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DIFFERENCES IN THE DEGREE OF INHIBITION OF NDP REDUCTASE BY CHEMICAL INACTIVATION AND BY THE THERMOSENSITIVE MUTATION *nrdA101* IN *Escherichia coli* SUGGEST AN EFFECT ON CHROMOSOME SEGREGATION

JOSÉ RIOLA, ESTRELLA GUARINO, ELENA C. GUZMÁN and ALFONSO JIMÉNEZ-SÁNCHEZ*
Departamento de Bioquímica y Biología Molecular y Genética, Facultad de Ciencias, Universidad de Extremadura, 06080-Badajoz, Spain

Abstract: NDP reductase activity can be inhibited either by treatment with hydroxyurea or by incubation of an $nrdA_{ts}$ mutant strain at the non-permissive temperature. Both methods inhibit replication, but experiments on these two types of inhibition yielded very different results. The chemical treatment immediately inhibited DNA synthesis but did not affect the cell and nucleoid appearance, while the incubation of an *nrdA*101 mutant strain at the non-permissive temperature inhibited DNA synthesis after more than 50 min, and resulted in aberrant chromosome segregation, long filaments, and a high frequency of anucleate cells. These phenotypes are not induced by SOS. In view of these results, we suggest there is an indirect relationship between NDP reductase and the chromosome segregation machinery through the maintenance of the proposed replication hyperstructure.

Key words: NDP reductase, Hyperstructure, Chromosome segregation, Hydroxyurea

INTRODUCTION

Ribonucleoside diphosphate reductase (NDP reductase) of *E. coli* is the prototype of the class I reductases common to most prokaryotes and eukaryotes from viruses to man. It is the only specific enzyme required, under aerobic growth, for the enzymatic formation of deoxyribonucleotides, the precursors of

* Author for correspondence; e-mail: a.jimenezsanchez@gmail.com

Abbreviations used: Cef – cephalexin; HU – hydroxyurea; NDP reductase – ribonucleoside diphosphate reductase

DNA synthesis. The active enzyme is a 1:1 complex of two subunits called proteins R1 and R2, each consisting of two polypeptide chains, coded by the genes *nrdA* and *nrdB*, respectively [1]. DNA replication requires a balanced supply of four dNTPs, which explains the complicated allosteric control of the enzyme. Although about 3000 nucleotides have to be consumed per second when a bacterium replicates its chromosome with two replication forks, only a very small pool of dNTP is accumulated in the cells. This pool would permit replication for no longer than half a minute [2, 3]. Channelling of the biosynthesis and compartmentation of the precursors have been proposed as explanations of how this shortage may be circumvented [4, 5]. To satisfy the changing demand for the four deoxynucleotides, NDP reductase must be closely associated with the replication machinery. In the aforementioned studies, Mathews *et al.* found evidence for the association of this enzyme with others related to precursor biosynthesis, and coined the term dNTP-synthesizing complex [4].

Chromosome replication and cell division are normally precisely coordinated events in the cell cycle. The best example of this coordination is the inhibition of cell division by the SulA protein as part of the SOS response to DNA damage or replication interruption [6]. During the SOS response, the induced SulA protein inhibits cell division by inhibiting the polymerization of FtsZ protein and the formation of the septal ring [7, 8]. Although there is much work showing that blocking cell division by interrupting chromosome replication is SOS dependent [9, 10], an SOS-independent inhibition of cell division by blocking replication has been reported on [11]. Here, after blocking replication by inhibiting the same enzyme in two different ways, we found no filamentation in one case and SOS-independent filamentation in the other.

