Published in final edited form as:

Methods. 2009 May ; 48(1): 8–13. doi:10.1016/j.ymeth.2009.02.010.

Cell cycle synchronization of *E. coli* using the stringent response, with fluorescence labeling assays for DNA content and replication

Daniel J. Ferullo, Deani L. Cooper, Hayley R. Moore, and Susan T. Lovett

Department of Biology and Rosenstiel Basic Medical Sciences Research Center MS029, Brandeis University, Waltham, MA 02454-9110

Abstract

We describe a method for synchronization of the cell cycle in the bacterium *E. coli*. Treatment of asynchronous cultures with the amino acid analog, DL-serine hydroxamate, induces the stringent response, with concomitant arrest of DNA replication at initiation. Following release of the stringent response, cells initiate DNA replication in synchrony, as determined by flow cytometry for DNA content, Southern blotting and microscopy. This method has the advantage that it can be used in fully wild-type cells, at different growth rates, and may be applicable to other bacterial species with replication control by the stringent response. We also elaborate other methods useful for establishing cell cycle parameters in bacterial populations. We describe flow cytometric methods for analyzing bacterial populations for DNA content using the DNA-specific dye PicoGreen, readily detected by most commercial flow cytometers. We also present an method for incorporation of the nucleotide ethynyl-deoxyuridine, EdU, followed by “click” labeling with fluorescent dyes, which allows us to measure and visualize newly replicated DNA in fixed *E. coli* K-12 cells under non-denaturing conditions.

Keywords

flow cytometry; stringent response; bacteria; serine hydroxamate; ppGpp; EdU; PicoGreen; FM4-64; click labeling; DNA synthesis

1. INTRODUCTION

The bacterium *Escherichia coli* has been a particularly useful model to study the repair of damaged replication forks [1-4]. Studies of DNA replication and other cell cycle events in bacteria are hampered by the asynchrony of the population, dispersed at various stages of the cell cycle. *E. coli* cells initiate replication from a single origin of replication, “*oriC*”, and forks proceed bidirectionally until they meet at the terminus regions “*ter*”. Under poor growth conditions, replication is completed before cell division and cells cycle between 1 and 2 chromosomes. Under optimal growth conditions, the cell cycle becomes faster than the period of time necessary to replicate the chromosome (approximately 40 min), and secondary rounds of initiation occur before completion of the previous round. In this more complex cell cycle pattern, *E. coli* cells are borne with partially replicated chromosomes and in rich medium can be as high as 16-ploid for the origin region of the chromosome.

lovett@brandeis.edu, (781) 736-2497.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Two main approaches have been used routinely to synchronize *E. coli* cells in culture. The first involves use of temperature-sensitive alleles of the essential DNA replication protein, DnaC [5], which interacts with the replicative DNA helicase, DnaB [6], and is required for initiation of DNA replication. The culture is shifted to the nonpermissive temperature and incubated for sufficient time to allow all replication forks to be completed; subsequent a short shift to permissive temperature allows initiation to occur in synchrony [7]. The advantage of this method is that it allows a large bulk of cells to be synchronized. However, this method requires that the *dnaC* allele (most commonly used is *dnaC2*) be present in the strain. The method is perturbing to physiology, including a heat shock. Initiation is not normal in *dnaCts* strains even at permissive temperatures, and cells appear to initiate secondary rounds of replication asynchronously [8]. In our hands, it has been difficult to move *dnaC2* to certain other genetic backgrounds and we suspect suppressor mutations have accumulated in the strain, PC2, that modify its phenotype. Moreover, DnaC is required for events other than initiation: the PriA and PriC-dependent replication restart pathways following DNA repair both require DnaC [9,10]. Because double mutants in PriA and PriC are inviable, restart must be a frequent need in the normal replication cycle. Stalled forks appear to accumulate in *dnaCts* strains ([11] and replication does not proceed to completion.

