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Chapter 2

**CELLULAR LOCALISATION OF oriC DURING THE CELL CYCLE OF *Escherichia coli* AS ANALYSED BY FLUORESCENT *IN SITU* HYBRIDISATION**

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2.1 Abstract

The origin of replication of *Escherichia coli*, oriC, has been labelled by fluorescent *in situ* hybridisation (FISH). The *E. coli* K12 strain was grown under steady state conditions with a doubling time of 79 min at 28 °C. Under these growth conditions DNA replication starts in the previous cell cycle at -33 min. At birth cells possess two origins, which are visible as two separated foci in fully labelled cells. The number of foci increased with cell length. The distance of foci from the nearest cell pole has been
measured in various length classes. The data suggest (i) that the two most outwardly located foci keep a constant distance to the cell pole and they therefore move apart gradually in line with cell elongation and (ii) that at the initiation of DNA replication the labelled origins occur near the centre of prospective daughter cells.

2.2 Introduction

The *Escherichia coli* chromosome has a circumference of about 1.6 mm, whereas the width of the cell is in the order of 1 mm. This indicates that considerable compaction is required to fold the bacterial chromosome into the so-called nucleoid (reviews: Pettijohn, 1996; Woldringh and Odijk, 1999). The extreme smallness of the nucleoid has to a large extent precluded the elucidation of its organisation by microscopic techniques, even by high-resolution transmission confocal-microscopy (Valkenburg et al., 1985). Recent advances in fluorescent labelling techniques, however, allow the visualisation of sub-nucleoid regions during DNA replication and cell elongation. Microscopic studies have led to the idea that the nucleoid is a polarised structure and, dependent on the replication status of the bacterial chromosome, sub-nucleoid regions occupy defined positions within the nucleoid and within the cell (Glaser et al., 1997; Gordon et al., 1997; Lewis and Errington, 1997; Lin et al., 1997; Mohl and Gober, 1997; Niki and Hiraga, 1998; Webb et al., 1997). Sub-nucleoid regions appear to move independently of cell elongation (Gordon et al., 1997; Webb et al., 1998), which has been interpreted to mean that motor-like proteins are involved in partitioning of the bacterial chromosome (Hiraga, 1992; Lewis and Errington, 1997; Niki and Hiraga, 1998).

The nucleoid as a whole is confined to a specific cellular position. For instance, during cell elongation of *E. coli*, the distance between the nucleoid border and the nearest cell pole remains constant (van Helvoort and Woldringh, 1994). Similarly, during a large part of the DNA replication cycle, there is a fixed distance between the polarly located origin of replication and the cell pole (Niki and Hiraga, 1998 and this paper).
Another important aspect relevant to the study of nucleoid organisation is that, in contrast to eukaryotic cells, the DNA replication-cycle is not always coincident with the cell division cycle. For instance, initiation of DNA replication can start in a previous cell division cycle. A special situation arises at fast growth, when the mass doubling time is smaller than the duration of DNA replication. In such a case, a new round of DNA replication is started on an already replicating chromosome (multifork replication, for a review see Helmstetter, 1996). Thus, to simplify the interpretation of sub-nucleoid microscopical data, it is important to use slowly growing cells and cells of which the temporal relationship between the DNA replication cycle and the cell division cycle is known.

Using such a defined culture we have carried out fluorescent *in situ* hybridisation (FISH) to label the origin of replication in an *E. coli* K12 strain growing with a doubling time of 79 min. We have addressed the following questions and we have compared the results with existing literature. (i) What is the temporal relationship between the DNA replication cycle and the cell division cycle, (ii) where are the replicating origins located in the newborn cell, (iii) where is the origin of replication located at initiation of replication, and finally, (iv) do the origins move within the nucleoid independently of cell elongation.

### 2.3 Materials and methods

#### 2.3.1 Bacterial strains and growth conditions

Cells of *E. coli* strain LMC500 (MC4100 (F-, araD139, Δ(argF-lac) U169, deoC1, flbB5301, ptsF25, rbsRelA1, rpsl150) lysA) (Taschner et al., 1988) were grown in steady-state in glucose minimal medium containing 6.33 g of K$_2$HPO$_4$.3H$_2$O, 2.95 g of KH$_2$PO$_4$, 1.05 g of (NH$_4$)$_2$SO$_4$, 0.10 g of MgSO$_4$.7H$_2$O, 0.10 g of MgSO$_4$.7H$_2$O, 0.28 mg of FeSO$_4$.7 H$_2$O, 7.1 mg of Ca(NO$_3$)$_2$.4H$_2$O, 4 mg of thiamine, 4 g of glucose and 50 μg lysine per liter (pH7.0) at 28 °C with a doubling time ($T_d$) of 79 min. Cells were
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harvested at OD$_{450}$ = 0.2 and fixed in 1ml 0.1% OsO$_4$ in TY-medium (1% tryptone, 0.5% yeast extract, 3mM NaOH, 0.5% NaCl) and stored at 4 °C.

