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Probing chromosome dynamics in *Bacillus subtilis*

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Key words

Microscopy, fluorescent protein, chromosome, DNA replication, DNA segregation, prokaryote

Summary

Research over the last two decades has revealed that bacterial genomes are, in fact, highly organized. The goal of future research is to understand the molecular mechanisms underlying bacterial chromosome architecture and dynamics during the cell cycle. Here we discuss techniques that can be used with live cells to analyse chromosome structure and segregation in the Gram-positive model organism *Bacillus subtilis*.

1. Introduction

Research into genome organization and dynamics was first established in eukaryotic cells where the condensation and segregation of chromosomes could be readily observed using microscopy techniques. Initial approaches with bacterial cells engendered the belief that chromosomes were compacted but largely unstructured, as they did not display the obvious hallmarks of cell cycle condensation and segregation detected in more complex organisms. However, advances in live cell fluorescence microscopy have revealed that, in fact, bacterial chromosomes are highly organized. Furthermore, recent evidence strongly

suggests that bacterial genomes are actively segregated and positioned within cells to facilitate accurate genetic inheritance. Bacterial cells, with their relatively small genomes and rapid generation times, provide excellent model systems with which to understand fundamental principles of DNA organization and dynamics.

In most bacteria the critical processes of DNA replication and segregation are coupled during the cell cycle so that following initiation of DNA synthesis the two daughter origin regions are rapidly segregated away from each other [1, 2]. Newly synthesized DNA molecules are thought to be compacted and organized into various domain structures to facilitate segregation [3, 4]. Although several factors have been identified that are important for bacterial chromosome organization and segregation (e.g. – condensin, ParABS), deletion of such systems often result in either mild or conditional phenotypes, presumably because the overlapping nature of these processes allow the remaining systems to compensate for each other [1, 5, 6, 7, 8]. Thus, the aims of future studies will be to identify unknown bacterial chromosome organization and segregation factors and to determine how these systems synergize to ensure accurate genome inheritance. In order to achieve these goals researchers will need tools for the characterization of DNA localization and movement within single cells. Below we will describe methods to analyze DNA dynamics in the bacterium *Bacillus subtilis*, a model organism that is particularly advantageous because its chromosome can be easily manipulated using genetic engineering methods and because it has the ability to differentiate into a dormant spore which requires specific developmental regulation of its chromosome copy number and localization (Figure 1A).

The tool used most frequently to study DNA localization in living cells is the green fluorescent protein (GFP), along with its derivatives that have distinct excitation and emission properties to allow multicolour imaging (cyan = CFP, yellow = YFP, red = RFP). These reporters can be genetically fused to a protein of interest in order to assess its position within the cell. No fixation or permeabilization is required for this approach, thus the signal detected can be observed during live cell growth and development. Although it is

possible to use fluorescent intercalating dyes to stain DNA in live cells (Figure 2A), we have found there are several drawbacks: (i) the most commonly used stain DAPI is excited using short wavelengths of light which can damage DNA upon prolonged exposure; (ii) alternative stains excited with longer wavelengths are available but more expensive; (iii) most significantly we have observed that staining is often heterogeneous within a population of cells, impeding quantitative analysis.

To easily visualize the entire bacterial nucleoid, FPs can be fused to the highly abundant nucleoid associated histone-like protein (HBSu) (Figure 2A) [9]. Coupling this with lipophilic membrane dyes (e.g. – FM5-95, Nile red) allow bulk chromosome localization and dynamics to be studied in individual cells.

For more specific labelling of the *B. subtilis* chromosome at the DNA replication origin and terminus regions, the endogenous Spo0J/ParB and replication terminator protein (RTP) proteins can be tagged with FPs, respectively. These DNA binding proteins localize to multiple recognition motifs within these regions and generate a punctate fluorescent signal within cells (Figure 3A and 3B) [10, 11, 12]. Furthermore, by measuring the position of the FP tagged origins / terminus or counting the number of origins within each cell; one could quickly determine the location of these tagged regions and the rate of DNA replication initiation respectively (Figure 3C-E).