In a previous study, we found evidence for an association of NDP reductase, and therefore of the proposed dNTP-synthesizing complex, with the replication machinery to constitute the replication hyperstructure [12, 13]. Molina and Skarstad [14] assumed this proposed replication hyperstructure to explain how the organization of the replication factory was dependent on nucleoid metabolism. In this study, we investigated the effects of inhibiting NDP reductase activity upon replication, chromosome segregation, and cell division. Based on our findings, we suggest an indirect relationship between NDP reductase and the chromosome segregation machinery through the maintenance of the replication hyperstructure.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Escherichia coli JS1018 (*nrdA101 thyA arg his thi malA rpsL mtl xyl su*) is a Pol^+ Thy^- low requirement derivative from strain E1011 (received from R. McMacken, Stanford University, Stanford, CA, USA). JK607 is JS1018 $nrdA^+$ made by P1 cotransduction from MG1655 yfaL::Tn5 (received from F. R. Blattner, Wisconsin,

USA). JK625 is JK607 Δ(*recA-srl*)::Tn10 and JS627 is JS1018 Δ(*recA-srl*)::Tn10; both strains were obtained by P1 transduction from JJC275. JJR750 is JK607 *sulA*::*MudAp*^{R}*lacZ* and JJR751 is JS1018 *sulA*::*MudAp*^{R}*lacZ*; both strains obtained by transduction from JC19008. Strains JJC275 and JC19008 were received from B. Michel, Jouy en Josas, France.

All the cultures were grown by shaking at 30ºC, unless otherwise indicated, in M9 minimal medium, containing M9 salts, 2 μg ml^{-1} thiamine, 0.4% glucose, 20 μg ml^{-1} of required amino acids, 0.2% casamino acids and 5 μg ml^{-1} thymidine. Cultures were grown overnight and diluted 200 times to obtain an exponentially growing population. Growth was monitored by following the optical density at 450 nm. All the treatments were initiated at an optical density of 0.05. Hydroxyurea (HU) was freshly prepared and used at a final concentration of 50 mM.

DNA synthesis measurements

DNA synthesis was determined by growing the cells in MM9 medium containing 1 μCi/ml of [methyl-^{3}H]thymidine (20 Ci/mmol) (ICN). The isotope was added to both the overnight and exponential cultures. TCA-insoluble material from 0.2 ml aliquots was measured using a Beckman LS3801 scintillation counter. All the measurements were made relative to the radioactive counting at the time of initiating the treatments; that always oscillated around 5000 dpm.

Microscopy and flow cytometry

Phase-contrast and fluorescence micrographs were obtained by treating the cultures with 100 μg ml^{-1} chloramphenicol for 15 min to enhance the visualization of nucleoids before fixing with ethanol and staining with DAPI. A Nikon Eclipse E600 microscope with a Hamamatsu C4742-95 camera were used to transfer micrographs to a Macintosh G4 computer. Phase-contrast micrographs were obtained in sufficient numbers to score at least three hundred cells for cell size measurements. These measurements were performed using IPLab 3.9. Beads of 2.5 μm (Bio-Rad) were used for size reference. Anucleated cells were counted by direct observation under a fluorescent microscope from a minimum of one thousand cells. Particle counting was carried out using a Neubauer chamber. DNA content and mass per cell were measured by fluorescence and light scattering, respectively, using a Bryte HS (Bio-Rad) flow cytometer essentially as described in the literature [15].

RESULTS

The effects of HU or the non-permissive temperature on DNA synthesis

Continual activity of NDP reductase is an essential requirement for continuous DNA synthesis, and its inhibition stops replication immediately. We studied the effects of NDP reductase inhibition by hydroxyurea and by non-permissive temperature incubation of a strain bearing allele *nrdA*101.

Exponentially growing cultures of JK607 (*nrdA*$^+$) and JS1018 (*nrdA*$_{ts}$) were treated with HU, and JS1018 was also shifted from 30ºC to 42ºC (Fig. 1). While the drug stopped DNA synthesis immediately in both strains at any temperature, the shift to the non-permissive temperature yielded residual DNA synthesis in JS1018. This residual synthesis can be explained by the inhibition of the NDP reductase after a roughly 50-minute thermoresistant period at the non-permissive temperature *in vivo*, randomly arresting elongation. By contrast, rifampicin addition inhibits the initiation of replication, but does not affect elongation and gives full replicated chromosomes [12].

