among related cells in the multicellular state. We show that the three–protein regulatory circuit governing the decision is modular, as initiation and maintenance of chaining are genetically separable functions. As stimulation of the same initiating pathway triggers biofilm formation, we argue that autonomous timing allows a trial commitment to multicellularity that external signals could extend.

Cell-fate decisions often result from explicit extracellular triggers1–3. It is now appreciated that internal stochastic fluctuations4–10 can also drive a cell to switch fates even in the apparent absence of external signals11–17. Neighbouring cells in the developing gonad of Caenorhabditis elegans compete to become ventral uterine or anchor cells18, and subpopulations of growing Escherichia coli cells probabilistically enter a quiescent, antibiotic-resistant state14,15. But whether occurring in the body of a nematode or in shaking culture, these decisions take place against a backdrop of environmental change driven by continued growth. With rising interest in quantitative properties of gene networks20,21, one central question is how much of a cell’s behaviour can be attributed to the environment and how much to the internal program, that is, the behaviour the network would implement were the environment fixed.

A prototypical situation arises in the conversion of bacteria from free-living, planktonic cells into sessile, multicellular communities known as biofilms22–23. Like many complex fates, biofilm formation is a product not just of a cell’s individual behaviour, but also of reinforcement by environmental cues created by nutrient depletion, the production of matrix24, quorum sensing25, and hypoxia26. Here we use a microfluidic device to investigate the earliest stages of multicellular growth by the soil bacterium Bacillus subtilis. Our approach removes confounding environmental influences while allowing for high-throughput quantitative imaging, thereby revealing the cell’s internal programs of development.

B. subtilis provides a natural model system for decision making. During the exponential phase of growth, it exists in two states: as individual, motile cells and as long, connected chains representing periodic attempts to settle down and start a colony. At the heart of the decision is a simple three–protein network between SinI, SinR and SlrR (refs 31, 32). Commitment to each state is controlled by a double-negative feedback loop in which SinR represses the slrR gene, and SlrR binds to and titrates SinR (Fig. 1a). Motility corresponds to the SlrROff state in which SinR represses the gene for SlrR and other chaining-associated genes. Chaining occurs during the SlrROn state in which SlrR forms a complex with SinR, both titrating its activity against chaining genes and redirecting it to repress motility-associated genes33. Although both states are present during exponential growth, the chained state is strongly reinforced during biofilm formation by further antagonism of SinR by SinI, which is produced in response to environmental signals34,35. This three-gene network thus supports a two-state process of decision making that can be influenced by environmental signals.

Visualizing fate switching in real time

Microfluidic systems that allow individual cells to be imaged over time as the growth medium is replenished provide an excellent opportunity to examine autonomous developmental programs. Extracellular signalling is removed, and cells cannot accumulate and starve themselves. Building on previous studies14,36–40, we constructed microfluidic channels from polydimethylsiloxane (PDMS, Fig. 1b) that were sized to accommodate chains of B. subtilis (75 μm long and 1.6 μm wide). A unique feature of our design is the shallow side channels that surround the cells, creating a ‘bath’ of medium that enables efficient feeding over long length scales41. The channels are closed on one end, and on the other they empty into a feeding channel that supplies fresh medium (by diffusion) and washes away excess cells as they are pushed out by growth. To prevent cells from swimming out of the channels, the ability of the flagellum to generate force was disrupted through a straight flagellum mutation42.

Only motile cells expressed the flagellin gene (Supplementary Video 1) as visualized with a Flag-mKate2 reporter (coloured green), and only chains expressed matrix genes as visualized with a TapA–cfp reporter (coloured red). We therefore used these reporters as proxies for the corresponding phenotypic states. B. subtilis interconverted between the motile and chained states while growing in the channels (Fig. 1c and Supplementary Video 2), leading to anticorrelated flagellin and matrix gene expression. In keeping with the premise that the chains had switched to the SlrROn state, imaging of slrR (visualized with a PsrlR–mKate2 reporter, artificially coloured green) and matrix coexpression

1Department of Systems Biology, Harvard Medical School, Boston, Massachusetts 02115, USA. 2Department of Molecular and Cellular Biology, Harvard University, Cambridge, Massachusetts 02138, USA.