In addition to visualizing the replication origin and terminus regions using endogenous proteins, any genetic locus of interest can be investigated by integrating arrays of operator sites that are recognized by fluorescently labelled DNA binding proteins. The two commonly used systems in *B. subtilis* are based on the repressor proteins TetR (binding *tetO*) and LacI (binding *lacO*) (Figure 4) [13, 14]. We wish to highlight the systems constructed by the Sherratt Lab in which the repeated operator sites within the arrays are separated by randomized 10 base pair sequences to reduce recombination and the tetramerization domains of the repressors have been removed to prevent DNA looping [15].

Moreover, by fusing DNA binding proteins to different FPs with distinct spectral properties (e.g. – GFP/RFP or CFP/YFP) allowed multiple chromosomal features to be visualized simultaneously (Figure 5) [6, 15].

At this point it is important to note that each of the DNA labelling strategies outlined above have potential shortcomings. First, FP-tags on endogenous proteins will always alter activity to a degree, so although cell growth and chromosome organization/segregation may appear normal in an otherwise wild-type strain background, combining multiple FP-tagged proteins in one strain or combining FP-tagged proteins with other mutants may result in synthetic phenotypes [16]. Second, the repressor/operator arrays have the capacity to block replisome progression [17]. This problem can be mitigated by using arrays containing a small number of binding sites or lowering the expression level of the FP-tagged repressor, although the trade-off is that the fluorescent signal will also be diminished. Since no single labelling technique is without caveats, it is advisable to utilize multiple approaches where possible.

To study chromosome dynamics it can be useful to synchronize DNA replication and segregation events. There are two readily available approaches to synchronize *B. subtilis* cells. First, the DNA replication cycle can be arrested at the initiation stage using temperature sensitive alleles of initiation proteins (e.g. – the helicase loader gene *dnaB134^{ts}*) [18]. After allowing ongoing rounds of DNA synthesis and segregation to be completed, dropping the culture to the permissive temperature produces a synchronous round of chromosome replication and segregation (Figure 6B) [19].

Second, a liquid culture of *B. subtilis* can be resuspended in medium that stimulates the sporulation developmental pathway (Figure 1B). During sporulation cells are arrested as diploids and the two chromosomes adopt an extended conformation (the axial filament) with the chromosome origin region anchored to the cell pole by RacA [20, 21, 22, 23]. Asymmetric cell division will then trap ~25% of the chromosome into the forespore. As

sporulation proceeds the remaining chromosome is pumped into the forespore by the translocase SpoIIIE before the spore is engulfed by the mother cell to complete maturation [23, 24, 25]. Thus, synchronizing sporulation initiation offers the opportunity to study unique aspects of chromosome organization and segregation (Figure 7B).

Here we describe methodologies in live cell imaging to study chromosome dynamics in *B. subtilis*, with a particular focus on the localization and separation of the DNA replication origin.

2. Materials

All solutions are prepared using ultrapure water and analytical grade reagents. All solutions and reagents are prepared and stored at room temperature unless specifically stated. All waste disposals were carried out using approved disposal procedures. All components are sterilized either by autoclaving at 15 psi for 30 min or for heat labile solutions by filtering (0.45 μm).

2.1 Origin separation, counting and positioning assay

1. Overnight culture Spizizen minimal medium (SMM) based media: 0.2% NH_4SO_4 , 1.4% K_2PO_4 , 0.6% KH_2PO_4 , 0.1% sodium citrate dehydrate, 0.02% MgSO_4 , 0.01 mg/ml Fe- NH_4 -citrate, 6 mM MgSO_4 , 0.1 mM CaCl_2 , 0.13 mM MnSO_4 , 1 μM ZnCl_2 , 2 μM thiamine, 0.1% glutamate, 0.02 mg/ml tryptophan, 200 $\mu\text{g/ml}$ casein hydrolysate, 2.0% succinate and inducers as needed (1 $\mu\text{g/ml}$ tetracycline, 500 ng/ml anhydrotetracycline or 1 mM IPTG).
2. SMM based media supplemented with the following carbon source: 2% succinate for slow growth, 2% glucose for medium growth or 2% glucose +

200 µg/ml casein hydrolysate for fast growth. Note that no inducer should be added into the fresh media.