The second set of methods involves physical attachment of cells, such that newborn cells are specifically released into the medium where they can be collected and analyzed. An early adaptation of this technique is the so-called “baby machine” [12-14], whereby cells are affixed to a membrane. Newborn cells are released to the medium, and are minimally perturbed, but it is difficult to collect large quantities of cells and there are strain variations to the stickiness of *E. coli* strains [14]. A variant on this technique is to express a sticky flagellin allele, *fliCst*, so that *E. coli* cells can be immobilized on a column; the *fliC* allele is turned off and newborn cells can be collected by elution [15,16]. This allows larger quantities of non-perturbed cells to be collected, although it requires special strain construction to introduce the *fliCst* allele.

Our studies of DNA replication during the stringent response of *E. coli* suggested another method for cell cycle synchronization. We observed that cells induced for the stringent response arrest their cell cycle at the level of DNA replication initiation [17]. This stringent cell cycle arrest can be evoked either by inhibition of tRNA charging by the analog serine hydroxamate [18,19] or by overexpression of the ppGpp synthetase, RelA from a plasmid construct [20]. When released from the stringent response, by washing in the case of treatment with serine hydroxamate, cells appear to enter the cell cycle synchronously. This method is similar to the *dnaC*-synchronization technique but does not require a specific genetic background, nor does it interfere with replication restart once the cells are released. Our method is a new version of an old technique using amino acid starvation to inhibit initiation and allow synchronous restart [21], which we have characterized more extensively by modern flow cytometric and microscopic measures. Unlike the old technique, amino acid auxotrophy is not required: serine hydroxamate can induce the stringent response in prototrophic *E. coli* strains, even in rich growth medium.

We use flow cytometry to determine DNA content in individual cells to ascertain their cell cycle status. This technique has been in use for some time and has been instrumental in our present knowledge of bacterial cell cycle [8,22,23]. We present methods for use of the DNA specific dye PicoGreen, which has many superior properties [24] to other fluors initially and more commonly used for bacterial cell cycle work. PicoGreen is a highly sensitive fluorescent dye for dsDNA, with high quantum efficiency and large molar extinction coefficient, which is optimally excited by Argon 488 nm lasers commonly available in commercial flow cytometers. PicoGreen binds double-strand DNA with high specificity, with linear fluorescence over a four-order of magnitude of DNA concentration, and has very low background fluorescence in the absence of DNA or in the presence of RNA. There have been a few reports of its use in *E.*

coli and other bacteria [8,25,26], and we describe here in more detail our method of staining fixed *E. coli* cells.

To determine if individual cells are replicating, we have developed an replication assay in *E. coli* based on the incorporation of nucleotide analog, EdU, followed by its labeling with a fluorescent dye azide derivative (a commercially available kit, marketed by Invitrogen as “Click-iT EdU”). In mammalian cells, this has been used as an alternative to labeling with ³H-thymidine or BrdU (bromodeoxyuridine) for detecting replicating cells, because of its ease and sensitivity [27,28]. The EdU-Click labeling method not only provides a quantitative assay for the extent of DNA synthesis, but this labeling also allows the visualization of newly replicated DNA in intact bacterial cells using fluorescence microscopy. Detection of analog bromodeoxyuridine (BrdU) requires immunofluorescence labeling with anti-BrdU antibody, which is not efficient unless the DNA is denatured [29]. The EdU-Click labeling can be performed in nondenaturing conditions and therefore allows visualization in relatively unperturbed samples.

DESCRIPTION OF METHODS

1. Using serine hydroxamate to arrest replication

The method to arrest *E. coli* cells with serine hydroxamate (SHX) used in our laboratory begins by growing cells in liquid cultures with aeration to mid-logarithmic phase (OD₆₀₀ 0.2 - 0.4) at 37°. We use defined but nutrient-rich minimal medium, M9 +0.4% glucose + 0.2% casamino acids (Bacto) and a wild-type strain of *E. coli* K-12, MG1655 [30,31]. In this growth medium, cells are cycling between 4N and 8N DNA content. We have also used more nutrient-poor growth media or rich broth (LB, Luria-Bertani), growth conditions in which DNA content per cell will be less or more, respectively.