*These authors contributed equally to this work.
revealed that slrR was expressed in chains (Fig. 1d), and that matrix and slrR expression were tightly correlated in time (Fig. 1e).

Several million cell divisions were imaged, but we only report data for the fates of the uppermost cell in each channel, as these could be monitored throughout the experiment without being washed away (Fig. 2a and Supplementary Video 2). We thus tracked the histories of thousands of individual bacteria through ~300 generations of growth. To define more precisely the motile and chained states, we found thresholds on the matrix reporter that coincided with onset of matrix expression and the subsequent return of motility, but similar results were obtained for a range of thresholds (Extended Data Fig. 1). All measured properties remained constant in time and across the device: a generation time of ~27 min was sustained for as long as 7 days (Extended Data Fig. 2), chaining occurred at a uniform rate (Extended Data Fig. 3), and within each lineage there was no correlation between the lengths of successive visits to the motile state (Fig. 2b) or the chained state (Extended Data Fig. 4). The switching behaviour was thus homogeneous throughout the device and experiment duration, reflecting a stochastic process at steady state. With the influence of environmental changes removed, we next set out to characterize the autonomous motility and chaining programs.

Memoryless motility and timed chaining
We monitored transitions between motile and chained states to determine whether cells exercise temporal control, or if they exit states independently of their history. The latter memoryless behaviour would imply exponentially distributed residence times between events and thus a coefficient of variation (standard deviation divided by mean) in residence times of CV = 100%, whereas other switching mechanisms could exploit history-dependence to produce narrower distributions. We
phenotype accumulates over time, where chaining for preclude both very short and very long commitments. The chained contrast, any decision that depends on coordination among progeny leaving no obvious reason to keep track of the residence time. In briefer, tightly timed transitions. This difference makes teleological and allow widely variable commitments, chains instead orchestrate and had a 28% relative standard deviation (Fig. 2f), resembling a tion or hundreds of generations.

Chains displayed a radically different behaviour. The residence time distribution was sharply peaked at a mean of 7.6 generations and had a 28% relative standard deviation (Fig. 2f), resembling a gamma distribution with a shape parameter of 13 and with an ageing curve prototypical of tight timing before eventually flattening out (Fig. 2g). Thus, whereas motile cells set long average residence times and allow widely variable commitments, chains instead orchestrate briefer, tightly timed transitions. This difference makes teleological sense given their respective lifestyles. As motile cells grow as individuals, their properties are insensitive to how long they remain motile, leaving no obvious reason to keep track of the residence time. In contrast, any decision that depends on coordination among progeny will require some degree of memory. Chains have strong incentives to preclude both very short and very long commitments. The chained phenotype accumulates over time, where chaining for T generations produces chains of length $2^T$. Relatively small differences in $T$ then translate into great differences in chain length. Memoryless exit from the chained state would in fact have extreme consequences, where many chains would break down almost instantaneously whereas others could contain millions of cells. The narrow time distribution guarantees a minimum chain length while preventing a high fraction of cells from effectively entering the chained state irreversibly.

Memoryless initiation from noisy antagonism

Slow and memoryless switching can arise from positive feedback loops, in which rare fluctuations allow the system to break out of the basin of attraction of each stable point. Indeed, one of the key features of the motility-chaining decision network is the SinR–SlrR double negative feedback loop. As expected, mutating slrR eliminated chaining: over the course of a 6-day experiment, we saw sustained high expression of flagellin in all cells and observed no morphological evidence of cells growing as connected groups. However, our sensitive time-lapse microscopy allowed us to detect exceedingly rare and weak expression signals, showing that an slrR mutant exhibited small and infrequent bursts of matrix expression (Fig. 3a and Supplementary Video 3). We refer to these events as pulses, to distinguish them from chains that pair high matrix expression with repression of flagellin. We note that they also appear in the wild-type data, but fail to trigger expression of slrR (Extended Data Fig. 5). Notably, the residence times between subsequent initiation attempts, whether resulting in chains or pulses, followed indistinguishable exponential distributions for wild-type cells and the slrR mutant (Fig. 3b). Removal of SlrR thus abolished the chaining phenotype, but left the memoryless process of initiation perfectly intact.