3. Test-tubes or 125 ml conical flasks.
4. Shaking incubator with temperature setting.

2.2 Sporulation assay

1. Casein hydrosylate media (CHM): 1% casein hydrosylate, 0.46% L-glutamic acid sodium salt, 0.13% L-alanine, 0.14% L-asparagine, 0.14% KH_2PO_4 , 0.05% NH_4Cl , 0.01% Na_2SO_4 , 0.01% NH_4NO_3 , 0.98 mg $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and adjusted to pH 7.0 with NaOH or HCl. After that add 0.1 mM CaCl_2 , 0.4 mM MgSO_4 , 0.13 mM MnSO_4 , 0.02 mg/ml tryptophan and inducers as needed (1 µg/ml tetracycline, 500 ng/ml anhydrotetracycline or 1 mM IPTG).
2. Sporulation salts solution (Solution A): 4 mM FeCl_3 , 40 mM MgSO_4 and 100 mM MgCl_2 .
3. Sporulation salts solution (Solution B): 1 M NH_4Cl , 75 mM Na_2SO_4 , 50 mM KH_2PO_4 and 120 mM NH_4NO_3 adjusted to pH 7.0 with HCl or NaOH. Sterile filter both solutions and store at 4°C.
4. Sporulation salts solution (Solution A+B): Mix 1 ml of solution A with 10 ml of solution B and top up with water to make 1 L. Autoclave the sporulation salts solution.
5. Sporulation media (SM): Sporulation salts (solution A + B) supplemented with 0.2% glutamate, 1 mM CaCl_2 , 40 mM MgSO_4 and 0.02 mg/ml tryptophan.
6. Test-tubes or 125 ml conical flasks.
7. Shaking incubator with temperature setting.

2.3 General microscopy

1. Prepare ~1.5% agarose gel in the media that was used for the cell culture.
(See Note 1 and 2)
2. Prepare 200 µg/ml of stock FM5-95 membrane dye and mix it with the molten agarose to a final concentration of 0.5 µg/ml. (See Note 3)
3. If desired prepare 10 µg/ml of stock DAPI stain and mix it with cells to stain the DNA to a final concentration of 0.6 µg/ml.
4. Multi-spot microscope slide (Figure 8A) or a Gene Frame (Life Technologies, cat no: AB-0578; 17 mm x 28 mm) mounted on a clear microscope slide (Figure 8B).
5. Glass coverslip
6. Surgical scalpel blades
7. Prepare 70% ethanol
8. Epifluorescence microscope with appropriate filter sets.
9. METAMORPH software (version V.6.2r6) for image capture.
10. ImageJ software (1.49n) can be downloaded at the following URL
<http://imagej.nih.gov/ij/>
11. ImageJ plugin for cell counter can be downloaded at the following URL
<http://imagej.nih.gov/ij/plugins/cell-counter.html>
12. ImageJ plugin for ObjectJ can be downloaded at the following URL
<https://sils.fnwi.uva.nl/bcb/objectj/>

3. Methods

3.1 Growth conditions for origin separation, counting and positioning assay

1. To visualize cells during exponential growth, 2 ml of starter cultures were grown overnight at either 30°C or 37°C in SMM based media. (See Note 4 to 8)
2. The next day, 1 ml cultures were washed twice with fresh SMM based media to remove any remaining inducer molecules (this step can be skipped if no inducer was used for the overnight culture).
3. Dilute culture 1:100 into fresh SMM based media supplemented with 2.0% succinate, 2.0% glucose, or 2.0% glucose + 200 µg/ml casein hydrolysate. (See Note 9 to 11)
4. Allow cultures to achieve at least three mass doublings (A_{600} 0.3-0.5) before observation by microscopy. (See Method 3.4 and 3.5)