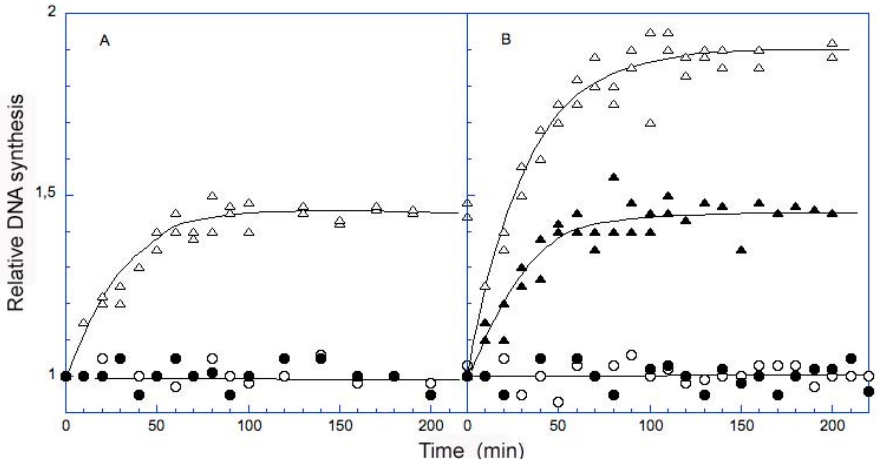

Fig. 1. Relative DNA synthesis of strains JK607, *nrdA*$^+$ (A), and JS1018, *nrdA*$_{ts}$ (B), after the addition of HU at 30ºC (open circles) or at 42ºC (closed circles), or after the shift from 30º to 42ºC (closed triangles), or after the addition of rifampicin at 30ºC (open triangles) at zero time.

There was a replication runout after the addition of 90% rifampicin to JS1018 growing at the permissive temperature with a mass and DNA doubling time of 70 min. This means that this strain has a long C period at 30ºC. As rifampicin inhibits the initiation of replication in this strain just after its addition, and flow cytometry shows that initiations are synchronic [12], the data permits the derivation of a C period of 154 min for JS1018 exponentially growing at 30ºC. This long elongation period, compared with mass doubling, means this strain has 2.2 overlapping replication cycles per chromosome.

The effects of HU or non-permissive temperature on cell division

To study the effect of the inhibitory treatments on cell division, we measured cell size after 4 hours in the presence of HU at 30ºC, and after incubation at 42ºC (Tab. 1). Even though the mass of the culture doubled in the presence of HU, the cell size in both strains was only slightly affected (Tab. 1). The obvious explanation for this result was the existence of cell divisions in the absence of

Tab. 1. Cell size (in μm) and percentage of anucleate cells obtained after 4 h of inhibition of NDP reductase by HU or by restrictive temperature.

Strain	Drug	30ºC		42ºC	
		cell size	anucleates	cell size	anucleates
JK607	-	2.0±0.6	<0.1	1.7±0.8	<0.1
	HU	2.4±1.6	0.7	2.2±0.9	0.6
	HU + Cef	6.1±3.4	<0.1	7.8±4.1	<0.1
JS1018 (*nrdA101*)	-	3.4±1.0	0.4	17.1±14.6	38.5
	HU	4.1±1.1	5.0	6.2±2.2	7.5
	HU + Cef	9.3±6.1	<0.1		
JK625 (Δ*recA*)	-	1.9±0.7	0.4	2.0±0.7	1.0
JJR750 (Δ*sulA*)	-	1.8±0.6	0.5	1.9±0.7	0.7
JS627 (*nrdA101* Δ*recA*)	-	3.2±1.0	2.0	15.9±16.1	42.5
JJR751 (*nrdA101* Δ*sulA*)	-	3.0±1.1	1.5	16.8±15.7	44.0

