Organization of ribonucleoside diphosphate reductase during multifork chromosome replication in *Escherichia coli*

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Ribonucleoside diphosphate reductase (RNR) is located in discrete foci in a number that increases with the overlapping of replication cycles in *Escherichia coli*. Comparison of the numbers of RNR, DnaX and SeqA protein foci with the number of replication forks at different growth rates reveals that fork : focus ratios augment with increasing growth rates, suggesting a higher cohesion of the three protein foci with increasing number of forks per cell. Quantification of NrdB and SeqA proteins per cell showed: (i) a higher amount of RNR per focus at faster growth rates, which sustains the higher cohesion of RNR foci with higher numbers of forks per cell; and (ii) an equivalent amount of RNR per replication fork, independent of the number of the latter.

INTRODUCTION

Slowly and rapidly growing bacterial cells face different organizational challenges during chromosome replication and segregation. In slowly growing bacteria, the cell cycle is similar to that of eukaryotic cells, with initiation and termination of chromosome replication taking place in the same cycle. In rapidly growing bacteria, replication can be longer than a generation time, and initiation and termination occur in different cycles. If replication initiates before the previous round is completed, replication cycles overlap and chromosomes have multiple replication forks assembled at different cell cycles (Cooper & Helmstetter, 1968). Accordingly, chromosome replication is controlled at its initiation step to ensure that only one initiation event takes place at each cell cycle, which, independently of the time required for replicating the entire chromosome, assures that only one chromosome termination takes place every cell cycle to allow cell division.

Colocalization of the DNA polymerases from the two forks originating from a single initiation event was initially proposed by Dingman *et al.* (1974). Other authors suggested that forks are paired for a time and then move in opposite directions (Bates & Kleckner, 2005; Hiraga *et al.*, 1998; Reyes-Lamothe *et al.*, 2010). Thus, organization of the replication apparatus is still controversial. Recent works have reported a dynamic organization of the replication forks and sister chromosomes: the extent of fork colocalization was found to be dependent on the replication pattern (Molina *et al.*, 2008; Morigen *et al.*, 2009), and origin cohesion has been described as being dependent on growth rate (Adachi *et al.*, 2008; Fossum *et al.*, 2007).

Chromosome replication requires synthesis of the four dNTPs at a high rate. Ribonucleoside diphosphate reductase (RNR) is an essential enzyme for biosynthesis of the four dNTPs in all living cells. The class I RNR, which predominates in aerobically grown cells, is a tetramer made of two homodimers: subunit R1, encoded by the *nrdA* gene, and subunit R2, encoded by the *nrdB* gene. There has been, for a long time, a discrepancy between the high demand of dNTPs for every replication fork and the low pools detected in bacteria. To resolve this discrepancy, the replication hyperstructure model postulates that all replicative proteins, including the dNTP-biosynthesis complex, are recruited at the initiation of the replication process. According to this model, RNR embedded in a hyperstructure would provide the dNTPs required for its associated replication fork (Guzmán *et al.*, 2002; Mathews *et al.*, 1993; Norris *et al.*, 2007).

We have recently published the first evidence for a close relationship between RNR and the replication proteins in slow-growing bacteria (Guzmán *et al.*, 2002; Sánchez-Romero *et al.*, 2010b). Here, we have performed the first study to our knowledge on the organization of RNR, a replisome protein, DnaX, and a replication-fork marker, SeqA, in cells with different patterns of chromosome replication and we have compared them with the replication forks.
METHODS

Bacterial strains and growth conditions. The strains used were derived from Escherichia coli K-12 CM735 (metE46, trp3, his4, thi1, galK2, lacY1, metII, ara9, tsx3, ton1, rps8, supE44). CM7931 contains a 3 × FLAG-tagged nrdB gene and CM7933 contains a haemagglutinin (HA)-tagged dnaX gene (Sánchez-Romero et al., 2010b). Bacteria were incubated at 37 °C in M9 minimal medium containing glycerol, glucose or glucose plus Casamino acids (CAA), and samples were taken at mid-exponential phase (OD550 0.1).

Cell-cycle parameters. Growth rates of exponentially growing bacteria were determined by the OD650 of the cultures. Cell-cycle parameters and the number of forks per cell were obtained for flow cytometry. For these measurements, cells were treated with 150 µg rifampicin ml⁻¹ and 50 µg cephalin ml⁻¹ to inhibit initiation of replication and cell division, respectively. After 3 h treatment, cells were fixed and the number of chromosomes per cell was measured by flow cytometry using a FACStar DIVA (Becton Dickinson) instrument, essentially as described previously (Molina & Skarstad, 2004). The time required for chromosome replication, , and the time from the termination of replication to cell division, , were determined from the number of cells before and after the initiation of replication in the initiation time equations (Jiménez-Sánchez & Guzmán, 1988).

