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Direct observation of type 1 fimbrial switching

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The defining feature of bacterial phase variation is a stochastic 'all-or-nothing' switching in gene expression. However, direct observations of these rare switching events have so far been lacking, obscuring possible correlations between switching events themselves, and between switching and other cellular events, such as division and DNA replication. We monitored the phase variation of type 1 fimbriae in individual *Escherichia coli* in real time and simultaneously tracked the chromosome replication process. We observed distinctive patterns of *fim* (*fimbriae*) expression in multiple genealogically related lineages. These patterns could be explained by a model that combines a single switching event with chromosomal *fim* replication, as well as the epigenetic inheritance of expressed *fim* protein and RNA, and their dilution by growth. Analysis of the moment of switching at sub-cell-cycle resolution revealed a correlation between *fim* switching and cell age, which challenges the traditional idea of phase variation as a random Poissonian phenomenon.

Keywords: fim; fimbriae; SeqA; single cell; phase variation

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INTRODUCTION

Phase variation is characterized by a stochastic switching between discrete gene expression states (Henderson *et al*, 1999; van der Woude & Baumler, 2004). This form of gene regulation contrasts with classical gene regulation, both in terms of the underlying molecular mechanisms and the effect on the population structure. Switching between phases typically involves a genetic or epigenetic mechanism such as a DNA inversion or a loss or gain of DNA methylation, resulting in 'all-or-nothing' ON or OFF expression levels. As the switch state is heritable over multiple generations, it allows the population to differentiate and stably maintain sub-populations. Phase variant macromolecules are typically exposed on the cell surface, and have a crucial function in bacterial virulence and immune evasion.

The regulation of phase variation switching rates has been studied in detail for several systems (Blomfield, 2001). However, the experimental methods used so far do not capture the switching event itself, but rather infer switching rates by monitoring the ratio between ON and OFF cells within a population (Gally *et al*, 1993). For this reason, the most elementary questions on the correlations and dynamics of phase variation have remained unaddressed. For example: is there a correlation between switching events in genealogically related cells? Does switching occur in particular phases of the cell cycle? What are the expression dynamics during switching? Here, we investigate these issues by following the expression of a phase-variable gene in single cells and in real time. In particular, we investigate the switching behaviour of type 1 fimbrial expression in *Escherichia coli*.

The mechanism of *fim* (*fimbriae*) switching is characterized by a reversible DNA inversion event, in which a *cis* regulatory element that contains the promoter of the *fim* operon changes its orientation (Fig 1A). The DNA inversion process involves the formation of a DNA recombinase (FimB and/or FimE) complex forming a loop of the 314-bp invertible region, the subsequent breaking and rejoining of single DNA strands, and the dissipation of the synapse complex to allow transcription of the *fim* operon.

To detect *fim* switching events in single cells, *GFPmut2* was inserted into the chromosomal *fimA*, which is the first gene of the *fim* operon. We followed cell growth and the level of green fluorescent protein (GFP) expression over time, and determined the genealogical relationships within the population by using image analysis. Chromosome replication was simultaneously monitored in some experiments, using a fusion between the DNA sequestering protein SeqA and the mCherry fluorescent reporter. SeqA forms visible foci at replication forks, as it has a binding affinity for hemimethylated DNA (Hiraga *et al*, 2000; Yamazoe *et al*, 2005).

RESULTS

Phase variation in real time at the single-cell level

To characterize the distribution of the *fim* operon expression level at a single time point, isogenic cells growing exponentially in defined rich medium were spread onto agar and then imaged using phase-contrast and fluorescence microscopy. Two distinct sub-populations—one with a low mean GFP brightness per unit area (0.77 ± 0.23 a.u.) and another with a high brightness (8.18 ± 1.88 a.u.; Fig 1B)—corresponding to the OFF and ON states of *fimS*, were observed. The ON cell fraction (4.6%) was in agreement with previous studies (Leathart & Gally, 1998). The low level of expression in the OFF state, which is indistinguishable from cells lacking the GFP fusion, was consistent with the tight orientational specificity of the promoter.