2.3.2 Probe and probe labelling

We used an oriC probe obtained from plasmid pOC162 (kindly provided by dr. W. Messer, Max Planck Institut für Molekulare Genetik, Berlin, Germany). This plasmid contains the 248bp oriC region with 3kbp on each side of oriC. Because pOC162 contains no plasmid sequences homologues to chromosomal sequences, the whole construct was labelled and used as a probe for FISH. The plasmid was labelled with digoxigenin-11-dUTP (DIG, Boehringer, Mannheim, Germany) by nick translation as described by Volkers (Volkers et al., 1988). DIG incorporation was checked by spot blotting and detection with mouse-anti-DIG alkaline phosphatase-conjugated antibodies and 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium (BCIP/NBT) as substrate. The specificity of the probe was checked by Southern hybridisation on digested genomic DNA of strain MC4100 (Sambrook et al., 1989).

2.3.3 Preparation of cells for fluorescence microscopy

Cells were washed in PBS (140mM NaCl, 27mM KCl, 10mM Na$_2$HPO$_4$ and 2mM KH$_2$PO$_4$, pH 7.2) and post-fixed in 0.5% formaldehyde and 0.04% glutaraldehyde in TBS (10mM Tris and 0.9% NaCl) for 15 min at room temperature. Fixed cells were centrifuged at 8,000 × g for 5 min and washed three times in PBS and subsequently incubated in 0.1% Triton X-100 in PBS for 45 min at room temperature. Cells were washed three times in PBS and incubated in PBS containing 100 mg/ml lysozyme and 5 mM EDTA (ethylene-diamine-tetra-acetate.2H$_2$O) for 45 min at 37 °C. After washing three times in PBS and once in 2xPBS/SSC (sodium salt citrate; 0.15M NaCl, 0.015M sodium citrate) the cells were incubated with 100 µl of 100 mg/ml RNase A (Boehringer, Mannheim, Germany) in 2xPBS/SSC for 60 min at 37 °C. After washing three times in PBS, 10 µl of cell suspension was applied to slides coated with 0.01% poly-L-lysine, covered with a coverslip (15 mm diameter) and left for 20 min at room temperature. Then the slides were washed in a coplin jar with 2xSSC and
put on top of coverslips with 10 μl of probe solution. The preparations were denatured for 5 min at 80 °C. Hybridisation occurred overnight in a moist chamber with 2xSSC and 50% formamide at 37 °C. Unbound probe was washed three times for 10 min at 40 °C in a coplin jar with 1xSSC and 50% formamide (pH7). Slides were subsequently washed in 2xSSC and TN buffer (100 mM Tris-HCl, pH 7.5, 150 mM NaCl).

Prior to immunofluorescence staining non-specific binding sites were blocked by incubating the cells in 0.5% (w/v) blocking reagens (Boehringer) in PBS for 60 min. at 37 °C. Digoxigenin-labelled probe detection was carried out in TNB with mouse-anti-digoxigenin (Boehringer 1333062), 1:500 in TNB, rabbit-anti-mouse conjugated Cy3 (Jackson ImmunoResearch Laboratories, Inc., West Grove, USA), 1:400 in TNB and goat-anti-rabbit conjugated Alexa 546 (Molecular Probes Inc., Eugene, USA), 1:600 in TNB. Slides with antibodies were incubated in a humid chamber at 37 °C for 60 min. Between incubations with antibodies, the slides were washed three times 5 min in TN with 0.05% (v/v) Tween 20. After immunodetection slides were rinsed in PBS/TNB and taken up in TE buffer (10 mM TrisHCl and 1mM EDTA). The preparations were mounted in 5 μl PBS with DAPI (500 ng/ml 2,4-diamidino-phenyl-indole).

