UNIVERSIDAD DE EXTREMADURA

TESIS DOCTORAL

PAPEL DEL INICIO DE REPLICACIÓN EN LA LETALIDAD CAUSADA POR LA CARENCIA DE TIMINA

ROLE OF DNA REPLICATION INITIATION ON THE LETHALITY CAUSED BY THYMINE STARVATION

Mª CARMEN MATA MARTÍN

BIOQUÍMICA Y BIOLOGÍA MOLECULAR Y GENÉTICA.

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Conformidad del Director: Dra. ELENA GUZMÁN CABAÑAS

Fdo: Dra. Elena Guzmán Cabañas

A Elena y Encarna

To Manu and Sachin

La presente Tesis es un esfuerzo en el cual, directa o indirectamente, participaron varias personas trabajando, leyendo, opinando, corrigiendo, teniéndome paciencia, dando ánimo o acompañando, sin ell@s esto no hubiera sido posible,

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I developed an obsession about TLD. And
I wanted to learn the mechanism.
I still do!
Dr.Prof. Philip C. Hanawalt

However, despite all this information, the molecular
pathway associated with thymineless death
is made up of several large black boxes.
Dr.Prof. Justin Courcelle

Esa mirada casual fue el ORIGEN de un
cataclismo de amor que medio siglo después
aún no había terminado
Gabriel García Márquez

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ΔG	Run-out DNA synthesis
Amp	Ampiciline
CFU	Colony Forming Units
Cfx	Cephalexin
ChIC	Chromosomal Initiation Capacity
Cm	Chloramphenicol
C period	Replication period
DAPI	4′,6-diamidino-2-phenylindole dihydrochloride
DHFR	Dihydrofolate reductase
D period	Cell Division period
dpm	Desintegrations per minute
DSBs	Double Strand Breaks
dUmp	Dexosiuridine monophosphate
EthBr	Ethidium Bromide
^{3}H-TdR	Tritium thymidine
^{3}H-Thy	Tritium thymine
Hu	Hydroxyurea
^{3}H-Uridine	Tritium uridine
i	Proportion of origins initiated under the different treatments.
LB	Luria Broth (rich medium)
n	Number of overlapped replication
N	Number of replication forks
NAL	Nalidixic acid
NAT	Nutrient Agar
nmDNA	"non-migrating DNA"
OD	Optical Density
ON	Overnight

oriC	Replication origin region in *E.coli*
PCR	Polymerase Chain Reaction
PFGE	Pulse field gel electrophoresis
Rif	Rifampicin
RNR	Ribonucleotide reductase
τ	Generation time
Tc	Tetracycline
TCA	Trichloroacetic acid
TdR	Thymidine
Thy	Thymine
ti	Time of initiation of the replication
TLD	Thymineless death
TMP	Thymidilate synthase
TS	Thymidilate synthetase
tt	Time of termination of the replication
UR	Uridine
wt	Wild type

El fenómeno *muerte por carencia de timina*, (*thymineless death*,TLD) es la perdida de viabilidad que tiene lugar cuando células se mantienen en carencia de timina. Esta letalidad se manifiesta desde bacterias hasta células eucariotas.

En este trabajo se han desarrollado diferentes aproximaciones experimentales para determinar y explicar los procesos moleculares o mecanismos desencadenantes de TLD. A partir de nuestros resultados se ponen de manifiesto importantes ideas: (i) existe una correlación entre la magnitud de TLD y numero de horquillas de replicación, (ii) el daño en el DNA y la formación de estructuras complejas están asociadas a TLD, pero no son suficientes para explicarla (iii) la inhibición de nuevos inicios de replicación durante carencia de timina suprime TLD. Entre estas condiciones están: la adición de rifampicina, inactivación de la proteína DnaA, delecciones parciales alrededor de *oriC* o del propio *oriC*; indicando que el inicio de replicación es un punto critico para desencadenar letalidad en carencia de timina, (iv) bajo carencia de timina se generan intermediarios de replicación en el origen de replicación, *oriC* asociadas a la letalidad (v) la carencia de timina acelera el proceso de división celular en células que han terminado un ciclo de replicación, lo que supone el establecimiento de un nuevo mecanismo de control entre la replicación cromosómica y la división celular no descrito hasta el momento. Los resultados presentados aquí muestran un papel esencial de los nuevos inicios de replicación en TLD, abriendo nuevas vías de desarrollo para quimioterapias o tratamientos antifúngicos y antibacteriano.

The *thymineless death* (TLD) is the loss of viability that take place when growing cells are maintained in a growth medium lacking of thymine but other wise sufficient for continued growth. This lethality is observed from bacteria to eukaryotic cells.

We have developed different approaches to study the mechanism underlying TLD. From the results presented in this work we can conclude: (i) that the magnitude of TLD correlates with the number of the replication forks (ii) DNA damage and complex DNA structures are associated to TLD but they alone can not explain TLD (iii) TLD was prevented under conditions in which new initiation events are inhibited such a rifampicin addition, inactivation of DnaA protein, abolishing or increasing the required transcription level of genes surrounding *oriC* or even partially deleted *oriC* sequence (iv) occurrence of abortive initiations of chromosome replication occurred at *oriC* under thymine starvation being associated to lethality; supporting initiation events are critical targets for the lethal injuries under thymine starvation concluding in TLD (v) were determined a very precisely study cell dimensions and nucleoid morphology under thymine starvation and recovery conditions. Surprisingly we observed instant cell division in short-term thymine-starved cells. This effect has important implications for the relationship between DNA replication and cell division, as results presented here establish a new mechanism never described until now.