Having determined that initiation arose from a factor upstream of the feedback loop, we examined the SinI protein that antagonizes SinR during biofilm formation. SinI was sufficient to drive chaining, as cells containing an IPTG-inducible sinI gene rapidly chained upon induction. It was also necessary: cells

Figures 3 | Memoryless initiation of chaining. a, An example trace of flagellin (P$_{lac-mKate2}$, green curve) and matrix (P$_{upA-cfp}$, red curve) reporter expression from slrR mutant cells (TMN1158). Seven matrix pulses are shaded. AU, arbitrary units. b, Log transformed cumulative distribution functions of times between subsequent initiations (of pulses or chains) in cells from wild type (blue curve, TMN1157, 399 events) or mutant for slrR (red curve, TMN1158, 296 events) strains. Plotted this way, exponential distributions yield straight lines. This result separately reproduced in a strain with different fluorescent reporter proteins. c, Example matrix expression traces in slrR mutant cells (blue curve, TMN1158), and in slrR mutant cells further deleted for the initiator (sinI) (red curve, TMN1198).

mutant for SinI did not chain, and pulses were absent in cells doubly mutant for SinI and SlrR (red curve in Fig. 3c). These results suggest that noisy antagonism of SinR by SinI drives spontaneous chaining in a way that is quantitatively independent of the SlrR feedback control system, as discussed below.

To test how cells control the duration of the chained state, we briefly switched (10 min) on the inducible sinI gene to provide a defined initiating signal (Fig. 4a and Supplementary Video 4). Notably, the ageing behaviour of the resulting chains was virtually identical to that of spontaneously occurring chains (see Figs 4b and 2g). Even switching on SinI synthesis a second time in cells that had started to revert from chaining (3 h after first pulse) or using a longer initiating signal led to no increase in the average duration of the resulting chains (Extended Data Fig. 6). The chained state is thus stereotyped: once a signal to chain is registered, the same program is executed in a way that is independent of the nature of the initiating signal or of the history of the cell. This tight timing is an intrinsic property of the SinR–SlrR feedback loop rather than the initiating event, as the spontaneous pulses seen in slrR mutant cells showed little evidence of temporal organization (red curve in Fig. 2g). Furthermore, chains lasted longer than pulses under both spontaneous and induced conditions (Figs 4c, d), suggesting that the feedback loop coordinated action after the initiating signal had faded. Indeed, adding an additional copy of slrR to strengthen feedback led to longer chaining events (Extended Data Fig. 7). Thus, we again see network modularity: just as the SinR–SlrR feedback loop did not affect the initiation of chaining, the duration of the chained state was independent of the initiation process.

To dissect how cells time their exit from the chained state, we analysed the temporal pattern of gene expression during hundreds of chaining events. Examining the rate of gene expression in these traces (Methods) revealed two distinct phases: a build-up phase of matrix expression was followed by a pure dilution phase when expression was negligible and levels exponentially decreased due to growth (Fig. 4e). Motility then reintroduced once levels fell below a threshold. The two phases were approximately equal in length, with the duration of the dilution phase more narrowly distributed than the build-up phase (CV$_{build-up} = 0.44$, CV$_{dilution} = 0.23$).
seemed high enough that random segregation of molecules between daughter cells at low numbers\textsuperscript{10,46} was made irrelevent. Second, the exponential nature of dilution—reducing levels twofold every generation—further tightend control by making the timing robust to heterogeneity in the initial level of protein. Specifically, the time spent diluting then depends logarithmically rather than linearly on the initial amount. Cells that, by chance, have much more or less protein initially, will then vary marginally in the time spent diluting. Indeed, the 30% deviations in matrix abundance at the onset of dilution was reduced to a 23% deviation in the dilution time, closely following the expectation from a noise-free exponential dilution process (Supplementary Information). Thus, by extending the build-up phase in chains, SlrR is responsible for translating widely variable initiating signals into a precisely timed pattern of gene expression.