3.2 Synchronization of DNA replication

1. To visualize a synchronized DNA replication and segregation cycle, 5 ml of starter culture was grown overnight at the permissible temperature of 30°C in CHM with inducer as needed. (See Note 4 to 8) (Figure 6A; flow chart)
2. The next day, 1 ml cultures were washed twice with fresh CHM to remove any remaining inducer molecule (this step can be skipped if no inducer was used for the overnight) before being diluted 1:100 into fresh CHM.
3. At an A_{600} of 0.1, 5 ml of fresh CHM that have been prewarmed to 70°C was added into the culture. This will rapidly equilibrate the culture temperature to the non-permissive temperature (~50°C) and allowed further growth at this temperature for 90 min to allow ongoing rounds of replication to complete.

4. To initiate DNA replication, the culture was shifted back to the permissible temperature (30°C).
5. Observe DNA replication by microscopy. (See Method 3.4 and 3.5)

3.3 Sporulation assay

1. To visualize cells during sporulation, 2 ml of starter cultures were grown overnight at 30°C in CHM media. (See Note 4 and 8) (Figure 7A; flow chart)
2. The next day the overnight cultures were diluted into 2 ml of fresh CHM media to achieve a starting A_{600} of 0.1, and then incubated at 37°C until they reached an A_{600} of 1.0.
3. The cultures were then centrifuged at 16000 x g for 10 min and the cell pellet was resuspended in 2 ml of fresh SM.
4. Transfer 2 ml of the resuspended culture back into the same test tube to induce sporulation according to the resuspension method of [26] and modified by [27] for at least 120 min before observation by microscopy. (See Method 3.4 and 3.5)

3.4 Preparation of multi-spot microscope slide

1. Clean the multi-spot microscope slide with 70% ethanol and ensure that it is dry and free of dust.
1. Pipette 750 µl of molten agarose onto the middle of the slide. Ensure that there is no air bubble in the agarose as this might interfere with how the agarose pad will harden. (See Note 12)
2. Quickly place a standard microscope slide on top of the molten agarose and squeeze any excess agarose out by gently pressing down on the slide. (See Note 13)

3. Air dry to allow the media to absorb into the pad. This normally takes about 5 min. (See Note 14)
4. Carefully remove the standard microscope slide on top of the multi-spot microscope slide to reveal the agarose.
5. Pipette ~1 μ l of cell culture onto the agarose pad. (See Note 15)
6. Air dry to allow the media to absorb into the pad. This normally takes about 5 min. (See Note 14)
7. Place a clean coverslip on top of the cells. (See Note 16)
8. The microscope sample is now ready for imaging (Table 1).

3.5 Preparation of microscope slide (Gene frame)

1. Clean the microscope slide with 70% ethanol and ensure that it is dry and free of dust.
2. Carefully remove the plastic cover from the gene frame (i.e. - the side with the solid plastic covering the whole gene frame) which will reveal the sticky pad.
3. Attach the gene frame with the sticky pad onto the middle of a standard microscope slide. Use the edge of a pen and move along the frame ensuring that there are no air bubbles between the frame and the slide (note that the upper side of the sticky gene frame will remain covered with a plastic mask).
4. Pipette 500 μ l of molten agarose onto the middle of the gene frame. Ensure that the agarose exceeds the volume of the gene frame and that there are no air bubbles in the agarose. (See Note 12)
5. Quickly place a standard microscope slide on top of the molten agarose. Squeeze excess agarose out by gently pressing down on the slide. (See Note 13)
6. To harden the agarose, place the gene frame horizontally into a petri dish that contains a small ball of wet tissue (to prevent desiccation). Cover the

- petri dish and store it in the refrigerator at 4°C for at least 30 min. (See Note 14)
7. To prepare the slides for microscopy, warm the microscope slides by shifting the petri dishes into the desired temperature (depending on experimental requirements) for at least 30 min. (See Note 12)
 8. Carefully remove the clear microscope slide on top of the gene frame to reveal the agarose. (See Note 14)
 9. To provide a source of oxygen create air pockets using a scalpel to cut out agar strips (Figure 8B).
 10. Pipette ~1 µl of cell culture onto the agarose pad. (See Note 15)
 11. Air dry to allow the media to absorb into the pad. This normally takes about 5 min. (See Note 14)
 12. Remove the mask from the gene frame and firmly attach a clean cover slip, ensuring no gaps are present. (See Note 16)
 13. The microscope sample is now ready for imaging (Table 1).
 14. For time lapse microscopy incubate the prepared microscope slide at the desired temperature for at least 30 min.
 15. The microscopy chambers should also be prewarmed to the desired temperature for at least 1 hr before commencing the time lapse experiment.