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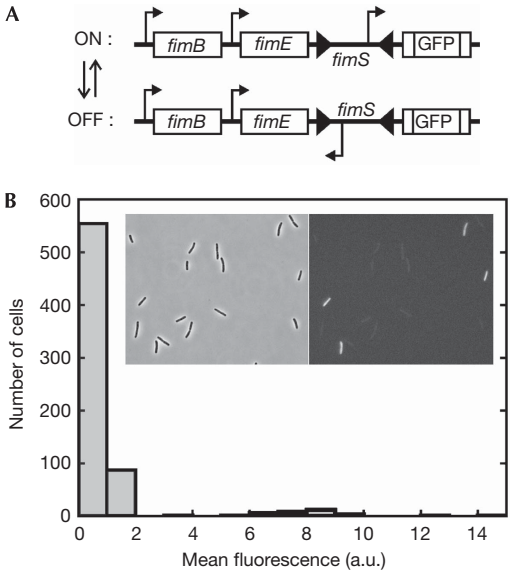

Fig 1 | The *fim* operon in ON and OFF phases. (**A**) In the ON orientation, the promoter positioned within the invertible element *fimS* drives transcription of the *fim* operon. GFP has been inserted into *fimA*, the first gene of the *fim* operon, allowing single-cell monitoring of the *fim* operon expression level. Transcription is turned OFF on inversion of *fimS*, which positions the promoter in the opposite direction. This reversible inversion process is performed by two recombinases, FimB and FimE. (**B**) Histogram of GFP fluorescence per cell. A phase-contrast image and its corresponding fluorescence image show ON and OFF cells deposited on agar. Both the images and the distribution of mean fluorescence level per cell show well differentiated ON and OFF states. *fim*, *fimbriae*; GFP, green fluorescent protein.

Cells were monitored as they continued to grow and divide. Microcolonies of about 500 cells were formed over a period of approximately 5 h (generation time, ~25 min; supplementary Fig S1 online). Some descendants of a non-fluorescent OFF progenitor spontaneously showed an increased fluorescence that signalled ON switching, resulting in a heterogeneous colony with patches of different phases (Fig 2A; supplementary Movies S1 and S2 online). Conversely, brightly fluorescent ON progenitors produced descendants with a decreasing fluorescence, indicating OFF switching (supplementary Movies S3 and S4 online). Cells grew exponentially throughout the experiment with a constant growth rate (supplementary Fig S1 online). In total, more than 400 progenitors were monitored producing more than 20,000 single-cell trajectories (see supplementary Table S1 online).

Distinct patterns of *fim* expression near switching

We constructed family trees detailing the genealogical relationships within the microcolonies, as well as the GFP expression history of each lineage (Fig 2). Some lineages showed a monotonic increase in brightness, whereas other lineages showed an increase followed by a decrease. These expression profiles developed over the course of several generations, which is significantly longer than the GFP expression and maturation time of about 6 min (supplementary Fig S2 online). The observed decreases in brightness might originate from ON-to-OFF switching events. However, as the bulk ON-to-OFF switching rate (of the order 0.1 per cell per generation; Gally *et al*, 1993) is too low to explain the data, one would have to invoke as-of-yet unknown correlations between switching events. Instead, we hypothesize that the observed pattern of expression is caused by the chromosome replication process in combination with a single OFF-to-ON switching event.

To synthesize a sufficient number of chromosomes for all progeny, rapidly dividing bacteria are thought to have multiple nested replication forks that operate simultaneously (Yoshikawa *et al*, 1964; Cooper & Helmstetter, 1968). Consequently, genes positioned near the chromosome origin (*oriC*) are amplified in number, whereas those near the terminus (*terC*) are not. Within the *E. coli* chromosome, the *fim* system is located near oriC (at ~610,000 bp from *oriC* or ~13% of the whole chromosome), and should therefore be present in multiple copies.