Cells are then treated with DL serine hydroxamate (Sigma-Aldrich, S4503) to a final concentration of 1 mg/ml (using a 10X stock of DL-Serine hydroxamate mixed in sterile water). After treatment, *E. coli* cells do not initiate a new round of replication, but do complete all ongoing rounds. To allow sufficient time for replication to complete to termination, cultures should continue to grow for at least an additional 90 minutes. Arrest of replication can be confirmed by examining the DNA content by flow cytometry (methods described below, in section 2). After 90 minutes of treatment, replication has ceased, causing the cells to contain an integer number of chromosomes (Fig. 1A). Though the number of chromosomes can vary depending on the nutrient quality of the medium being used, the population will synchronize using serine hydroxamate addition to most common media formulations, rich or minimal. This method will not work for cells that are known to be defective in eliciting the stringent response, such as *relA* mutants. Our strain, MG1655, is prototrophic for serine metabolism and serine is present in the casamino acids added to the growth medium. We have used similar concentration of SHX in medium without added source of amino acids.

1.1 Release from serine hydroxamate for synchronous restart of replication—

To initiate replication synchronously, cells are washed of serine hydroxamate and introduced to drug-free medium. Cells from cultures with serine hydroxamate can be pelleted by centrifugation at 6000 × g. The supernatant is decanted, and the cells resuspended in the same growth medium lacking serine hydroxamate. It may be advantageous to pre-warm the growth medium to allow rapid restart of replication.

1.2 Ascertainment of cell cycle synchrony—Detection of replication restart is apparent by flow cytometry for DNA content (as by the methods described in section 2 below). In M9 Glucose CAA medium, initially the population consists of primarily 4N cells, (arrested after completion of replication and cell division, but prior to initiation) with a small subpopulation

at 8N (which arrest after completion of replication but prior to cell division). A similar subpopulation is seen with synchronization with *dnaC2* [7]. Using quantitative Southern blots for markers near the origin relative to the terminus [32], duplication of *oriC* in the bulk population is detected as early as 6-10 minutes after release (data not shown), indicating that replication is initiated rapidly after removal of serine hydroxamate. Within 30 minutes after the introduction of new medium (Figure 1AB) DNA content in the population is notably increased, indicating ongoing replication. Closer examination of the time period from 30-44 minutes post-release (Figure 1B) shows that the DNA content peak gradually shifts from 4N toward 8N. The broadening of DNA content in the population at 44 minutes after release may indicate a secondary round of initiation, as is seen in cells released from *dnaCts* arrest [7].

Cell size, as determined by microscopic analysis of FM4-64 strained cells (see section 3.1 below) increases until 45 min. after release (Figure 2). At 60 minutes after release, we begin to see maximal signs of cell division, as evident from shift to lower DNA content (Fig. 1AB) and by microscopic examination of cells, showing FM4-64 stained septa. (Fig. 2, see methods described in section 3.1 below). We have not followed cells through their second cell cycle after release to determine the synchrony of subsequent rounds.

1. 3 Limitations of this method—As with other methods of synchronization, the achieved synchrony is not perfect. In this growth condition, although most cells arrest at 4N, we do detect 2N and 8N cells by flow cytometry. Our data suggest that 73 % of the cells are arrested at 4N. Some of this heterogeneity could be reduced by growth of cells in poorer medium such as M9+ 0.2% succinate, where cells cycle between 1N and 2N. Treatment with serine hydroxamate and induction of the stringent response does interfere somewhat with protein translation [33]) although the block is not complete. Although the stringent response is usually considered a “stress response”, it is important to note that bacteria cells in culture naturally induce the response in the late logarithmic phase of growth in culture. Levels of ppGpp, the signaling molecule that elicits the stringent response, rises dramatically as bacterial cultures deplete the growth medium, prior to entry into stationary phase of growth [33].