Immunofluorescence microscopy. Subcellular location of RNR was analysed by using a FLAG-tagged RNR and immunolocalized by treating ethanol-fixed cells with mouse monoclonal anti-FLAG M2–Cy3 antibody (Sigma-Aldrich) in CM7931. Mouse monoclonal anti-HA antibody and secondary anti-mouse antibody conjugated to FITC were used to immunolocalize the subunit of DNA polymerase III (encoded by the dnaX gene) in strain CM7933. Immunostaining of the SeqA protein was performed using a specific SeqA antibody as described previously (Fossum et al., 2007; Sánchez-Romero et al., 2010b). Nucleoids were stained with Hoechst 33258 and micrographs were obtained by using an Olympus IX-70 Delta Vision fluorescence microscope equipped with a 100 × UPLS Apo objective. Pictures were taken using a CoolSNAP HQ/ICX285 camera and deconvolved using the Delta Vision constrained iterative deconvolution software (Applied Precision). The deconvolved images were saved in TIF format and imported into ImageJ software (Wayne Rasband, Research Services Branch, National Institute of Mental Health, MD, USA). Any significant differences in focus scoring were found between deconvolved and not-deconvolved images (see Supplementary Fig. S1, available with the online version of this paper).

Western blot. Whole-cell protein extracts in each medium were analysed by Western blotting using anti-FLAG–Cy3 mouse monoclonal antibody (Sigma-Aldrich) for RNR and specific antibodies against the SeqA and OmpA proteins (gifts from K. Skarstad’s and I. Henderson’s laboratories, respectively). Western blotting was repeated three times.

RESULTS AND DISCUSSION

Organization of RNR depends on the replication overlapping

The aim of this work was to investigate whether the organization of RNR relies on the type of cell cycle, i.e. the number of replication forks per cell. To this end, we studied the distribution of the number of replication forks and that of RNR foci per cell under different growth conditions.

E. coli strain CM7931 was grown at 37 °C in medium containing glycerol, glucose or glucose with CAA and samples were taken for flow cytometry and fluorescence microscopy. Cell-cycle parameters and the distribution of replication forks per cell were obtained from flow-cytometry DNA histograms (Fig. 1). As expected, the overlapping of replication rounds increases as growth rate increases and so does the number of replication forks. Whilst slowly growing cells show no overlapping of replication cycles (Fig. 1d), fast-growing cells show an extensive overlapping of consecutive replication cycles (Fig. 1f) and carry up to 12 replication forks for a short period of the cell cycle (Fig. 1i).

Fluorescence microscopy images show that RNR is located in discrete foci in cells growing at any growth rate (Fig. 2, Supplementary Fig. S1, Supplementary Movie S1). The subcellular position of RNR foci in slow-growing cells reveals that there are cells with a single focus localized at the middle position of the cell, with two RNR foci localized at the one-quarter and three-quarter positions of the cell, with three foci at one-, two- and three-quarter positions, or with four RNR foci distributed along the cell length (Fig. 3). This distribution is essentially the same as has been described for the replication-fork foci (Hiraga et al., 1998; Ohsumi et al., 2001).

Previous works have reported that as the overlapping of replication cycles increases, so does the cohesion of replication-fork and replication-protein foci. To assess the degree of cohesion of RNR foci under different replication patterns, the mean number of replication forks per RNR focus was studied as a cohesion index. As foci from coupled forks are detected as a single focus, fork focus ratio has been used as a way to understand the organization of fork-associated proteins (Molina et al., 2008). To compare the cohesion of RNR foci with that of replication forks and other replication-protein foci, fork focus ratios corresponding to cultures with different replication patterns from previously published data (Adachi et al., 2008; Bates & Kleckner, 2005; Brendler et al., 2000; Fossum et al., 2007; Molina & Skarstad, 2004; Sánchez-Romero et al., 2010b; Sunako et al., 2001) and from our results were contrasted (Fig. 4). In cells with one replication cycle per chromosome, i.e. two forks per cell, the number of RNR foci corresponds to the number of replication forks throughout the cell cycle. Therefore, the sister replication forks, each associated with an RNR focus, move away from each other and show the lowest degree of cohesion (Fig. 4). An increase in the number of forks per cell is correlated with an increase in the cohesion degree of forks and foci, with values even higher than full cohesion (i.e. more than two forks per focus), suggesting the transitory grouping of forks from two consecutive replication cycles. The observed cohesion of RNR foci shows a high correlation with published data when cells were grown in glycerol or glucose medium, but this cohesion was lower than expected for the fast-growing bacteria in glucose plus CAA (fork: focus ratio of approx. 1.5, when a ratio of approx. 2 would be expected).