How could multiple chromosomal copies of *fim* explain the observed pattern of expression? We consider a single OFF-to-ON event, in which initially all *fimS* copies are in the OFF state, of which one turns ON. In Fig 2C, this moment could coincide with the first fluorescence increase seen in the orange lineage at about 100 min. On division, this single chromosomal ON copy is inherited by one of the daughters (red lineage), which continues GFP expression. By contrast, the second daughter (orange lineage) inherits several *fimS* copies in the OFF state, as well as GFP proteins and messenger RNA, which will eventually be diluted due to growth resulting in a slow decrease in mean fluorescence. After the replication of *fimS* and division in the red lineage, one daughter (red) decreases in brightness, whereas the other daughter (green) continues to increase, suggesting that the latter inherited both ON copies and the former only OFF copies. This grouped redistribution of *fimS* copies agrees with a nested arrangement of replication forks, in which the most recently replicated DNA remains physically linked with the formerly replicated DNA. No other lineages with decreases were observed, suggesting that the green lineage inherited only ON *fim* copies. An inheritance of two *fimS* copies at birth is in accordance with the Helmstetter–Cooper model of chromosome replication at these growth rates (Cooper & Helmstetter, 1968). This pattern has parallels with the idea of gene dosage, which has been used to denote differences in the expression level owing to different copy numbers of the corresponding gene, for example due to gene duplication.

The model described above provides two predictions. First, the pattern of GFP expression should alter for different growth rates, as the chromosome replication scheme is expected to change with growth rate. Slow-growing cells with generation times greater than 60 min are expected to have one chromosome copy at birth and two at division (Cooper & Helmstetter, 1968). This would imply that one lineage at maximum would show one expression peak, instead of the two peaks observed for fast growing cells. Second, the pattern of expression should depend on the moment of switching within the cell cycle (Fig 3). In slow-growing cells, if switching occurs after birth but before replication of the *fimS* switch, then all descendants would show an increase in monotonic fluorescence. If, conversely, switching occurs after the replication of *fimS*, only one of the two *fimS* copies is ON at division, resulting in one daughter lineage with an expression peak.

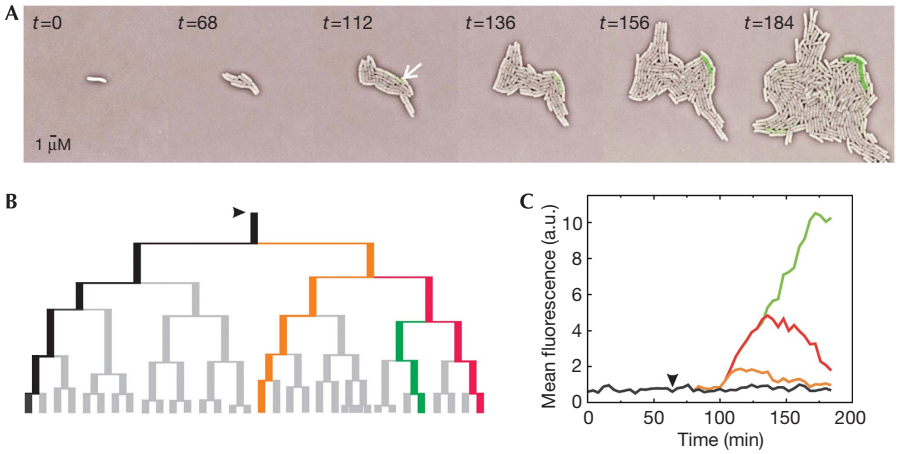

Fig 2 | ON switching in a growing microcolony. (**A**) Overlay of fluorescence and phase-contrast images showing a single OFF cell developing into a microcolony. A cell in this isogenic population starts to fluoresce spontaneously (white arrow), and develops into a brightly fluorescent sub-colony, indicating an OFF-to-ON *fimS* inversion event. A later switching event is seen to occur in the bottom left corner of the microcolony. (**B**) A section of the family tree of the microcolony in (**A**). The length of the bars represents the division time. The black arrowhead represents the start of the family tree. The fluorescence of the coloured lineages is followed in (**C**). (**C**) GFP expression history of the four lineages labelled in (**B**). Cells in the black lineage show a low mean fluorescence per unit area, which corresponds to the OFF phase. The orange and red lineages show first an increase in fluorescence, and then a decrease. The green lineage shows an increase in monotonic expression. *fim*, *fimbriae*; GFP, green fluorescent protein.

