Phase variation of type 1 fimbriae: a single cell investigation
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To observe *fim* switching events at the single cell level, an allelic exchange method was used to place GFPmut2 under the control of the native *fimS*. In addition, the SeqA protein was labeled by fusing it with fluorescent protein mCherry to follow the progress of the replication process. The states of *fimS* over time were monitored by growing single cells into microcolonies on various agar media and subsequently by performing automatic timelapse microscopy. Imaging was carried out using both phase contrast and fluorescence microscopy. Movies were then analyzed with segmentation software to extract various cell parameters and to construct family trees.
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2.1 Genetic Manipulation

All genetic manipulation was performed according to general microbiology protocols [103]. A more extended explanation for GFP insertion into the chromosome by means of allelic exchange is explained below.

2.1.1 Allelic Exchange

To differentiate between ON and OFF states of fimS, a method called allelic exchange [12] was used to insert the fast folding GFPmut2 into the HpaI site of chromosomal fimA under the control of the native E.coli K-12 fimS. The main steps involved were two homologous recombinations, one of plasmid integration into the chromosome of an intermediate strain, and the other of plasmid excision and curing (fig. 2.1).

In this method, an intermediate strain derived from wild type E.coli MG1655, in which the destined locus was replaced with a sacB-kan cassette (strain Δ3’fimE-5’fimA ΔSacB-Kan8), was transformed with a temperature-sensitive plasmid containing homologous fragments of the destined locus (fimE–fimD) and GFPmut2 downstream of fimS. The plasmid was also carrying a chloramphenicol resistance gene. By growing the transformants at the non-permissive temperature for plasmid replication (42°C) with chloramphenicol, the plasmid was selected for integration into the chromosome.

Once integrated, a second recombination for plasmid excision was selected. This second recombination could result in either a successful allelic exchange or an abortive one yielding the original strain. The excised plasmid was then cured from both strains by growing the cells in medium without antibiotics at 42°C. The cells that had successful allelic exchange were then selected by growing the cells at 28°C on medium with sucrose (without sodium chloride). Expression of sacB under these conditions is lethal for the original, untransformed strain. The resulting cells were then plated and re-checked for newly acquired susceptibility for chloramphenicol and kanamycin.

2.1.2 Construction of a mCherry-SeqA fusion

To follow progress of the replication fork in vivo, we fluorescently labeled the SeqA protein with mCherry (a generous gift from R. Tsien). We cloned the SeqA gene from pGAP40 (a generous gift from T. Brendler) into the multiple cloning sites in pSAV047 (kindly provided by S. Alexeeva), in between BsrGI and EcoRI.
Figure 2.1. Allelic exchange (adapted from [12]).
The grey colored block represents the GFPmut2 gene. The crosses represent homologous recombinations. The resulting strain is ASC 129.
sites, to place it under the control of the IPTG inducible promoter and fuse it with mCherry (fig. 2.2 B). Five amino acids were acting as a linker (Glu-Phe-Asn-Asn-Asn). The resulting plasmid (pASC215) was then transformed into strain ASC129 resulting in strain ASC215. Upon imaging, we were able to observe our red fluorescently labeled SeqA as discrete foci.

In order to investigate the possible adverse effects of SeqA-mCherry expression, we characterized its effect on growth rate and cell length. High overexpression of SeqA had previously been reported to result in filamentous cells [5], consistent with its role as a negative regulator for DNA replication initiation. The fusion expression was therefore kept at a minimal level, by omitting the inducer IPTG. We note that the fusion was expressed in addition to the endogenous unlabelled SeqA, which was expressed at wild-type levels in both cells. Expression leakage from the promoter appeared sufficient to produce visible SeqA foci. The interdivision time of the rapidly growing cells appeared similar with (Luria Bertani medium, Td=29.5±1 min) and without (Td=29.9±0.8 min) expression of the SeqA-mCherry fusion (fig. 2.3). Histograms of cell lengths were similar for both type of cells, as determined by a Kolmogorov-Smirnov test (D=0.068, P=0.39) (fig.2.4). For both strains, the length distributions were skewed as expected for exponentially growing culture, which is composed more of younger cells than older ones [69]. Filamentous cells were only rarely observed (less than 1 in 100). These results indicate no significant adverse effect of SeqA labeling on cell growth.

2.1.3 Strains and plasmids
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The bacterial strains used in this study are derivatives of *E. coli* K-12. An intermediate strain AAEC112 (Δ3’fimE-5’fimA ΩSacB-KanΔ), plasmid pIB315 (CmR, temperature sensitive) containing fimE-fimD genes and *E. coli* strain MG1655 were generous gifts from I. Blomfield [11]. GFPmut2 [30] as a reporter for the Fim switch was cloned into the HpaI site of the fimA gene within pIB315. By means of allelic exchange (as described in section 2.1.1) the fimA gene with

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**Figure 2.3** Growth rate determinations
(A) Optical density at 600nm of control cells (ASC129, carrying no plasmid) and cells carrying expressing SeqA-mCherry (ASC215) in LB Lennox medium at 37°C.
(B) Optical density data on a log scale (A) fitted with linear regression lines. The interdivision time for ASC129 is 29.9 minutes and ASC215 is 29.5 minutes. The symbols are the same as image A.