3.6 Measurement of focus position within cells with two foci (replication origin and terminus positioning)

1. Cells are grown as described in (Method 3.1).
2. ImageJ software with ObjectJ plugin was utilized for determining the focus position within the cell (Figure 3C).

3. For each cell, the focus closest to an arbitrarily selected pole was designated “near”, and the other focus was designated “far”. Three measurements were made: (i) the distance from the pole to the center of the proximal focus (near); (ii) the distance from the same pole to the center of the distal focus (far); and (iii) the distance between the two poles (cell length).
4. To determine the position of the near focus within the cell, the measured distance was divided by the cell length and multiplied by 100 to give the focus position as a percentage of cell length. To determine the position of the far focus within the cell, the measured distance was subtracted from the cell length before being divided by the cell length, then being multiplied by 100 to give the focus position as a percentage of cell length. These numbers were averaged for all cells in a sample. The 95% confidence intervals for the mean were calculated.
5. Interfocal distance was calculated by subtracting the position of the near focus from the far focus, then dividing by cell length and multiplying by 100 to give the interfocal distance as a percentage of cell length. These numbers were averaged for all cells in a sample. The 95% confidence intervals for the mean were calculated.

3.7 Counting the number of different class of cells (Origin separation) and the number of origin per cell (Origin counting)

1. Cells are grown as described in (Method 3.1).
2. ImageJ software with cell counter plugin was utilized to determine the number of different type of cells or the number of origins per cell.

4. Notes

1. Minimise the use of complex media such as PAB, LB and CH, which produce high autofluorescence. While minimal media has a lower autofluorescence level.
2. Always prepare fresh agarose solution on the day of the experiment and ensure that the agarose is complete dissolved to prevent agarose crystallization and store the solution at ~60 °C to prevent it from solidifying.
3. Mix the membrane dye with the agarose solution ~1 hr before the anticipated microscopy session; in our hands this produces the most uniform straining. Note that exposing the dye to light and high temperature will reduce its efficacy over time.
4. *B. subtilis* requires thorough aeration for optimum growth and development; therefore ensure vigorous shaking of the culture. Normally a >1:20 ratio (culture volume:container volume) is desired.
5. Maintain and grow overnight culture of strains that harbour the large ~150 *tetO* array with 1 µg/ml tetracycline (only if the strain is resistance to tetracycline) or 500 ng/ml anhydrotetracycline and large ~150 *lacO* array with 1 mM IPTG to inhibit TetR and LacI binding to its operator sites, respectively.
6. Use fresh colonies streaked onto nutrient agar plates for each experiment.
7. Allow strains that exhibit slower growth rates a longer overnight incubation period to allow the overnight culture to achieve a high density.
8. Prewarm media before use to prevent temperature shock to the bacteria.
9. Care should be taken to prevent *B. subtilis* from entering the sporulation developmental pathway as this could interfere with the positioning of the chromosome and thus the origin.
10. There is a positive correlation between DNA replication initiation and nutrient-mediated growth rates. Therefore in complex media such as PAB, LB and

CH, cells undergo faster growth rate as compared to minimal media supplemented with the various type of carbon sources.