2. Flow cytometric analysis of bacterial cell cycle by staining for DNA content

DNA content as determined by flow cytometry is a useful means of tracking replication. Flow cytometry can be performed on relatively large populations (we routinely assay 30,000 cells), for which DNA content in each individual cell can be determined by fluorescence detection of DNA-specific dyes. In concert, other physical parameters such as size, proportional to the forward light-scattering signal, can be ascertained for each cell. Flow cytometric data are commonly expressed as histograms, with fluorescence intensity on the X-axis and number of events on the Y-axis. As discussed earlier, the fluor PicoGreen has many properties that make it particularly useful for bacterial DNA content determination, including sensitivity, dsDNA specificity, large linear range and excitation by Argon lasers commonly available in commercial flow cytometers.

This type of analysis is useful for determination of cell cycle stages and, in the case of *E. coli*, how much replication is supported by the current growth conditions. Inference of chromosome number based on determination of DNA content may be achieved by preparing non-replicating control cultures in which cells have integer DNA content. Stationary phase cells assume integer DNA content but have been reported to be a mixture of 1N (cells containing 1 chromosome) and 2N (cells with 2 chromosomes) [7], or, alternatively, 2N or 4N [23]; a comprehensive study concluded that chromosome number at stationary phase is influenced by growth conditions and can change over time [34]. Another reference value that has been used is the DNA content of mutants in conditional replication initiation mutants shifted to their nonpermissive temperature. However, we and other others observe mixed 1 and 2N

subpopulations ([7,35] and data not shown); in our hands, cultures in certain growth media consist of primarily 2N cells (data not shown). A better normalization control is to culture cells in poor growth medium, such as M9+0.2% succinate, where 1N and 2N cells, which have not yet initiated replication or have completed replication and not yet divided, are apparent in the asynchronous population. All other “N” positions can be inferred due to the linear scale of the fluorescence intensity X axis. Using the same labeling and growth conditions, we have found the absolute fluorescence for populations with integer DNA content highly reproducible in day-to-day determinations.

2. 1. Methods for replication “run-out”—Additionally, a culture grown in “runout” conditions is a useful control to examine the amount of replication supported by the medium, in particular strains [22]. This involves dual treatment with rifampicin, a transcription inhibitor that blocks initiation of DNA replication, and cephalixin, which inhibits septum formation and cell division. With this combined treatment, cells complete ongoing replication forks and arrest in their D period, or G2-like state (Fig. 3). For achieving run-out conditions, we add 300 µg/ml rifampicin (USBiological #13292-46-1) and 30 µg/ml cephalixin (Sigma C4895) to early logarithmic cultures and grow for at least 3 hours. (We have experienced difficulty with run-out experiments using certain sources of rifampicin, which appeared to be more toxic and interfere with the completion of replication, so take care with this reagent.) A complement to run-out treatment is use of DL-serine hydroxamate, as above, which arrests cells in the B period, or G1-like state, of the culture. Using these treatments, positions of the peaks on this histogram can be used to infer the chromosome number that is supported by the current nutrition conditions. For example, in M9 + 0.2% glucose cells are cycling between 1 and 2N DNA content; in M9 + 0.4% glucose + CAA, cells primarily initiate replication from 4 origins to 8.

2. 2. Method for PicoGreen analysis of DNA content by flow cytometry—

Preparation of samples for flow cytometry with PicoGreen first requires cell fixation. Our laboratory adds 1 ml of culture to 9 mls of 70% ethanol. The samples can be stored for at least 12 hours, up to three weeks, at 4°C. The samples are then centrifuged at 3300 × g for 10 minutes. The ethanol is decanted and the pellets kept a room temperature for an additional 20 minutes for the residual ethanol to evaporate. Complete removal of ethanol is necessary to avoid clumping of the cells after resuspension. We resuspend the cell pellet in 1 ml 1X phosphate-buffered saline (PBS) pH 7.4. We then add 200 µl of 100X PicoGreen (Invitrogen, P7581) in 25% DMSO (Sigma) to each ml of sample. We allow the samples to incubate for at least 30 minutes and up to 6 hours in the dark at room temperature. After incubation with PicoGreen, the samples are diluted with the addition of 1 ml of PBS. Unlike propidium iodide (another fluorescent dye used for cell cycle analysis), PicoGreen does not fluoresce with RNA [24], so RNase treatment of cells is not required.