2.3.4 Microscopy and image analysis

Preparations were photographed with a cooled Princeton CCD camera mounted on an Olympus fluorescence microscope (BH2-RFC; Olympus, Tokyo, Japan) equipped with a 100 W mercury lamp. Images were made using the program IPlab spectrum 3.0 (Signal Analytics Co., Vienna, USA). Cells were first photographed in phase contrast mode, then with an Alexa filter (illuminated at 510-550 nm with an emission filter of 590 nm) and finally with a DAPI fluorescence filter (illuminated at 300-400 nm with an emission filter of 420 nm). Length, positions of foci and nucleoid length of each cell were determined interactively. Interactive measurements were performed as 'structured point collection' on a Macintosh 7100 computer using the public domain program Object-Image.
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2.3.5 Shift correction

The position of a single object imaged using different filter combinations can be slightly different. To correct for this shift in position, we determined the centre of gravity of spheres (1.01 μm diameter) that are visible in phase contrast, Alexa and DAPI images, respectively. Preparations of these spheres were made and from a single position on the slide ten times three images were made: a phase contrast image, an Alexa-image and a DAPI-image. As correction factors we used the average distance between the centre of gravity of corresponding spheres in a phase contrast image and an Alexa image and the average distance between corresponding spheres in a phase contrast image and a DAPI image.

2.4 Results

2.4.1 Temporal relationship between DNA replication cycle and cell division cycle

Under certain conditions E. coli initiates replication before cell division resulting in multiple copies of oriC during a cell cycle (Helmstetter, 1996). Therefore, we estimated the number of oriC copies per cell length using data from Huls et al. (Huls et al., 1999). These authors determined the C and D period by measuring the DNA content per cell by integrated-density cytometry (Vischer et al., 1999). We used the same strain growing under the same conditions as Huls et al. did (Huls et al., 1999). Of this steady state culture with a \( T_d \) of 79 minutes, the C period was approximately 70 minutes and the D period was approximately 43 minutes (Huls et al., 1999). A value for the C period of 70 min for cells grown at 28 °C is comparable to the period of 55 min for 37 °C-culture of E. coli cells as determined by Bipatnath et al. (Bipatnath et al., 1998). By subtracting the sum of C and D from \( T_{dl} \), the time of initiation of DNA replication was calculated to be \(-33\) minutes, indicating that replication
Position of oriC in relation to cell length

To study the position of the origin of replication in relation to cell length we fixed cells from the aforementioned culture of strain MC4100 and used a 6kbp sequence containing oriC for a FISH experiment. Of 871 cells analysed, 692 (79%) contained fluorescent foci. We saw one focus in 312 cells (36%), two foci in 261 cells (30%), three foci in 77 cells (9%), four foci in 381 cells (4%), and five foci in 4 cells (<1%). In cells with fluorescent foci we measured the distance from each focus to the nearest cell pole (fig. 2.2). We found foci close to a cell pole and foci closer to mid-cell. Of foci close to a cell pole, the average distance between focus and pole was 0.5 μm (CV = 47%). As can be judged from the regression lines through the foci positions with respect to the two cell poles (fig. 2.3) the focus-to-pole distance remains largely constant. This indicates a gradual and continuous separation of the most outwardly positioned replicated origins.
2.4.3 Position of oriC in newborn cells and at initiation of DNA replication

The average length of a newborn cell was estimated using the cell length distribution of constricting cells (cf. Koppes, L. H. et al., 1978). This value is indicated as an interrupted horizontal line in figure 2.3. At this length newborn cells have two copies of oriC (see above). The location of the two regression lines in the newborn cell indicates that the origins have separated and that their relative positions are at approximately $\frac{1}{2}$ and $\frac{2}{3}$ of cell length. Where is oriC located at initiation of DNA replication? From the calculated C-period, its temporal position in the cell cycle (fig. 2.1) and from the known relationship between cell age and cell length ($l = e^{T_d \cdot \ln 2}$, Helmstetter, 1996), initiation of DNA replication is expected to start at an average cell length of 1.1 µm. (Note that this is the length of the prospective daughter cell in the previous cell cycle; see fig. 2.1). One approach to assess oriC position at this cell length is to extrapolate the regression lines to the point where they cross (fig. 2.3). This occurs at a cell length of 0.82 µm. The estimated cell length at which replication initiated was within the 95% confidence interval around this length (i.e., between 0.5 and 1.2 µm). Though oriC thus seems to be located at the centre of the prospective daughter cell upon initiation of DNA replication, determination of its precise position is hampered by the limitations of the cytological technique.