11. Media such as minimal media which support slower growth rate will result in cells having fewer origins/chromosomes per cell, therefore making analysis less complicated.
12. Reduce exposing the sample and the microscope slide to rapid temperature changes. Try to do all steps in the desired experimental temperature.
13. Ensure that the agarose pad is evenly spread onto the microscope slide to achieve best imaging for the field of cells. To achieve this, it is recommended that the gene frame be used.
14. Avoid exposing the agarose pad to the external environment for too long as this will cause excessive drying.
15. Agitate the culture before removing samples for imaging to reduce cells from clumping together.
16. To prevent air bubbles from accumulating under the coverslip, use the edge of the pen and moves it firmly from one end of the cover slip to the other. This will force any air pockets between the cover slip and agarose pad out.

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Figure 1: *Bacillus subtilis* developmental pathways

Cartoon representation showing the different growth patterns of *Bacillus subtilis*. **(A)** Under favourable conditions, *B. subtilis* undergoes vegetative growth and divides by binary fission (Green dotted box). **(B)** During conditions of nutrient deprivation and high cell density, *B. subtilis* undergoes a differentiation process that involves the formation of a stress resistant spore. **(i and ii)** Sporulation requires a diploid state where the chromosomes form an axial filament and the replication origin regions (green circle) are anchored to the cell poles by RacA and DivIVA. Formation of the asymmetric septum (orange dotted ring) near one pole leads to the capture of ~25% of the chromosome surrounding *oriC* in the forespore. The remaining ~75% of the chromosome in the mother cell is then pumped into the forespore by the translocase, SpoIIIE. **(iii)** As sporulation proceeds, the forespore is engulfed by the mother cell. **(iv)** Following spore maturation the mother cell will lyse, releasing the spore into the environment. **(v)** Spores remain dormant until conditions favourable for growth are encountered, whereupon they will germinate and resume vegetative growth.

Figure 2: Bulk chromosome localization within the cell.

Strains were grown at either 30°C or 37°C until mid-exponential phase. Cell membranes were stained with FM5-95 dye to distinguish individual cells. **(A)** Bulk chromosome morphology was visualized with **(i)** the nucleoid associated DNA binding protein HBsu-GFP or **(ii)** the DNA stain DAPI. **(B)** The origin region and the nucleoid were visualized simultaneously using TetR-YFP binding to a *tetO* array (~150 operator sites) inserted near the replication origin (359°) and the nucleoid visualized with HBsu-RFP.

Figure 3: Visualization of the replication origin and terminus with fluorescently labelled proteins.

Strains were grown at 37°C until exponential phase. Cell membranes were stained with FM5-95 dye to distinguish individual cells. **(A)** The origin can be visualized using Spo0J-GFP binding to origin proximal *parS* sites (grey dots). The *spo0J-gfp* fusion was expressed from its native locus at 359°. **(B)** The terminus can be visualized using RTP-GFP binding to nine *ter* sites located at the terminus region (green dots). The *rtp-gfp* fusion was expressed from its native locus located at 178°. **(C)** The origin/terminus localization assay was used to determine the position of each chromosome region within the long axis of the cell. The origin position was determined by visualizing the *oriC* region using either Spo0J-GFP binding to origin proximal *parS* sites or TetR-GFP binding to a *tetO* array near the replication origin. Schematic diagrams illustrate the measurements for either the **(i)** origin or **(ii)** terminus position in cells with two foci. For a detailed explanation of the measurement, please see method 3.6. **(D)** Quantification of origin and terminus localization within the cell. Approximately 100 cells were measured for each reporter strain. The 95% confidence intervals for the mean were calculated. **(E)** The origin counting assay was used to determine the average rate of DNA replication initiation. Reporter strains using Spo0J-GFP binding to origin proximal *parS* sites were grown at 37°. Cell membranes were stained with FM5-95 dye to distinguish individual cells. Qualification of Spo0J-GFP foci per cell in the wild-type strain (green bar) and a mutant that overinitiates DNA replication (red bar) reveals the mutant phenotype (for each strain approximately 500 cells were analyzed). For a detailed explanation of the measurement, please see method 3.7.

Figure 4: Construction of the TetR/*tetO* and LacI/*lacO* reporter system in *B. subtilis*.