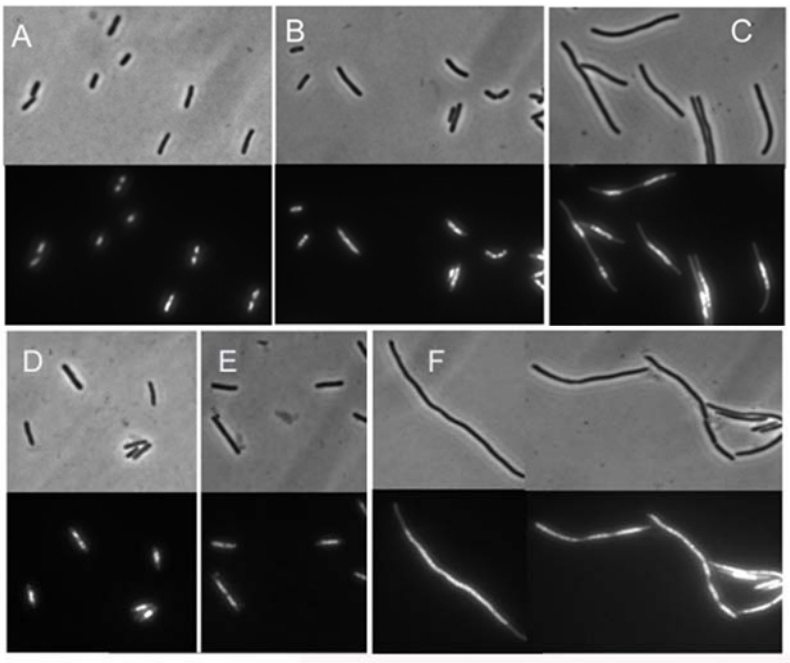

Fig. 2. Phase-contrast and fluorescence micrographs of DAPI-stained JK607, nrdA+ (A, B, C) or JS1018, nrdAts (D, E, F), growing exponentially at 30ºC (A, D), after 4 h with HU (B, E), or after 4 h with HU and cephalexin (C, F).

DNA replication. To check this explanation, cells were treated with HU and cephalexin, an inhibitor of cell division. Under this treatment, a three-fold increase was observed in the size of cells of both strains, as was to be expected if inhibition of replication ended in inhibition of cell division (Tab. 1, Fig. 2).

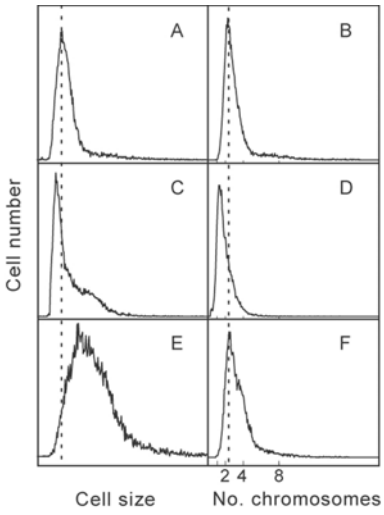

Fig. 3. Cell size and DNA content distributions measured by light scattering (A, C, E) and fluorescence (B, D, F) of JK607, *nrdA*$^+$, growing exponentially (A, B), after 4 h of treatment with HU (C, D), or after 4 h of treatment with HU and cephalexin (E, F).

These cell divisions in the presence of HU were confirmed by the increase in the number of particles counted after the treatments (data not shown). Furthermore, cytometry of JK607 showed that, in the presence of HU, cell size underwent very little variation, and DNA content per cell dropped by half (Fig. 3), but when cell division was inhibited by cephalexin in addition to HU, cell mass increased considerably and DNA content did not change. Analogous results were obtained with JS1018 (data not shown). Similar results were obtained with JK607 when the drug was added together with a temperature shift-up, indicating that the various alterations in cell metabolism caused by the heat were of no consequence for the effects being studied in here.

Incubation of JS1018 at 42ºC showed a severe effect upon cell division concluding in long filaments on average five times longer than cells grown at 30ºC, and with some filaments as much as ten times the normal length (Tab. 1, Fig. 4). At this non-permissive temperature, some cell division still occurs, most frequently near the cell poles, as was observed in the micrographs, generating a great number of anucleate cells with an average size of 2.3 μm. When JS1018 was treated with HU at the time of the shift to the non-permissive temperature, cell size increased 1.8-fold (Tab. 1). This result confirms the instantaneous effect of HU as against the retarded action of heat on the *nrdA101* mutant strain.