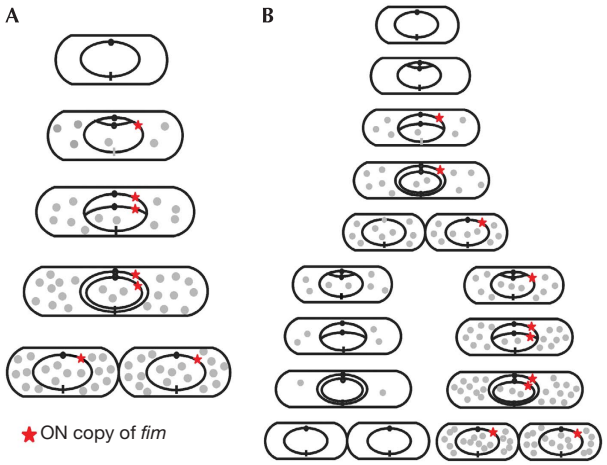

Fig 3 | Fimbrial expression and DNA replication. Model for slow-growing *Escherichia coli* cells inheriting a single chromosome (replicated in a bi-directional manner) with an OFF *fim* switch. At a certain moment, the switch spontaneously switches to the ON state (asterisk). (**A**) OFF-to-ON switching occurring before *fim* replication. Both daughter cells inherit ON copies of *fim*, resulting in two lineages with monotonically increasing fluorescence. (**B**) OFF-to-ON switching occurring after *fim* replication. One daughter cell inherits only GFP protein and messenger RNA, which are diluted by growth and thus decrease in concentration. The other daughter inherits one chromosomal *fim* system in the ON state, resulting in a monotonic increase in fluorescence. *fim*, *fimbriae*; GFP, green fluorescent protein.

Dependence on growth rate and cell age

To test the predictions outlined above, cells were grown on succinate minimal medium with a mean generation time of approximately 120 min (fraction of ON cells, 7.4%). The number of SeqA foci within one cell cycle showed a regular 0-1-2-1-0 pattern (Fig 4E,F). The appearance of a single SeqA focus indicated the start of chromosome replication (Fig 4G). The single focus then split into two separate foci, indicating a bi-directional movement of the two replication forks, which later merged again into one focus, suggesting that the forks were near termination. The disappearance of the focus indicated the complete methylation of both chromosomes and the end of replication. The delay in methylation of nascent DNA has been reported to be about 1.5 min (Lu *et al*, 1994). The moment of *fimS* replication was determined at 26% of the replication process, based on a bi-directional replication and a constant replication rate along the chromosome. The moment of *fimS* switching was determined by the intersection of the background fluorescence signal and the rising fluorescence signal, while correcting for the GFP maturation time.

In this manner, we were able to determine the number of inherited chromosomes, to pinpoint the start and end of replication (and consequently the moment of *fimS* replication), and to correlate it with *fim* switching time and the ensuing pattern of GFP expression of individual cells. The data showed that all *fimS* switching events occurring after the replication of *fimS* resulted in a single lineage with transient GFP brightness (Fig 4A,C,E). All *fimS* switching events occurring before the replication of *fimS* resulted in no lineages with transient brightness (Fig 4B,D,F). The results at slow growth verify the essential feature of our proposed model: the pattern of expression is determined by the growth rate and switching moment relative to the moment of *fimS* replication. For ON-to-OFF switching, a similar but inverse pattern of GFP expression was observed (supplementary Fig S3 online), which showed decreases in brightness followed by increases (back to the ON fluorescence level), as well as monotonic decreases in the fluorescence level.