Cartoon representation showing the construction of the reporter systems. **(i)** Organization of the *lacO/tetO* operator sequence (up to 240 operator repeats) arranged into arrays that contains 10 basepairs of randomized nucleotide sequence between each operator sequence. **(ii)** Plasmids containing either the *tetO* array or *tetR-gfp* are stably integrated into the chromosome by double-crossover. **(iii)** Successful construction allows visualization of the *tetO*-proximal locus bound by TetR-GFP using epifluorescence microscopy.

Figure 5: The origin separation assay.

Strains were grown at 30°C in minimal growth media to avoid overlapping rounds of DNA replication. **(i)** Schematic diagram showing the location of the *tetO* array (~150) inserted near the replication origin (359°) (visualized using TetR-YFP; green dot) and the location of the *lacO* array (~150) inserted ~150 kb down the left chromosome arm at 345° (visualized using LacI-CFP; red dot). **(ii)** A newborn cell that recently underwent cell division. **(iii)** A cell has initiated DNA replication and segregated the duplicated origin regions. **(iv)** DNA replication and segregation proceeds normally. **(v)** Successful formation of two identical daughter cells. **(vi)** A cell displaying a defect in separation of sister origins.

Figure 6: Synchronization of DNA replication using a *dnaB134^{ts}* mutant and localization of the replication origin using Spo0J-GFP.

(A) Experimental overview showing the major steps involved in synchronizing DNA replication in a *dnaB134^{ts}* mutant. For a detailed explanation of the procedure, see method 3.2. **(B)** The origin region was visualized using Spo0J-GFP binding to origin proximal *parS* sites in a *dnaB134^{ts}* mutant after synchronizing DNA replication. Cells were imaged every 15 min after shifting to the permissible temperature (30°C), displaying a synchronous increase in Spo0J-GFP foci per cell as replication proceeds. A white line (-) denotes boundary between cells.

Figure 7: The replication origin trapping assay during sporulation using the TetR/*tetO* reporter system.

(A) Experimental overview showing the major steps involved in the sporulation assay. For a detailed explanation of the procedure, see method 3.3. **(B)** The origin region was localized by visualizing TetR-GFP binding to a *tetO* array (~25) inserted near the replication origin (353°). TetR-GFP localization was determined during sporulation of *B.subtilis* cells 120 min post resuspension in sporulation medium. Cell membranes were stained with FM5-95 dye to identify individual cells undergoing spore development. Enlarged images highlight cells displaying either **(i)** normal origin localization or **(ii)** origin trapping defects (denoted with an asterisk *).

Figure 8: Layout of a multi-spot microscope slide and a gene frame on a microscope slide

(A) Image showing a multi-spot microscope slide. **(B)** Schematic diagrams showing the layout of a gene frame microscope slide for either **(i)** two or **(ii)** four different strains. Note that the agarose pads have been stained with brilliant blue dye to allow their visualization in the images shown.

Table 1: Suggested growth conditions and exposure times for fluorescent reporters

Table showing the suggested growth conditions and exposure times for each fluorescent reporter used in this paper.

Figure 1

(C)

Fluorophore/Stain	Array size	Average exposure time	Optimum growth temperature	Repressor/Stain Concentration
TetR-GFP	~25 <i>tetO</i>	3 - 5 s	30°C - 37°C	Tetracycline (1 µg/ml) or Anhydrotetracycline (500 ng/ml)
TetR-YFP	~150 <i>tetO</i>	5 s	30°C	
LacI-CFP	~150 <i>lacO</i>	5 s	30°C - 37°C	IPTG (1 mM)
Spo0J-GFP	<i>parS</i> sites	3 - 5 s	30°C - 37°C	nil
RTP-GFP	<i>ter</i> sites	5 s	30°C - 37°C	nil
DAPI	chromosome	3 - 5 s	30°C - 37°C	DAPI (0.2 µg/ml)
HBsu-GFP	chromosome	3 - 5 s	30°C - 37°C	nil
HBsu-RFP	chromosome	3 - 5 s	30°C - 37°C	nil
FM5-95	cell membrane	3 s	nil	Add FM5-95 (0.4 µg/ml) to agarose
brightfield	whole cell	100 ms	nil	nil

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8