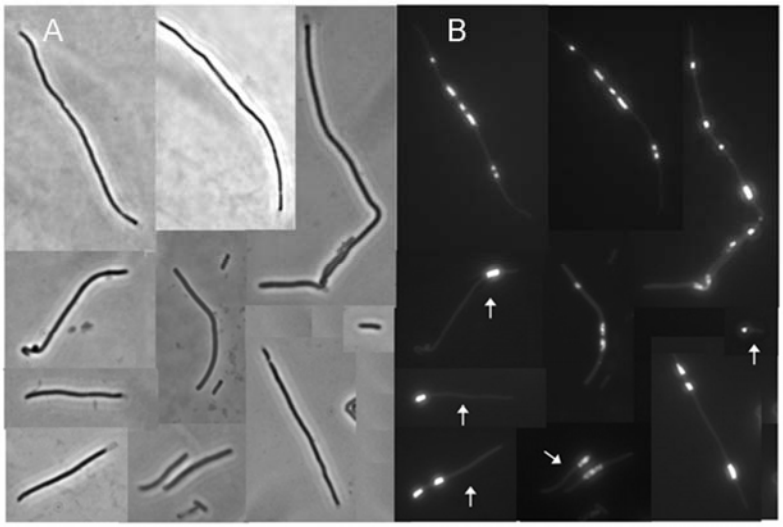

Fig. 4. Phase-contrast (A) and fluorescence micrographs (B) of DAPI-stained JS1018, *nrdA*$_{ts}$, after 4 h of incubation at 42ºC. Cells with one or two nucleotides located at the cell pole are marked. The figure is a composite of different fields and over-represents the number of cells with altered nucleoids in the population.

In a study using a different *nrdA* allele, Taschner [16] found a slight filamentation that was explained by the induction of *sulA* gene expression as an SOS response. To determine whether the filamentation observed in our work was due to an SOS response, JK607 and JS1018 strains were constructed lacking *sulA* or *recA* genes. Both the *nrdA101* Δ*sulA* and *nrdA101* Δ*recA* strains showed the same filamentation at the non-permissive temperature as the single *nrdA101* mutant strain (Tab. 1, Fig. 5). These results allow us to conclude that the observed filamentation was not induced by the SOS response.

The effects of inhibitory treatments on chromosome segregation

The effects of HU treatment or of incubating at the non-permissive temperature on chromosome segregation were measured by the emergence of anucleate cells and by the analysis of nucleoid distribution by the fluorescence of DAPI-stained cells. HU treatment did not alter nucleoid position in JK607 or JS1018 (Fig. 2) and produced an indetectable or low fraction of anucleate cells (Tab. 1). The incubation of JS1018 at 42ºC produced 38% anucleate cells (Tab. 1), and most of the nucleated cells showed severe nucleoid segregation and position defects, such as cells having one or two nucleoids located at the cell pole (Fig. 4). For comparison purposes, JS1018 was treated for 4 h with cephalexin at 30ºC to observe the possible effect on the nucleoid structure of a filamentation that does