Other details of the expression traces could also be understood within the framework of the replication model. For example, as

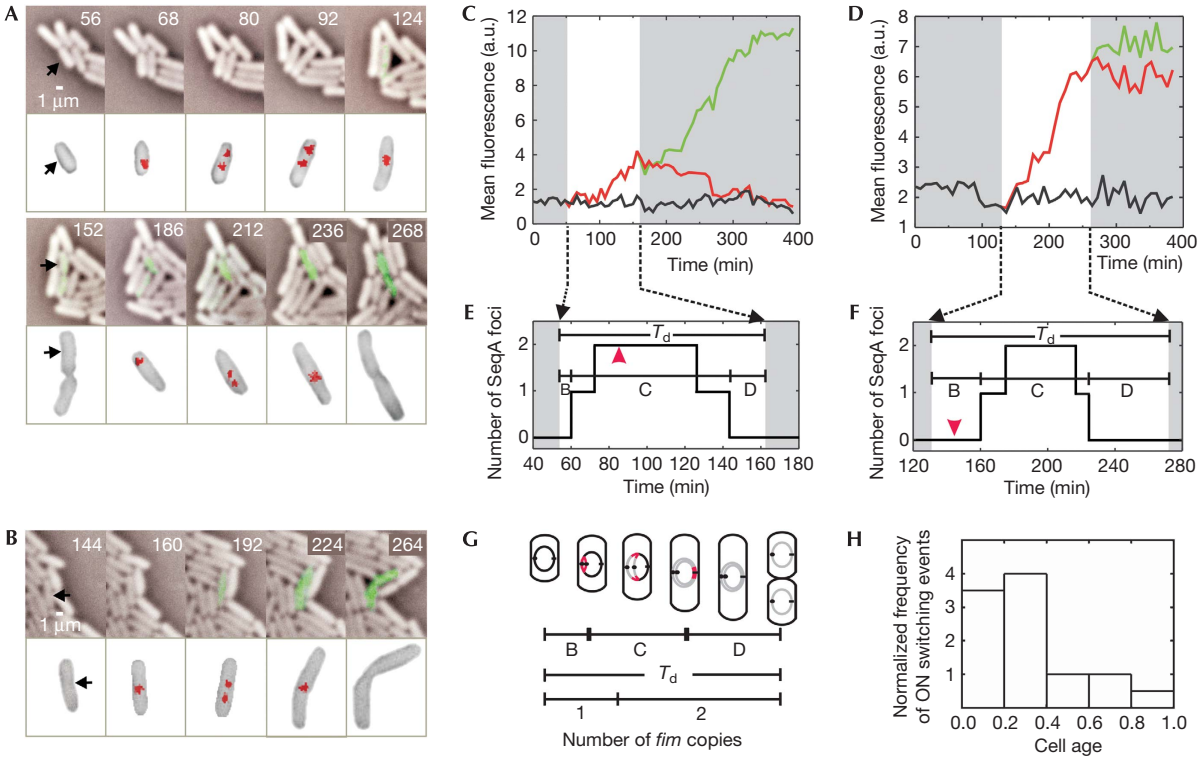

Fig 4 | ON switching events in slow-growing cells. (**A**) OFF-to-ON switch with transient expression increase. (**B**) OFF-to-ON switch with only increases in monotonic expression. (**A,B**) Top panels show phase-contrast images overlaid with GFP fluorescence signal reporting for *fim* expression. Bottom panels show phase-contrast images of *fim*-switching cells overlaid with mCherry fluorescence signal reporting for SeqA, which labels chromosome replication forks. Black arrows indicate the start of the lineage shown at the lower panel. (**C,D**) GFP expression history of three lineages, from the measurements as shown in (**A,B**), respectively. Cells in the black lineage show a low fluorescence level (mean fluorescence per unit area), which corresponds to the OFF phase. The red lineage shows the first fluorescence increase, which either subsequently decreases (**C**), or continues to increase monotonically (**D**). The green lineage corresponds to a daughter branching from the red lineage, and shows a monotonic expression increase in both (**C,D**). (**E,F**) Pattern of SeqA foci within the cell cycle. The number of SeqA foci, which label the replication forks, is plotted as a function of time from the measurements shown in (**A,B**), respectively. Red arrowheads indicate the start of GFP fluorescence. B period represents the time before the chromosome replication process (C period). D period represents the time from the end of replication to the end of cell division. T_d is the cell doubling time. (**G**) A schematic representation of the proposed chromosome replication process at slow growth, indicating the SeqA foci and *fimS* location along the chromosome. *fimS* is replicated at about 26% of the C period. (**H**) Frequency of observed OFF-to-ON switching events, normalized to the number of *fim* copies, as a function of cell-cycle phase ($n = 13$). The cell age is indicated along the x axis, from birth (0) to division (1). *fim*, *fimbriae*; GFP, green fluorescent protein.