These results lead us to conclude that the organization of RNR foci is dependent on the replication pattern and
reproduces that of other replication proteins in slowly growing cells, whereas in fast-growing cells, the number of RNR foci is somewhat greater than the number of foci of replication proteins, which could indicate pre-assembly of the required proteins before initiation of replication.

**Correspondence among the number of foci of RNR, DnaX and SeqA**

To study the relationship between RNR foci and replication-protein foci, we studied the number of foci of the tagged replisome protein DnaX, the DNA polymerase III \( \tau \) subunit and the SeqA protein, a binding protein for hemimethylated GATC sites on newly replicated DNA, which has often been used as a marker of replication forks (Molina *et al.*, 2008; Sánchez-Romero *et al.*, 2010a).

Comparison of the mean number of RNR foci per cell with those of DnaX and SeqA, and with replication forks shows that, in cells growing in glycerol, the numbers of foci per cell of the three proteins are very similar to the number of forks (Fig. 5). Furthermore, the mean number of foci of all proteins and forks increases with increasing growth rate, although not to the same extent (Fig. 5). The higher ratio of fork number to RNR, SeqA and DnaX foci at high growth rates suggests an increased grouping or cohesion of the three protein foci with increasing number of forks per cell.

**The amount of RNR protein per fork is constant at any growth rate**

As the number of replication forks per RNR focus was found to increase with growth rate, we wanted to find out how the amount of NrdB protein correlates with the number of RNR foci and replication forks. For this purpose, the levels of NrdB, SeqA and OmpA (an outer-membrane protein) from bacteria growing in medium with glycerol, glucose or glucose plus CAA were measured. As
expected, the total amount of these proteins per cell increased with increasing growth rates (Fig. 6a). To estimate whether the amounts of NrdB and SeqA are related to the number of forks under different growth conditions, we analysed protein:fork ratio at different growth rates. These results show that, essentially, the amounts of NrdB and SeqA proteins per fork do not vary with growth rate, whereas OmpA protein:fork ratio increases with growth rate (Fig. 6b) and, consequently, the amount of NrdB and SeqA proteins per focus increases.

Conclusions

Results reported in this work suggest a variable cohesion of RNR foci that depends on the number of forks per cell, in a similar way as has been reported for other replication proteins.

Comparative analysis of foci from RNR, DnaX (a replisome protein) and SeqA (a marker for replicating forks) shows that: (i) at low growth rate, most of the cells have the same number of RNR foci as DnaX and SeqA protein foci; (ii) the number of forks per cell increases with growth rate at a higher level than the number of the protein foci, denoting a higher cohesion of the foci at increasing number of forks per cell; and (iii) the numbers of RNR and DnaX foci are moderately higher than those of SeqA foci, which might be explained by pre-assembly of the replication proteins before initiation of replication (den Blaauwen et al., 2006).

Measurement of the amounts of RNR, of the replication-fork marker SeqA and of OmpA, a protein independent of replication, demonstrates a constant amount of RNR per fork, independent of the number of forks per cell and of the degree of RNR focus cohesion. Furthermore, the increased amount of both proteins per focus supports the existence of focus cohesion without disturbance of the composition of every replication hyperstructure.

Fig. 2. Selected fluorescence microscope images of strain CMT931 growing in minimal medium containing glycerol (a), glucose (b) or glucose plus CAA (c). RNR foci were detected by Cy3 red fluorescence. Nucleoids were stained with Hoechst 33258 (blue). Bar, 1 μm. Constrained iterative deconvolution was used for these images in order to highlight the position of the fluorescence. Therefore, intensities are not quantifiable.

Fig. 3. Subcellular localization of RNR foci in strain CMT931 growing exponentially at 37 °C in minimal medium containing glycerol. Cells containing one, two, three or four foci (a–d, respectively) are displayed separately. Each part of the figure is a superimposition of the positions of the RNR foci from individual cells.
These conclusions support the organization of the RNR protein into a higher-order structure, or replication hyperstructure, that does not depend on the association between forks. Consequently, each replication fork would incorporate its own machinery for the synthesis of its required precursors that would run independently of other hyperstructures, hence maximizing replication efficiency, facilitating the cell’s ability to adapt in a changing environment and leading, eventually, to cell viability.

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