**Figure 2.4** Histogram of cell lengths.
Length data of control cells expressing no SeqA-mCherry (ASC129, left panel) and cells expressing low amount of SeqA-mCherry protein (ASC215, right panel) grown exponentially in MOPS succinate medium. The data show similar cell length distribution. A Kolmogorov-Smirnov test indicated that there was no significant difference between the two data sets (D=0.068, P=0.39).
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*gfpmut2* was inserted within the original chromosomal wild-type *fimA* site, resulting in strain ASC129 (*fimA::gfpmut2*).

Into vector pSAV047 (Amp\(^R\), pBR322 ori; rop\(-\); a generous gift from S.Alexeeva), *seqA* gene from pGAP40 (kindly provided by T.Brendler [19]) was fused to the N-terminus of mCherry fluorescent protein under the control of a repressed trc-down promoter resulting in plasmid pASC215. Strain ASC215 is ASC129 transformed with pASC215. Strain ASC181 is ASC129 transformed with pGAP40.

### 2.1.4 Materials

The medium used for cloning and allelic exchange included LB Lennox broth (Difco), LB Lennox agar (Difco) and sucrose agar. Sucrose agar is LB agar (5 g of sodium chloride, 5 g of yeast extract and 10 g of tryptone per liter without sodium chloride and supplemented with 6% sucrose (Sigma)). Whenever necessary chloramphenicol or kanamycin, or ampicillin was supplemented to the medium to a final concentration of 30, 25 and 100µg/mL. Cells were grown at 28, 37 and 42\(^\circ\)C accordingly.

Restriction enzymes and polymerase were purchased from New England Biolabs, and DNA ladders were purchased from Fermentas and Promega.

### 2.2 Microscopy

#### 2.2.1 Sample preparation

We modified a method previously developed by E. Stewart [113] to grow cells on agar slab. Before imaging, cells were grown overnight in the appropriate medium with the addition of antibiotics when necessary. The next day, the overnight culture was diluted with pre-warmed medium and grown for another 4 hours to be in the exponential phase (OD between 0.05 and 0.1). When a rich medium was used, the dilution was done twice, each 1000X and grown for 2 hours. When a minimum medium was used, the dilution was done once and such that the OD of the culture did not exceed 0.1 in 4 hours of growth.

Two microscope slides, one of which had a hole of size 18 mm x 55 mm, were attached to each other by use of silicon grease (Dow Corning) and heated to 80\(^\circ\)C. Next, 10X concentrated medium of interest was mixed with hot agaroseMP solution to its final concentration (total volume is 1 mL) and spread horizontally across the center of the microscope slides cavity (fig. 2.5). Immediately afterwards, the microscope slides were transferred to a cool flat surface, and covered with a 24
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Figure 2.5 Sample holder for timelapse microscopy. The agar strip is shown in grey.

mm x 60 mm silanized coverslip (a silanized coverslip was made by dipping normal coverlips into RepelSilane, and then left to dry at room temperature) by dropping it right on top of the liquid agar so that the agar surface would become flat upon cooling. The agar solidified at room temperature within 2-3 minutes.

When the color of the agar had become less transparent, the slides were incubated in 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 incubation chamber, the silanized coverslip was carefully removed, revealing a thin, flat agar slab. Excess agar slab was trimmed to leave a thin strip of agar of dimension 2.5 mm x 55 mm across the center of the cavity. The rest of the cavity served as an air reservoir for cell growth. Using a pre-warmed pipette tip, 2-3 tiny droplets of exponential liquid culture were dropped on top of the agar strip. The sample was then closed immediately by dropping a pre-warmed normal coverslip on top of the cavity and the gap between the coverslip and the microscope slide was sealed with silicon grease. The cells could then spread evenly across the agar strip and become immobile. Over time, each single cell grew into microcolonies.

2.2.2 Microscopy

Imaging was performed with a Nikon Eclipse TE2000 inverted microscope equipped with a 37°C incubation chamber, automated stage (Marszhauser) and CCD camera (Coolsnap, Roper Scientific). Phase contrast and green fluorescence images were taken automatically by imaging software Metamorph (RoperScientific) with a 100X magnification objective lens. For SeqA experiments, an additional 1.5X magnification was used. Light from a xenon lamp (Lambda LS) was filtered by a GFP filter (Nikon) and an HCRed filter (Chroma) for GFP and mCherry detection. mCherry images were taken every timestep for SeqA.
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experiments and every other timestep for fim experiments. There were two kinds of time steps used, 4 minutes (chapter 3 and 4) and 10 minutes (chapter 5). Depending on the growth medium and the experimental purpose, typical experimental times ranged from 4 to 16 hours.