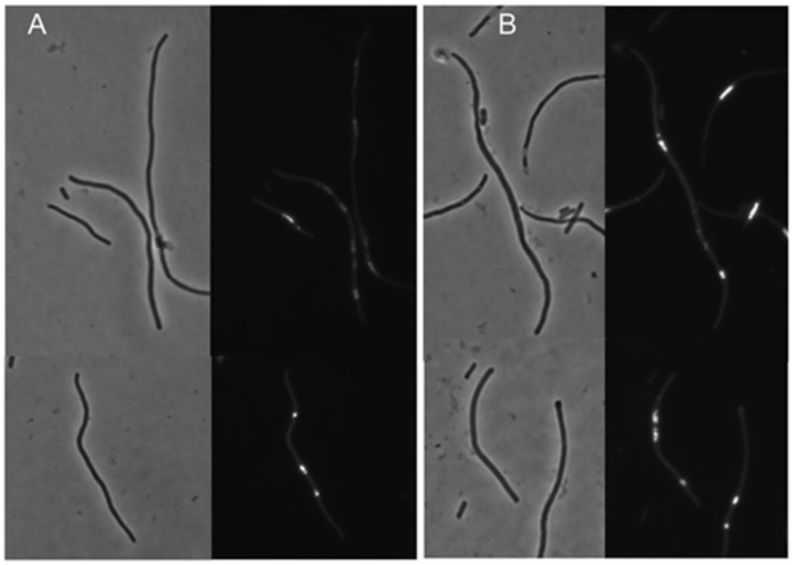

Fig. 5. Phase-contrast and fluorescence micrographs of DAPI-stained JS627, $nrdA_{ts}$ Δ*recA* (A) and JJR751, $nrdA_{ts}$ Δ*sulA* (B) after 3 h of growth at 42ºC.

not result from an effect on DNA replication. We observed a normal nucleoid segregation within the filament (data not shown). Despite the insufficient nucleoid condensation by chloramphenicol of the HU treated cells, no alterations in nucleoid structure were observed in HU- and cephalexin-treated cells (Fig. 3C and F). These results allow us to conclude that the extensive nucleoid alterations observed when JS1018 was incubated at the non-permissive temperature were not due to the enzymatic inhibition of the NDP reductase but to the alteration of its structure. If this enzyme were only related to DNA precursor biosynthesis, no effect on chromosome position, structure, or segregation should be observed. We therefore suggest that the effects of the high temperature upon nucleoid disturbances can be explained by the physical interaction of NDP reductase with the proteins responsible for nucleoid location and segregation.

DISCUSSION

In this study, we investigated the effects of inhibiting NDP reductase activity by inactivating its enzymatic activity with the specific inhibitor HU, or by altering its structure with the incubation of an *nrdA* thermosensitive strain at its non-permissive temperature. We were able to show a clear-cut difference in their consequences.

The HU treatment inhibited NDP reductase activity, and, as a consequence, replication forks arrested without any delay (Fig. 1). This result is in agreement with pool analyses of deoxynucleotides that suggest that the extremely low

concentrations of precursors are insufficient to permit replication for longer than a few minutes [2, 3]. By contrast, incubation of JS1018 at the non-permissive temperature, even though its NDP reductase is inactivated in less than two minutes *in vitro* [17], permits *in vivo* DNA synthesis for more than 50 min. This and other results led us to propose that NDP reductase has not just a functional but a structural implication in the replication process. Extending Norris' theoretical model [18], we call it the replication hyperstructure [12, 13], resulting from the association of the replication factory and Mathews' dNTP-synthesizing complex [4, 5].

The greatest difference in the effects caused by the chemical or thermal inactivation of NDP reductase was observed in the cell size and nucleoid structure. The HU treatment of different strains did not affect cell size. As the mass of the culture increased by more than one doubling after three hours (data not shown), this must mean that the cells divide. That division was not inhibited after inhibiting replication was observed by particle counting (data not shown). To look further into this idea, treatment with cephalexin, an inhibitor of cell division, together with HU showed the size the cell should have if division were inhibited (Tab. 1, Fig. 2C and F). The occurrence of cell division without replication caused a decrease in the amount of DNA per cell (Fig. 3D) and the appearance of some anucleate cells (Tab. 1) when cells were treated with HU. Inhibition of cell divisions by cephalexin when the bacteria were treated with HU circumvented both effects. The apparent contradiction between cell size and particle number increase in JS1018 at the high temperature is due to the high number of new small anucleated cells that appear at this temperature. The appearance of this number of small cells is the origin of the high standard deviation found at the non-permissive temperature. Fluorescence micrographs showed that after 4 h at the high temperature, JS1018 exhibited 38.5% anucleate cells. This high level of appearance of small cells with no nucleoid is most likely due to the formation of the septum close to the cell poles, which was frequently observed in the filaments. This filamentation, contrary to what was observed by Taschner *et al.* [16], most likely using a different allele, does not depend on *sulA* or *recA* gene expression, and therefore is not induced by the SOS response.