2.5 Discussion

2.5.1 Replication starts before cell division and newborn cells have two origins

The $C+D$ period of the E. coli strain we used was 33 minutes longer than the doubling time (Huls et al., 1999), which means that a large part of the chromosome is replicated before cell birth. Hence, we should expect two origins of replication in newborn cells and four origins of replication in cells before division. Indeed, we found newborn cells with two foci and we found that the number of foci per cell increased with cell length.
2.5.1 Replication starts before cell division and newborn cells have two origins

Figure 2.2 - Analysis of oriC localisation in E. coli using FISH

Cell length and distances of foci from a cell pole were measured as shown in A: one of the poles was arbitrarily chosen as reference pole. Distances of foci closest to this pole were measured directly (cyan and blue arrows). Distances of foci closer to the other pole were derived by subtracting the distance to the nearest pole (grey arrows above cells) from cell length (grey arrows below cells). B: plot of polar distances, measured as shown in A, against cell length of 692 cells. Black crosses represent polar distances of cells with a single focus, filled cyan triangles represent distances of the foci closest to the reference pole, filled magenta circles represent distances of the foci furthest from the reference pole, open blue triangles and open red circles represent distances of the remaining foci in cells with more than 2 foci.

Figure 2.3 - Regression analysis of oriC localisation in E. coli during steady state growth

Data points from figure 2.2 of foci close to a cell pole were used for regression analysis. The regression equation of the cyan line is: \( d1(l) = 0.37 + 2.5 \times 10^{-3} l \) µm, where \( d1(l) \) is the distance of a focus from the nearest pole and \( l \) is the cell length. The slope was statistically not significant (\( p>0.05 \), standard error: \( 1.3 \times 10^{-3} \)). The equation of the magenta regression line is: \( d2(l) = 1 - d1(l) \). The extrapolated cross-point of the two regression lines represents the putative separation point of two replicated origins. The grey area represents the 95% confidence interval of this cross point. Black and grey horizontal lines indicate the cell lengths at which replication initiates and terminates, respectively. The interrupted horizontal lines represent the cell lengths at birth (1.5 µm, CV=12%) and division, respectively.
However, the number of foci did not always correspond to the number of origins expected. We presume this to be due partly to a less than 100% labelling efficiency of the FISH technique and partly because cells could have been impermeable to probe DNA and/or fluorescent antibodies.

The resolution of our method is limited by the optical resolving power and by biological variation. Part of this variation can be explained by variation in cell length (Koppes, L. H. et al., 1978), but variation in the position of oriC itself cannot be excluded. To understand this variation we could speculate that two sources of oriC movement exist. The first source of movement is related to chromosome separation, which we believe is gradual and continuous. The second source might be Brownian motion, which would result in movement within a confined region of the nucleoid, similar to what has been found in eukaryotic cells (Marshall et al., 1997). Our regression lines correspond to the first source of movement; part of the variation in oriC position will correspond to the second source of movement.

2.5.2 Localisation of oriC at initiation of DNA replication

The position of oriC when DNA replication starts could, in principle, be assessed by estimating the cell length at which four foci first appear, or by attempting to draw regression lines through the innermost pairs of oriC foci and estimate where they cross the regression lines through the outward ones. However, such an analysis appeared not sufficiently reliable because of less than hundred percent FISH efficiency, variation in cell length and in oriC position as well as the limited resolution of light microscopy. Nevertheless, our findings indicated that on average the distance between oriC foci and cell pole is almost constant for all cell lengths (fig. 2.2). This would mean that initiation of DNA replication starts (by definition) in the centre of a prospective daughter cell. This is in line with electron microscopic auto radiography data on slow growing E. coli B/r K showing that DNA replication takes place in the cell centre (Koppes, L. J. et al., 1999).
2.5.3 Separation of origins

During the cell cycle the origins move apart gradually (fig. 2.4) In other words, the interpretation of our data does not require the assumption of a relatively fast movement of a just-duplicated oriC copy to the opposite pole (cf. Niki and Hiraga, 1998).

Gordon and coworkers visualised the vicinity of the origin region by binding of GFP-LacI fusion proteins to multiple lac operator sequence insertions (Gordon et al., 1997). How do our data compare to theirs? These authors used a rich medium (L broth) and a growth temperature of 20 °C. The DNA replication cycle under those conditions was not determined. In both cases (Gordon et al., 1997 and see above) two fluorescent foci occurred in newborn cells and four in older cells and the four foci population seemed to arise gradually from the two-foci ones.

A different picture emerged when applying time-lapse microphotography (Gordon et al., 1997). In this case a duplicated focus appeared to move at a speed exceeding that of cell elongation. However, under time-lapse conditions, cell growth became severely impaired. It is unclear whether this affects oriC partitioning and therefore we have no adequate explanation for the discrepancy with respect to the speed of oriC movement between our results and those of Gordon et al. (Gordon et
2.6 References


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