Performing PicoGreen flow cytometry for DNA content in *E. coli* cultures can be done with most commonly available brands of flow cytometers using fluorescence in the green wavelengths. Our laboratory uses a Becton Dickinson FACSCalibur with a 488 nm Argon laser. We collect 30,000 events. In our experiments, the rate of events/second is critical for accurate readings, with an ideal rate is between 200 – 1000 events per second. Rates that exceed this are caused by samples too concentrated with cells. To overcome this, the sample can be diluted with additional PBS and rerun to attain a more desirable rate. To perform analysis of the acquired data, we use the FloJo software, version 6.4.1 (Tree Star, Inc.). We plot each sample as histogram vs. the green channel (FL1-A, voltage=458, gain=1, mode=linear). Another useful plot is DNA content (fluorescence, channel FL1-A) as a function of cell size (forward scatter signal, FSC, gain=4.59, mode=linear). In such blots, bacterial cell filaments (such as those induced for the SOS response to DNA damage) are apparent as subpopulations of large size, usually with excessive DNA content. DNA-free cells can also be apparent from such analysis, although care should be taken that this is not acellular debris. Inclusion of a

secondary cellular stain, such as those for membranes like FM4-64 (see below), is useful here, since true cells will stain with this dye. Windows for cell size (forward scatter, FSC channel) can be restricted to eliminate small or large debris.

3. Microscopic analysis of synchronized cells

Cells can also be followed microscopically, for replication status (incorporation of labeled nucleotides) and for increases in cell size and for achievement of landmark events such as cell septation. Signs of cell division, at the early stages, can be difficult to ascertain by phase contrast microscopy; visualization of septation is aided by fluorescence membrane stains, such as the red fluorescent dye FM4-64 (*N*-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino) phenyl) hexatrienyl) pyridinium dibromide, with excitation/emission maxima at 515/640 nm, Invitrogen, T1366), which stains the inner membrane of *E. coli* [36]. Measurement of cell length or volume is also aided by FM4-64 to delineate the cell periphery. We describe methods for FM4-64 staining, as well as for replication incorporation of nucleotide, via EdU-click fluorescence labeling.

3.1 FM4-64 staining of *E. coli* cells—We transfer 0.5 mL of cell culture to an opaque, darkly colored microcentrifuge tube and add FM 4-64 (Invitrogen, T1366, 200 $\mu\text{g mL}^{-1}$, in water) to a final concentration of 5 $\mu\text{g mL}^{-1}$. The sample is then incubated at 37°C with aeration for 10 minutes and fixed with cold 0.5 mL 3:1 methanol: acetic acid on ice for at least 10 minutes. Samples are concentrated by microcentrifugation at 13000 rpm for 5 minutes, all but about 100 μL of the supernatant is decanted, and cells are resuspended in the remaining dye-medium. To affix cells to microscope slides, 10 μL poly-L-lysine (0.01% solution, Sigma-Aldrich P4832) is applied to the slides, allowed to air-dry, followed by 20 μL of the concentrated culture. An antifade-mounting medium is spotted on the samples (5 μL Vectashield Mounting Medium for Fluorescence, Vector Laboratories, H-1000) and coverslips are applied. Our laboratory uses an Olympus BX51 microscope equipped with an 100x objective and a Retiga Exi (Qimaging Inc.) camera. For FM4-64 stained cells, we use a rhodamine filter and exposures are typically 1 s. Images are analyzed with either Openlab Darkroom or Volocity imaging software (Perkin-Elmer Improvision) and edited with Adobe Photoshop Elements 4.0 for MacIntosh.