A further major difference between the chemical and thermal inactivation of NDP reductase was in the effect on nucleoid segregation. Micrographs showed that nucleoid organization was not affected by the addition of either HU or cephalexin to any strain at 30ºC, but it was affected by the incubation of JS1018 at 42ºC; in this latter case, the micrographs showed a broad range of alterations in the location and number of nucleoids. Both chemical and thermal inhibitory treatments affect the same enzyme, with the effect of the chemical treatment being more radical. Nevertheless, the thermal treatment produced a greater disturbance in the structure and distribution of nucleoids, which can only be explained in terms of the way the two treatments affect the enzyme. Whereas chemical inhibition does not affect protein structure, and is fully reversible in the absence of protein synthesis (data not shown), thermal inactivation irreversibly

modifies protein structure. This structural modification can affect other proteins in the complex that could influence activities not directly related to the mutant protein.

Other mutations in proteins more directly related to the replication process give very similar results. Mutation *dnaE486*, which codes for a thermosensitive DNA polymerase III, has residual DNA synthesis at the non-permissive temperature and induces abnormal nucleoid structure and filamentation very similar to what is observed with *nrdA101* (data not shown). Thermosensitive mutations in the *dnaA, dnaB*, or *dnaC* genes, which respectively code for the DNA initiation protein, the replicative DNA helicase, and the protein required for loading DnaB helicase, induce filamentation and anucleate cells at the non-permissive temperature [11]. Alleles *parB* and *dnaG2903*, which code for a thermosensitive DNA primase, have residual DNA synthesis at the non-permissive temperature and induce abnormal nucleoid structure [19]. Based on the hyperstructure proposed by Norris [18], we suggest a replication hyperstructure that would include the replisome or replication factory, and the dNTP-synthesizing complex in which NDP reductase would be directly integrated. The anchoring of the chromosome to this structure while replicating and the union of the hyperstructure to the membrane and to septum proteins could give the structural basis for the chromosome positioning and segregation required for cell division. This explanation may shed some light on how replication activity influences positioning and division sites in *E. coli*, as observed by Mulder and Woldringh [20]. A prediction of this model is that any inhibition of chromosome replication not affecting the conformation of the replication hyperstructure will not affect segregation and cell division; the inhibition of DNA synthesis with alteration of the replication hyperstructure, as with any physical interruption due to DNA damage, will end in the loss of its structural integrity and thus affect chromosome segregation and cell division. As DNA damage also induces the SOS response, which at the same time ends in the inhibition of cell division [6, 9, 11], this prediction could only be tested in treatments altering the replication hyperstructure but not inducing the SOS response, as was observed with JS1018 in this study.

We suggest that the association of NDP reductase within this hyperstructure could be a requirement not only for the synthesis of precursors for replication but also for the correct conformation of the hyperstructure required for the location of the replication fork and for chromosome segregation. In this sense it is interesting to mention the connection of topoisomerase IV (required for decatenation of sister chromosomes and segregation) with SeqA (a hemi-methylated DNA-binding protein located at initiation and at the replication fork [21]), and with FtsK (a translocase required to facilitate chromosome dimer resolution, to clear DNA from the closing septum, and to form septa), and with DnaX (which codes for the γ and τ subunits of DNA polymerase III holoenzyme [22]). Studies on *B. subtilis* support the idea that the replication factory, for most of the replication cycle with a fixed mid-cell location [23], is likely to be co-

ordinated with the Z-ring assembly [24]. More recently, the finding relating a heat-shock protein, such as GroEL, with FtsZ, supports the idea of a correct macromolecular conformation for cell division [18]; the appearance of an altered protein in the hyperstructure, such as the thermosensitive NDP reductase at high temperatures, might induce either effective sequestration or degradation of this protein, and hence prevent the Z-ring assembly. This evidence points to a connection of the putative replication hyperstructure with the segregation complex and with the septal machinery. The correct conformation and functionality of every part of this macromolecular assemblage would be a pre-requisite for the correct conclusion of the chromosome replication and cell division process.

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