**Memory enables multicellular cooperation**

The choice between motility and multicellularity is central to the lives of many bacteria, as cells must relinquish their autonomy to benefit from living together\textsuperscript{22,23}. The chaining program may underlie the earliest steps of multicellularity: by coordinating behavious across many generations, the tight timing provided by SlrR enforces cooperation among the progeny of a cell that initiates a new sessile community. The long-term commitment to chaining seen during biofilm formation could in turn rely on continued initiation or on feedback mechanisms that lock cells into the multicellular state. Although we saw no evidence that SlrR feedback could provide the requisite comitmitment, the initiator SinI is indeed strongly expressed both in response to desirably niches (for example, plant polysaccharides)\textsuperscript{47} and growth-related stresses (for example, starvation or hypoxia)\textsuperscript{24,26}. Our results show that different environmental signals are channelled into the same robust chaining behaviour, and cessation of the stimulus ultimately leads to coordinated exit. Maintenance is thus contingent on continued stimulation, but even small signals will suffice to renew commitment. The role of SlrR feedback may thus be to provide a well-defined ‘trial period’ of multicellular growth, the continuation of which is periodically re-evaluated.

Regulation of chaining weaves together stochastic gene expression, transcriptional feedback and post-translational regulation. Any quantitative property of the decision could therefore have been a product of several factors acting together. Yet observation of thousands of chaining decisions free from environmental influences revealed a modular network that separates initiation from control of the residence time; eliminating one function leaves the other intact in quantitative detail, allowing the overall behaviour to be explained in terms of these two pieces. This type of excitable dynamics, in which the system is randomly kicked out of a stable state but returns after a well-defined excursion, is often explained in terms of linked feedback loops, and has been implicated in other B. subtilis decision networks\textsuperscript{16,17}. In this case, however, an exceedingly simple alternative mechanism may explain most of the behaviour. SinR and SinI are known to form an inactive complex with binding constants in the nanomolar range\textsuperscript{48}. Because more SinR is produced than SinI, SinR typically titrates out all free SinI molecules, thereby acting as a buffer against small fluctuations. However, a rare persistent accumulation of SinI levels temporarily kicks SinR out of the active pool, leading to a buffering pool of free SinI instead. This mechanism can generate long periods of virtually no free SinI (corresponding to the motile state) followed by long stretches of SinI dominance, which induces chaining. The memory in the chained state is in turn largely explained by the production-dilution mechanism above, in which feedback could have a role in narrowing the probability distribution of time spent producing matrix proteins.

Other systems may also display memory and memorylessness for the times spent sessile and motile, respectively\textsuperscript{49}, but we suspect any broader principles will follow from the sensitivity of a phenotype to the time spent in the state. Decisions that aim only to set the occupancy of a particular state\textsuperscript{14,15,19} do not require explicit timing, and
may therefore randomize commitments with memoryless switching. In contrast, when the effectiveness of a cell-fate choice is tied to population size\(^{46}\), timed decision making could again be used to ensure cooperation among progeny. In metazoans, stochastic cell-fate decisions are often stabilized after the fact by lateral inhibition\(^1\). Timing the adopted state could provide an initial window of commitment to give extracellular feedback time to take hold. Our approach—observing the cell’s intrinsic dynamics while keeping everything else static for extended periods of time—may reveal that many complex developmental choices can be explained by surprisingly simple dynamical principles in individual cells.