3. 2 EdU-Click labeling of newly replicated DNA in *E. coli*—Our laboratory has developed a method suitable for fluorescence labeling of newly replicated DNA in *E. coli* using a commercially available kit, “Click-iT EdU” (Invitrogen). In this assay, EdU (5-ethynyl-2'-deoxyuridine) is incorporated in vivo; subsequently an number of different Alexa fluor azides can be conjugated to the EdU moiety after cell fixation and permeabilization. Although this technique has been used with mammalian cells and tissues [27,28], its utility for bacterial labeling has not been reported. Our laboratory most commonly uses green fluorescence labeling, (Invitrogen Click-iT Edu Alexa fluor 488 imaging kit C10083) and fluorescence microscopy for its detection.

3. 2.1. Incorporation of EdU—To label live *E. coli* cells with EdU, 2 ml aliquots from the cultures are removed and added to 15 ml conical tubes containing 24 μL EdU, giving a final concentration of 30 $\mu\text{g/mL}$. After 15 minutes of incubation with EdU at 37°, incorporation was terminated and cells fixed by adding 13 ml 90% methanol. Fixed cells can be stored at 4° at this point.

3.2.2. Click-labeling with fluor—Fixed cells were pelleted by centrifugation at $5,000 \times g$ at 4°. The supernatant was decanted and the cells were resuspended in 1.5 ml filtered PBS and transferred to microcentrifuge tubes. After pelleting, the supernatant was again removed and the pellet was resuspended gently in 100 μL 0.5% Triton X-100 in PBS solution and incubated

at room temperature for 30 minutes. After incubation, cells were centrifuged and washed once in 1.5 ml PBS. The cell pellet was resuspended in 200 µl Click-iT reaction cocktail/ ml cell culture as detailed in the Invitrogen protocol. Reaction buffer additive (Invitrogen EdU Click-iT kit component) was added last and the mixture was used within 15 minutes. Cells were incubated at room temperature (protected from light) for 30 minutes and then collected by centrifugation. The pellet was washed once with 1.5 ml PBS and then finally resuspended in 0.5-1.0 ml PBS.

Labeled cells were spotted onto slides coated with poly-lysine and visualized as described above. We use a GFP filter and exposure times of 2.4 seconds for image acquisition of Alex Fluor 488-labeled cells.

3.2.3. Results of EdU-Click labeling of untreated, serine hydroxamate-arrested and released cells—Using the above protocol, incorporation of EdU in 15 min pulses produces labeled foci, visible in proliferating *E. coli* K-12 cells in M9 + 0.4% glucose + CAA medium (Figure 4). The individual foci represent the spatial segregation of newly replicated DNA from the replicating sister chromosomes. This appearance is similar to that observed after incorporation of nucleotide BrdU, followed by indirect detection by anti-BrdU antibodies [37]. After treatment with SHX, labeling is greatly decreased and undetectable at 90-105 min (Fig. 4 Panel A), consistent with the completion of all ongoing forks and the inhibition of initiation of new ones. Upon release from serine hydroxamate (Fig. 4, Panel B), labeling is detected in the first 0-15 min pulse, increases over time and is bright by 40-65 min, consistent with the rapid resumption of replication.

3.2.4. Limitations, potentials and further questions—The potential to shorten the EdU pulse period, while retaining detection, has not been fully explored. With a 15 min labeling period and assuming 40 min for replication of the entire 4.5 Mb *E. coli* chromosome, one is visualizing a stretch of 1-2 Mb of DNA, a considerable portion of the chromosome. Likewise, we have not fully developed flow cytometric analysis of Edu-Click labeled *E. coli* cells. We do not know how readily *E. coli* DNA polymerases incorporate EdU, nor how perturbing to *E. coli* replication or physiology is EdU incorporation into DNA. Click-labeling cannot be performed in live cells, although the reactions conditions are considerably milder than those for BrdU detection, possibly aiding dual detection with other probes.