2.2.3 Materials

For microscopy experiments, cells were grown in EZ Defined Rich medium (Teknova) or MOPS medium (Teknova) supplemented with 0.4% sodium succinate (Sigma) or 0.4% glucose (Teknova) and 0.8 mM L-leucine (Merck). AgaroseMP was purchased from Roche. Whenever SeqA was visualized, 100µg/mL ampicillin (Sigma Aldrich) was added to the medium. For cell immobilization and microcolony growth, MOPS agar was made by mixing 10X concentrated medium (Teknova) with hot 2% agaroseMP solution to a final volume of 1 mL. Cells were grown at 37ºC. All liquid cultures were grown aerobically. To make silanized coverslips, Repel Silane was purchased from Amersham Biosciences.

2.2.4 Data analysis

Simple image manipulation and viewing was done with Metamorph, ImageJ and IrfanView. Cell fluorescence quantification, cell parameters extraction and lineage tree construction was performed with Schnitzcell, software written in MATLAB kindly provided by M. Elowitz [100]. First, cells from the phase contrast images were segmented automatically, in which the border of each cell was determined and dividing cells were separated accordingly (fig. 2.6). The segmentation result was then manually checked. Next, frame-by-frame, each cell was tracked through the following frames to establish familial connections. The resulting cell lineage connections were then manually checked. Finally, the SeqA foci positions were indicated manually when available and then all parameters for each cell were extracted from both the phase contrast and fluorescence images. Those included cell size, age, background corrected mean fluorescence (cell’s fluorescence divided by its pixels area), relatives, etc. Family trees and growth profiles of each cell could then be plotted. An example of an analyzed image of a growing ASC129 microcolony on LB agar is shown in figure 2.5. The mean interdivision time is about 29 minutes, in accordance with our previous growth measurement in liquid culture for the same strain.

Cell growth on the agar slab typically proceeded exponentially for a significant amount of time, and then it decreased over time until an abrupt decrease of growth occurred. The observed brightness of GFP expressing cells also decreased moderately at the end of each experiment. We excluded these data with decreased
growth rate obtained late in the experiments. The time period a certain microcolony stayed in the exponential phase varied from microcolony to microcolony and also varied with colony size and type of medium. Presumably, this was partly due to microenvironment nutrient or oxygen exhaustion and partly due to phototoxicity. Exposure times were chosen to be as short as possible to minimize cell damage, while fluorescence images were binned 2x2 to improve the signal-to-noise ratio and enhance visibility. Generally, a rich medium sustained longer exponential growth as well as a smaller sized microcolony.

Good quality images are beneficial for better success in automatic cell segmentation. The dryness of the agar surface played an important role in image quality. In some experiments where the agar surface was more slippery the cells were able to spread better, thus preventing multiple layered cells to occur earlier.

Upon growth of cells with a labeled fim switch (ASC129) in LB medium, we observed a sudden increase in background fluorescence in large microcolonies. Interestingly, stationary cells from an overnight LB culture were also very bright.

Figure 2.6 Data analysis
(A) A phase contrast image of a microcolony growing on LB agar slab (left), image where each cell is an individual segment (middle) and its corresponding fluorescence image (right).
(B) A part of family tree of the microcolony in A.
(C) Cell length versus time for cells in figure A showing exponential growth.
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We propose that autofluorescence is a sign of a cell entering a stationary phase. This observation is only observed in LB medium, which has its own independent autofluorescence properties.

2.3 Determination of GFPmut2 maturation time

To be able to optimally pinpoint the moment of switching, a fast folding version of GFP is used to label the state of fimS. GFPmut2 is a triple mutant (S65A, V68L, S72A) of the wild type Aequorea Victoria GFP, and is about 100 times brighter [30]. We investigated its maturation time in our strain MG1655, by performing IPTG induction experiment with a plasmid containing the GFPmut2 under the control of a LacI repressor (pTHV048, a generous gift from Tanneke den Blaauwen, UVA).

Cells containing pTHV038 plasmid (ASC401) were grown overnight in MOPS medium supplemented with 0.4% succinate at 37°C and then diluted 400 times the next morning to promote exponential growth. After about 4 hours of growth, IPTG was added to the medium (end concentration of 1 mM) and immediately spread on agar slab containing the same medium and IPTG. Microscopy images of cells were taken over time (exposure time 100ms for both fluorescence and phase contrast).
As a measure of background fluorescence level, non-induced control cells from the same culture were also imaged.

During the time necessary for microscope and sample preparations directly prior to imaging, the induced cells became much brighter than the control cells (fig. 2.7). On average, we found that increase in brightness started 5.7 minutes after IPTG was added, indicating a fast GFP maturation time.