**METHODS SUMMARY**

Strains were grown to high density and loaded into freshly cast and bonded microfluidic chips. A straight flagellum mutation in all strains (flagG\(^{A233V}\)) prevents the flagellum from generating force so that motile cells cannot swim out of the channels. Fresh LB medium was continuously supplied using syringe pumps, and an automated fluorescence microscope maintained at 37 °C was used to image cells every 10 min. When needed, 10-nl pulses of 100 μM IPTG were used to induce chaining. The top cell in each channel was segmented (Extended Data Fig. 10) and its fluorescence was quantified using a Matlab analysis pipeline. Resulting reporter traces were used to produce residence time distributions by finding thresholds on the matrix reporter that identified when the signal was first distinguishable from background, and when motility reporter expression subsequently returned. The time between these two points was defined as the duration of a chain or pulse, and the time between subsequent peaks was defined as the time spent motile. The log transform of a cumulative distribution function \(F(t) = -\log[1 - F(t)]\), which for exponential distributions yields a straight line. For a distribution of times \(T\), the ageing curve is \(m(t) = E[T - t | T > t]\). Average chain and pulse profiles were compiled by normalizing each peak height to 1, registering the leading edges and averaging the aligned peaks. This normalization removes variation due to peak height but leaves variation due to timing behaviour intact. Chain ‘build-up’ and ‘dilution’ phases were identified by fitting matrix reporter traces to a kinetic model and extracting expression rates at each point. The build-up phase extends from beginning of the chain to the point where the dilution rate is fivefold larger than the matrix expression rate, and the dilution phase comprises the remainder of the chain.

**Online Content** Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

**Received 20 June; accepted 29 October 2013.**

**Published online 20 November 2013.**
Supplementary Information is available in the online version of the paper.

Acknowledgements We thank A. Lindner for sharing an early version of the microfluidic mother machine with our groups. We thank Y. Chai for discussions and C. Saenz, V. Lien, S. Hickman, J. Tresback and J. Deng for technical help with microfluidic fabrication. This work was performed in part at the Harvard Medical School Microfluidics Facility and in part at the Center for Nanoscale Systems (CNS), a member of the National Nanotechnology Infrastructure Network (NNIN), which is supported by the National Science Foundation under NSF award no. ECS-0335765. CNS is part of Harvard University. This work was supported by grants from the NIH to R.L. (GM18568) and J.P. (GM081563).

Author Contributions T.M.N. and N.D.L. designed and fabricated the microfluidic device, cloned strains and collected the data. All authors were involved in conceiving the study, analysing results and writing the paper. J.P. and R.L. are corresponding authors.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to J.P. (Johan_Paulsson@hms.harvard.edu) or R.L. (losick@mcb.harvard.edu).
METHODS

Strain construction. All strains were derived from Bacillus subtilis NCIB3610 using standard molecular biology techniques. Strain genotypes, full construction details and a list of primer sequences are provided in the Supplementary Information. To prevent motile cells from swimming out of the channels, all strains bore a hagA233V straight flagellum mutation, which impairs the ability of the flagellum to generate force while leaving its construction intact41.

Microfluidic device fabrication. The master mould for the device was fabricated in four layers by ultraviolet photolithography using standard methods (for detailed protocol, see Supplementary Information). For each layer, Shipley or SU-8 (Microchem) photoresist was applied to a silicon wafer by spin coating to appropriate thickness (corresponding to the channel height) and patterns were then created by exposing the uncured photoresist to ultraviolet light through custom quartz–chrome photo-masks (Toppan Inc.).

Microfluidic devices were fabricated by moulding channel features into a polydimethylsiloxane (PDMS) slab and then bonding that slab to a glass coverslip. To produce the slab, dimethyl siloxane monomer (Sylgard 184) was mixed in a 5:1 ratio with curing agent, poured onto the silicon wafer master, degassed under vacuum, and cured at 65°C overnight. Holes to connect the feeding channels to the external tubing used for medium perfusion were then introduced using a biopsy punch, and individual chips were cut and bonded onto KOH-cleaned cover slips using oxygen plasma treatment the day of the experiment. Bonded chips were baked at 65°C for at least an hour before use.