Concluding remarks

Fluorescence detection has greatly aided the study of bacterial cell biology. As a fluorescence-based incorporation assay for DNA replication, we have developed EdU-Alexa Fluor click-labeling for application to *E. coli*. Flow cytometry of PicoGreen-stained cells provides an additional sensitive means to study the complex replication cycles of *E. coli*. We describe a method for the synchronization of replication of *E. coli* cells using the natural stringent response to amino acid starvation, which temporarily blocks the initiation of DNA replication. When cells are released from the stringent response, they appear to rapidly resume replication in synchrony, as assayed by flow-cytometry and microscopy. We hope to use this synchrony to examine in further detail events that accompany replication fork repair. Because our method can be used with fully wild-type strains, this method may be more broadly applicable to studies of DNA replication in many other bacterial species.

References

1. Cox MM. Annu Rev Genet 2001;35:53–82. [PubMed: 11700277]
2. Kreuzer KN. Annu Rev Microbiol 2005;59:43–67. [PubMed: 15792496]

3. Michel B, Boubakri H, Baharoglu Z, LeMasson M, Lestini R. DNA Repair (Amst) 2007;6:967–980. [PubMed: 17395553]
4. Persky NS, Lovett ST. Crit Rev Biochem Mol Biol 2008;43:1–24.
5. Carl PL. Mol Gen Genet 1970;109:107–122. [PubMed: 4925091]
6. Wickner S, Hurwitz J. Proc Natl Acad Sci U S A 1975;72:921–925. [PubMed: 1093174]
7. Withers HL, Bernander R. J Bacteriol 1998;180:1624–1631. [PubMed: 9537356]
8. Boye E, Lobner-Olesen A, Skarstad K. Biochim Biophys Acta 1988;951:359–364. [PubMed: 2850013]
9. Sandler SJ. Genetics 2000;155:487–497. [PubMed: 10835375]
10. Sandler SJ, Marians KJ. J Bacteriol 2000;182:9–13. [PubMed: 10613856]
11. Maisnier-Patin S, Nordstrom K, Dasgupta S. Mol Microbiol 2001;42:1371–1382. [PubMed: 11886566]
12. Helmstetter CE, Cummings DJ. Proc Natl Acad Sci U S A 1963;50:767–774. [PubMed: 14077509]
13. Helmstetter CE, Cummings DJ. Biochim Biophys Acta 1964;82:608–610. [PubMed: 14148828]
14. Helmstetter CE, Eenhuis C, Theisen P, Grimwade J, Leonard AC. J Bacteriol 1992;174:3445–3449. [PubMed: 1592802]
15. Bates D, Epstein J, Boye E, Fahrner K, Berg H, Kleckner N. Mol Microbiol 2005;57:380–391. [PubMed: 15978072]
16. Bates D, Kleckner N. Cell 2005;121:899–911. [PubMed: 15960977]
17. Ferullo DJ, Lovett ST. PLOS Genetics 2008;4:e1000300. [PubMed: 19079575]
18. Tosa T, Pizer LI. J Bacteriol 1971;106:966–971. [PubMed: 4934071]
19. Tosa T, Pizer LI. J Bacteriol 1971;106:972–982. [PubMed: 4934072]
20. Schreiber G, Metzger S, Aizenman E, Roza S, Cashel M, Glaser G. J Biol Chem 1991;266:3760–3767. [PubMed: 1899866]
21. Marsh RC, Hepburn ML. J Bacteriol 1980;142:236–242. [PubMed: 6246063]
22. Skarstad K, Boye E, Steen HB. Embo J 1986;5:1711–1717. [PubMed: 3527695]
23. Steen HB, Boye E. Cytometry 1980;1:32–36. [PubMed: 7023880]
24. Singer VL, Jones LJ, Yue ST, Haugland RP. Anal Biochem 1997;249:228–238. [PubMed: 9212875]
25. Foti JJ, Schiend J, Sutera VA Jr, Lovett ST. Mol Cell 2005;17:549–560. [PubMed: 15721258]
26. Marie D, Vaultod D, Partensky F. Appl Environ Microbiol 1996;62:1649–1655. [PubMed: 8633863]
27. Buck SB, Bradford J, Gee KR, Agnew BJ, Clarke ST, Salic A. Biotechniques 2008;44:927–929. [PubMed: 18533904]
28. Salic A, Mitchison TJ. Proc Natl Acad Sci U S A 2008;105:2415–2420. [PubMed: 18272492]
29. Gratzner HG. Science 1982;218:474–475. [PubMed: 7123245]
30. Bachmann BJ. Journal 1996;2:2460–2488.
31. Blattner FR, Plunkett G 3rd, Bloch CA, Perna NT, Burland V, Riley M, Collado-Vides J, Glasner JD, Rode CK, Mayhew GF, Gregor J, Davis NW, Kirkpatrick HA, Goeden MA, Rose DJ, Mau B, Shao Y. Science 1997;277:1453–1474. [PubMed: 9278503]
32. Nyborg M, Atlung T, Skovgaard O, Hansen FG. Mol Microbiol 2000;35:1202–1210. [PubMed: 10712700]
33. Potrykus K, Cashel M. Annu Rev Microbiol 2008;62:35–51. [PubMed: 18454629]
34. Akerlund T, Nordstrom K, Bernander R. J Bacteriol 1995;177:6791–6797. [PubMed: 7592469]
35. Boye E, Blinkova A, Walker JR. Biochimie 2001;83:25–32. [PubMed: 11254971]
36. Fishov I, Woldringh CL. Mol Microbiol 1999;32:1166–1172. [PubMed: 10383758]
37. Adachi S, Kohiyama M, Onogi T, Hiraga S. Mol Genet Genomics 2005;274:264–271. [PubMed: 16133165]