switching before replication quickly results in two *fimS* copies in the ON state, the expression rises faster, leading to a more rapidly established steady-state expression level (corresponding to 100 min for the first case compared with ~200 min for the second case).

Higher *fim* switching rate at the beginning of cell cycle

Next, we investigated how the *fimS* switching events are distributed within the cell cycle. The null hypothesis we aimed to test is that for one given environmental condition, *fim* switching occurs randomly in time with a constant switching probability per *fimS* throughout the cell cycle. We found that replication of *fimS* occurs at an average cell age of $\varphi = 0.36 \pm 0.11$ (average value of each cell cycle's B-period plus 0.26 of its corresponding C-period, divided by its T_d). Here, cell age denotes the progression within the cell cycle—running from 0 at birth to 1 at division—and is thus distinguished from the cell-pole age that can run over multiple divisions (Stewart *et al*, 2005). Given the doubling of *fimS* copy number on replication and the null hypothesis, the expected fraction of switching events that occur before the replication of *fimS* would be $\varphi/(\varphi + 2(1-\varphi)) = 0.22$. Our data show that the measured fraction is 0.44 (24 out of 55 OFF-to-ON switching events in slow-growing cells), which is significantly higher than expected (χ^2 test, $P < 0.001$). The distribution of switching probability in time (Fig 4H) shows that the switching probability is approximately two times higher in the first part of the cell cycle.

DISCUSSION

By following single cells in real time, we have shown not only a distinctive pattern of expression of the *fim* operon on *fim* switching by DNA inversion, but also that they are explained by

accounting for the number of chromosomes and the moment of *fim* switching with respect to the moment of replication of the *fim* switch. The observed patterns are consistent with independent switching of each *fim* copy, as expected when local events at the *fim* switch, such as DNA looping or the actual inversion reaction, are the origin of the switching stochasticity. In a possible alternative pattern, a fluctuating global cellular parameter such as the concentration of recombinase is the origin of the stochasticity. In this case, switching would occur only after the recombinase concentration exceeds a critical value. Consequently, the multiple *fim* copies in one cell would then switch in a time-correlated manner. However, the data are not consistent with such a pattern. We did not observe switching events after *fim* replication in which both daughters showed monotonically increasing fluorescence. In addition, it has previously been found that when recombinase FimB is overexpressed, switching remains a rare stochastic process ($\sim 10^{-2}$ events per cell per generation; Dove & Dorman, 1996), which also suggests that processes other than fluctuations in recombinase expression level are the causes of the large switching timescales.

Unlike other phase variation control mechanisms such as slipped-strand mispairing or specific Dam methylation sites, the inversion of *fimS* is not expected to require DNA replication as an essential step in the switching process (Low *et al*, 2001; Davidsen & Tonjum, 2006). However, a crucial finding of this study is that OFF-to-ON *fim* switching occurs preferentially at the beginning of the cell cycle, before replication of the *fim* genes. What might be the cause of this observed switching bias? The known molecular mechanisms underlying *fimS* switching do not provide a convincing answer, and we might only speculate on possible causes. One might consider variations in the level of recombinase *fimB* (required for OFF-to-ON switching). The expression of *fimB* is diminished considerably in a *dam* mutant (supplementary Fig S4 online), and the generation of unmethylated—or hemimethylated—DNA in the vicinity of *fimB* following passage of the replication fork might temporarily reduce *fimB* expression. Although this pattern would suggest a more active and regulated link between cell cycle and gene regulation, at present we do not understand how Dam affects *fimB* expression. However, we have ruled out the possibility that this is due to changes in methylation protection associated with the interaction of activator proteins, NanR and NagC, with their operator sites upstream from *fimB* (Sohanpal *et al*, 2004; data not shown).