Cell preparation and device loading. Immediately before use, the microfluidic device was passivated with a 10 mg ml⁻¹ solution of bovine serum albumin (BSA). B. subtilis cells were grown to late stationary phase in LB to decrease their size and thus increase efficiency of loading. They were then passed through a 5 μm filter (Pall Acrodisc) to remove chains, concentrated by centrifugation, and injected into the feeding channel. The chip was mounted on a custom-machined platform that could be inserted into a standard bench-top centrifuge, and cells were forced into the cell channels by centrifugation. Syringes containing LB medium with 0.1 mg ml⁻¹ BSA were connected to the device using Tygon tubing (VWR), and were pumped at a flow rate of 3 μl min⁻¹ using syringe pumps (New Era Pump Systems). BSA was provided as a lubricant to prevent cells (and chains in particular) from adhering to the surface of the main feeding conduit as they are pushed out of the device.

Microscopy and image acquisition. The microfluidic device was mounted on a fluorescence microscope immediately after loading. We used a Nikon Eclipse Ti inverted microscope equipped with an Orca R2 (Hamamatsu) camera, a ×60 Plan Apo oil objective (NA 1.4, Nikon), an automated stage (Ludl), and a Lumencor SOLA fluorescent illumination system. Image acquisition was performed using Matlab scripts interfacing with μManager39. The microscope was encased in a custom-built incubator that maintained it at 37°C throughout the experiment. The following filter sets were used for acquisition: GFP (Semrock GFP-1828A), mKate2 (Semrock mCherry-B), CFP (Semrock CFP-2432C), YFP (Semrock YFP-2427B). The slr/tapA co-expression experiment was performed on an almost identically configured microscope that instead had a Lumencor SPECTRA fluorescent illumination system. Exposures were done at very low illumination intensities with 2 × 2 binning (CCD chip dimension of 1,344 × 1,024 pixels, pixel size of 6.45 μm × 6.45 μm) and typical acquisition periods of 200–500 ms. The Lumencor light sources produce little ultraviolet or infrared light, obviating the need for supplementary filters to block these wavelengths.

Cells were allowed to equilibrate in the device for several hours before imaging, and all data before the first chain or pulse in each lineage was ignored in subsequent analysis. Images were acquired every 10 min and saved as 16 bit TIFFs. Focal drift was controlled through the use of the Nikon PerfectFocus system and a custom-built, image-based autofocus that imaged a sacrificial position over many planes.

Induction of chaining with IPTG. To induce chaining, two syringes carrying either LB with 0.1 mg ml⁻¹ BSA or LB with 0.1 mg ml⁻¹ BSA and 100 μM IPTG (isopropyl β-D-1-thiogalactopyranoside) were connected via soft tubing to a Y-junction connector that fed into a common line connected to the device. The line that was not in use was clamped shut with a binder clip. Each syringe was loaded into an independently controlled syringe pump, and a pulse of IPTG was produced by switching to the IPTG-bearing syringe for 10 min.

Image processing and lineage construction. All data analysis was based on a custom Matlab image processing pipeline described in detail in the Supplementary Information. For each image, the top cell in each channel was identified as summarized in Extended Data Fig. 10. The mean fluorescence intensity within these cells was then calculated for each fluorescence channel. A simple tracking algorithm was used to follow cells as they grew and divided, producing long lineages lasting the duration of the experiment. Cell division events were identified by looking for instances where a cell’s calculated area dropped to less than 60% of its previous value. If a tracked cell died spontaneously, the algorithm continued the lineage from the dead cell’s closest relative.

Measuring residence times in the two states. Motility and chaining durations were called by examining the trace of P_{GFP-CFP} fluorescence within a lineage. To identify the level of background fluorescence, rough peaks were identified using a peak-finding algorithm (N. C. Yoder, available at http://www.mathworks.com/matlabcentral/fileexchange/25500-peakfinder) on traces smoothed with a Savitzky–Golay filter, and the average fluorescence outside these peaks was subtracted from all traces. Final peak boundaries were called where the matrix reporter signal crossed pre-defined thresholds. These thresholds were chosen to correspond to phenotypic transitions: onset of matrix gene expression defines the beginning of the peak, and onset of motility gene expression defines the end (Extended Data Fig. 1). We note that the main conclusions of the paper are insensitive to the threshold values (Extended Data Fig. 1). All peaks were manually curated before calculating statistics.