Figure 1.

Synchronous DNA replication following release from the stringent response. A. Flow cytometry for DNA content in *E. coli* K-12 cultures treated with serine hydroxamate (SHX) for 90 minute followed by release into medium without SHX for the indicate period of time in minutes. This panel is from our previously published work [17] B. Additional timepoints following release show synchronous replication, as evident by flow cytometric determination of DNA content in the population (black traces). The overlaid red trace is that prior to release.

Figure 2.

Cell division after release from the stringent response. A. Fluorescence microscopy images of wild type *E. coli* treated with serine hydroxamate (SHX) for 90 minutes and subsequently released into M9 minimal medium with casamino acids without SHX for the indicated length of time and stained with the lipophilic dye FM 4-64. Cells have an increase in cell length by 20 minutes with septa first visible in the 30 minute image. Percentage of cells with visible septa, as indicated, increases with time after release. Bar length = 3.20 μm. B. Distribution of cell lengths observed at each time point after release from SHX. Cell lengths increase from 0-45 minutes after release.

Figure 3.

Flow cytometric analysis of DNA content for *E. coli* K-12 cultures in three different growth media (reproduced from [17]). Untreated cells yield a distribution of DNA content, increasing with growth rate. Doubling times (T_d) in each medium are indicated. Cultures treated with serine hydroxamate (SHX) show quantized DNA content, variable with the growth medium, consistent with DNA content in the B period (G1-like) of the cell cycle. Cultures treated with rifampicin and cephalixin (Rif + Ceph) show integer DNA content, with cells arrested in the D period (G2-like).

Figure 4.

EdU-Alexa 488 click-labeling during and after serine hydroxamate treatment. Cultures in the exponential phase of growth were divided and either mock-treated (-) or treated with 1 mg/ml serine hydroxamate.(+). Panel A. After 30, 60, or 90 minutes of treatment EdU was added to aliquots from each culture. Following a further incubation of 15 minutes, cells were fixed in methanol and then processed as described in the text. Panel B. After treatment for 90 minutes with serine hydroxamate, cultures were centrifuged and resuspended into fresh medium without serine hydroxamate. At 0, 20, and 40 minutes after release aliquots were incubated with EdU for 15 min, after which they were fixed and click-labeled. Bottom image: control culture with no EdU treatment.