Alternatively, the observed bias for switching at the beginning of the cell cycle might be caused by changes in DNA topology during replication. The site-specific recombination process that is at the basis of *fimS* switching has been shown to be sensitive to changes in DNA topology (Dove & Dorman, 1994). During DNA replication, the separation of complementary DNA strands by helicase results in a relaxed (or possibly positive supercoiled) region in front of the replication fork, and a negative supercoiled region behind it (Kornberg & Baker, 1992). The *fim* switching rates might thus be modulated by these changes in supercoiling density or, instead, by the disruption of the recombinase synapse with the looped *fimS* by the passing replication fork. A possible advantage of switching early in the cell cycle might be that it reduces the number of transiently expressing, ambiguous lineages, thus limiting the wastage of resources on unused *fim* protein and RNA.

By investigating the behaviour of single cells, this study has shown a correlation between the cell-cycle dynamics and *fim* switching behaviour. It shows that multiple genetic *fim* systems per cell ought to be taken into account in mechanistic descriptions of *fim* switching. The observed modulation of the *fim* switching rate with the cell cycle challenges the common idea that *fim* switching is a straightforward Poissonian process, in which the switching probability is constant in time for fixed external conditions. The methodologies presented here can be applied to explore further the boundary between randomness and correlations in phase variation, as well as to reveal the dynamics of other phase variation systems at the level of discrete events.

METHODS

Strains and plasmids. Derivatives of *E. coli* K-12, strain ASC129 (MG1655 *fimAΩGFPmut2*) and strain ASC215, which is ASC129 bearing pASC215 (Amp^R, pBR322 ori; *rop*$^-$, *mcherry-seqA*), were used for experiments with and without SeqA foci. For microscopy experiments, EZ Defined Rich medium and MOPS medium (Teknova, Hollister, CA, USA) supplemented with 0.4% sodium succinate (Sigma Aldrich, St Louis, MO, USA) were used. Ampicillin 100 μg/ml (Sigma Aldrich) was added to the medium when appropriate.

Microscopy sample preparation. A fresh overnight culture was diluted with pre-warmed medium (such that the optical density of the culture would never exceed 0.02) and grown for a further 5 h to be in the exponential phase.

Two microscope slides, one with a hole of size 18 mm × 55 mm, were attached to each other by using silicon grease (Dow Corning, Midland, MI, USA) and heated to 80 °C. Next, 10 × concentrated medium was mixed with hot 2% agaroseMP solution to its final concentration (total volume is 1 ml) and spread horizontally across the centre of the cavity of the microscope slides. Immediately afterwards, the slides were transferred to a cool flat surface, and covered with a 24 mm × 60 mm silanized coverslip (a normal coverslip dipped into RepelSilane and then left to dry). The coverslip was placed directly on top of the liquid agar so that its surface would become flat on cooling (at room temperature).

Once the agar had solidified, the slides were moved to the microscope's 37 °C incubation chamber (to avoid any cold shock to the cells when they were deposited on the agar surface). Inside the chamber, the coverslip was removed. The now flat-surfaced agar was trimmed into a thin strip of about 2 mm × 55 mm across the centre of the cavity. The rest of the cavity acted as an air reservoir for cell growth. By using a pre-warmed pipette tip, 2–3 tiny droplets of the culture were dropped on the agar strip. Then, the sample was closed immediately with a normal pre-warmed coverslip and sealed with silicon grease. The cells spread evenly and became immobile. Over time, each single cell grew into microcolonies.

Microscopy and data analysis. Imaging was performed with a Nikon Eclipse TE2000 inverted microscope. Cell fluorescence quantification and lineage tree construction were performed with Schnitzcell, a program written in MATLAB, kindly provided by M. Elowitz. A further description of Materials and methods is provided in the supplementary information online.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org)

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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