With the cell-fate history of each lineage in hand, we compiled statistics describing residence time in the chained state (chain/pulse periods) and residence time in the motile state (subsequent initiation times and motility periods). We define a chain or pulse period as the duration of matrix expression within a peak (identified as described above) and the motility period as the duration of uninterrupted motility gene expression between chaining events. In Fig. 3b, we instead measured the time between the start times of consecutive peaks (‘subsequent initiations’), meaning either chains or pulses. Owing to the long average residence time in the motile state, long motility periods are difficult to sample adequately. We account for this issue in the calculation of motility-related statistics, and include a complete discussion of the correction in the Supplementary Information.

Log transformation. We define the log transformation of a cumulative distribution function F(t) as −log[1 − R(t)]. This transformation facilitates comparisons, as exponential distributions are transformed to straight lines.

Memory (mean residual lifetime). We measured the memory associated with each state using the mean residual lifetime, defined as m(t) = E[T − t | T > t] for a distribution of residence times, T. The mean residual lifetime at time t is the average amount of time a cell will remain in its current state given that it has already spent t time units there.

Average expression profiles. Average profiles of matrix gene expression during pulses and chains were created by normalizing all measured events’ heights to 1, aligning the events’ leading edges, and averaging the expression values at each time point. This procedure normalizes away variability in peak height so that variation between average traces derives primarily from differences in timing.

Identifying chain build-up and dilution phases. Each chaining event was decomposed into ‘build-up’ and ‘dilution’ phases based on rates of matrix reporter synthesis and dilution that were calculated from each trace. Briefly, traces were smoothed using a Savitzky–Golay filter, the resulting polynomial was differentiated, and the rate of expression was inferred from a kinetic model of gene expression (see Supplementary Information) that assumed a time varying synthesis rate and exponential degradation of reporter. The build-up phase was defined as the time over which the synthesis rate of reporter was at least 20% of the dilution rate, and the dilution phase was the remaining time in which dilution dominated.

51. Edelstein, A., Amodaj, N., Hoover, K., Vale, R. & Stuurman, N. Computer control of microscopes using microManager. Curr. Protoc. Mol. Biol. Ch. 14, Unit14.20 (2010).
Extended Data Figure 1 | Ageing behaviour is independent of choice of threshold. Initially, the duration of a chaining event was called as the time between when matrix expression was first detectable to when flagellin expression began to increase. However, to compare chains (in strain TMN1157) and pulses (in strain TMN1158), we examined whether it was possible to call the end point using only the matrix reporter, as flagellin expression does not fall during pulses. In both methods, the beginning of a chain was called as the time when the matrix signal was first detectable above background fluctuations (~0.033 arbitrary fluorescence units, AU; see Supplementary Information). a, To call the end of a chain using only the matrix signal, various thresholds were applied. The figure plots the difference in chain duration between this single reporter method for different thresholds and the two reporter method. A threshold of 0.15 AU called the duration of chaining to within 20 min of the two-reporter method and was used throughout the text to call the end of the events. b, To show that the primary conclusions are unchanged by the choice of threshold, the ageing curves for the chained state are plotted for all thresholds shown in the previous panel. As the motile state is extremely long in comparison to the chained state, properties of the motile state are completely insensitive to how we called chains.
Extended Data Figure 2 | Cell growth is homogeneous in time. Sliding window average of division time plotted as a function of time (in strain TMN1158). Each point in the curve represents the average of all division times that occurred within a 250-min window. The grey shaded area denotes ±1 standard deviation, whereas the red shaded error denotes ±1 standard error of the mean. A flat trend indicates that conditions in the device do not change in time.
Extended Data Figure 3 | Chaining incidence is constant in time. Histogram of the number of chaining events observed in successive 330-min windows in the experiment described in Fig. 2 of the main text. As the number of observed lineages was constant throughout the experiment, these measurements reflect the average chaining rate in each window. A flat trend occurs when this average rate is constant in time, and thus that the factors controlling the switching decision have reached stationarity. Chains occurring early in our experiments were excluded from subsequent analysis to avoid any transient effects associated with adapting to growth in the device (Supplementary Information).
Extended Data Figure 4 | Successive visits to the chained state are uncorrelated. Scatter plot of the durations of sequential visits to the chained state within each wild-type lineage (440 events), analogous to Fig. 2b for the motile state. Note that some points fall on top of each other owing to the discrete nature of the measurements.
Extended Data Figure 5 | SlrR is expressed strongly only in chains. Average expression traces of slrR during chains (blue curve, 25 events) and pulses (green curve, 14 events) seen in strain TMN1180 (P_{tapA}\text{-cfp} PslrR\text{-mKate2 hagA233V}). AU, arbitrary units.
Extended Data Figure 6 | Chaining program is independent of cellular state.

To test whether the initial state of the cell influenced the chaining program, cells (of strain TMN1195) were forced to chain with a burst of expression from an IPTG-inducible sinI gene (created by switching to medium containing 100 μM IPTG for 10 min). When some cells began to return to the motile state (3 h later), a second IPTG treatment was administered. a, Average matrix expression profiles in chains induced by single pulses of IPTG (blue curve) or two consecutive IPTG pulses (red curve). The average amount of time spent as a chain after the second IPTG treatment was similar to the time seen in the chained state after a single treatment (260 min versus 280 min, 177 and 28 events, respectively). b, Scatter plot comparing matrix expression level (in arbitrary fluorescence units, AU) at the time of the second IPTG treatment to the duration of the ensuing chain, indicating that the state of the cell at the time of treatment had no effect on the subsequent chain duration. c, 10 min (blue curve, 84 events) and 20 min (red curve, 99 events) IPTG treatments were used to induce chaining, resulting in near identical distributions of chain durations. Note that the 10-min data set contained two exceptionally long chaining events that explain the slightly higher average duration.
Extended Data Figure 7 | Strongly enhanced commitment to the chained state in strains overexpressing *slrR*. The figure shows an example trace of a chain made by the strain TMN1206 (P*tapA*-cfp P*hag*-mKate2 hagA233V ywrK::P*slrR*-slrR), which bears an additional copy of the gene for SlrR under its native promoter. In this strain, most chains last long enough that they are eventually pulled out by the flow of fresh medium running through the device. Using the time to fall-out as a lower bound for the average duration of the chaining state suggests that the chained state lasts at least ~420 min (~15.5 generations) in these cells. AU, arbitrary units.
Extended Data Figure 8 | Variation in matrix expression rate over time during build-up phase. As described in the main text, chaining events can be naturally broken down into a build-up period, when new synthesis dominates, and a subsequent dilution period, where new synthesis is minimal. The rate of matrix reporter expression was calculated at each time point during the build-up period for all chaining events, producing a time-varying distribution of possible expression rates. The figure plots the coefficient of variation of this distribution, showing that expression rates show a roughly constant CV of $-0.5$ over much of the build-up period. Note that most chains have ceased the build-up phase by about 250 min in, so the end of the graph is less informative. This figure should be compared with Fig. 4f, which shows that the CV in the abundance of the matrix reporter decreases over the same period due to the time averaging principle described in the main text.
Extended Data Figure 9 | Dilution phase is well described by a deterministic model for exponential decay. Scatter plot comparison of observed and predicted dilution phase durations in spontaneous chains. Expected dilution times were derived from a deterministic model for exponential decay of the reporter (Supplementary Information). Close proximity to the line $y = x$ (red line) indicates that the data are well described by the model.
Extended Data Figure 10 | Image processing used for image quantification. 

a, Cells are identified using a constitutively expressed YFP construct. 
b, Images are rotated so that channels are oriented vertically. 
c, Images are contrast enhanced to better identify cell boundaries. 
d, Cells are preliminarily identified by edge detection. 
e, The mask identifying cells is improved by morphological processing. 
f, Mother cells are identified (highlighted in white). 
g, Superposition of segmented cell boundaries and rotated data YFP image.