The relationship between cell death and the initiation of replication events in thymine-starved cells may provide new avenues for the development of improved either chemotherapies or antibacterial or antifungal treatments based on thymine starvation.

The goal of this work has been to elucidate the molecular mechanism by which thymine starvation kills the cell; phenomenon best-known as *thymineless death* (TLD). Given that thymine is exclusively incorporated during the DNA replication we studied several points mostly related to replication forks during or after the recovering from thymine starvation:

1. Relationship between TLD and the replication forks.
2. Fate of stalled replication forks generated under thymine starvation.
3. Role of chromosomal initiation on TLD.
4. Modulation of TLD by different approaches.
5. Morphological changes occurred in thymine starved cells and recovering after restoring the thymine.

Introduction

1. *Escherichia coli* as a model organism

Before going deeper into the ideas about thymineless death (TLD) phenomenon, I introduce *Escherichia coli*, our model organism. *E. coli* is a bacterium that normally grows in the lower intestine of warm-blooded organisms that the german scientist Theodor Escherich discovered in 1885. It is a gram-negative, non-spore forming, facultative anaerobic, rod-shaped bacterium of about 2 μm long and 0,5 μm wide (Neidhardt 1987) from the family *Enterobacteriaceae*. It serves as a model organism in biology, in particular, the laboratory strain K-12 because it grows rapidly, has undemanding growth requirements, is metabolically versatile, has tractable genetic (Hobman 2007). Much is known about its physiology, genetics, molecular biology, and biochemistry (Neidhardt 1987, Ingraham *et al*. 1990, Neidhardt 1990, Higgins 2005) and its complete genome sequence is known (Blattener *et al*. 1997). Standard experimental methods for manipulating *E. coli* are readily available, including procedures for modifying the DNA and performing biochemical analysis (Miller 1972, Sambrook and Russell 2001). It is an ideal experimental platform for quantitative studies of living cells (Alon 2007, Kondew *et al*. 2009, Locke and Elowitz 2009, Phillips 2009) and constitutes a unique potential for understanding the physiology of other prokaryotic and eukaryotic cells.

E. coli cells grow differently under different conditions. The *E. coli* cell culture after inoculation its growth can be graphically described by a curve comprised of four major phases (Fig. 1)

At the *lag phase* the culture density increases slowly, the population remains temporarily unchanged. Although not apparent cell division seems to occur, cells may be growing in volume or mass, synthesizing enzymes, proteins, RNA, etc., and increasing in metabolic activity.

The *log phase,* also called the exponential growth phase, it is a pattern of balanced growth wherein all the cells are dividing regularly by binary fission growing by an exponential progression. Cells divide at a constant rate depending upon the composition of the growth medium and the conditions of incubation. The rate of the exponential growth is expressed by the generation time or doubling time, denoted by τ.

Under the *stationary phase* the population growth is limited by exhaustion of available nutrients or accumulation of inhibitory metabolites or end products.

The *death phase* is also known as the decline phase, where the nutrients become so limited, and toxic metabolites are so high, that the perished cells exceed to those newly formed cells and cell density decreases.

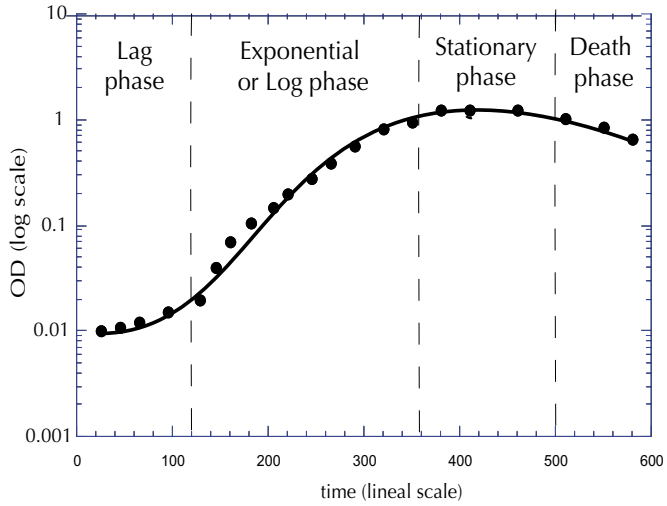

Figure 1. *E.coli* Growth curve. Phases of a typical bacterial growth curve (Lag-phase, exponential phase, stationary phase and die phase). The stationary phase often lasts much longer than all the preceding phases together. Growth experiments in a laboratory course usually get only to the slow-down phase or at best to the beginning of the stationary phase. But what counts is the exponential phase. There are theoretical approaches to describe also the slow-down phase, but this is not part of a basic growth experiment.

1.1. The *E. coli* cell cycle

A cell cycle can be defined as time span from one cell division to the following one. The baterial cell cycle is divided into three periods, called B, C and D (Cooper and Helmstetter 1968) (Fig. 2A).

B Period (Period Before Chromosome Replication) is the time from cell birth to the initiation of replication. It is a period of cellular growth characterized by the increment in cell mass due to new protein synthesis (Donachie 1968), which is required for the cell to undergo DNA replication, cell division, and other physiological and metabolic functions. Time span of the B-period varies according to growth conditions.

C Period (Period of Chromosome Replication) during this phase cell duplicates its chromosome via new DNA synthesis.

D Period (Period of Cell Division) is the time expanded between termination of chromosomal replication and cell division. During this period new chromosomes segregate and followed by the formation of a septum and cell division such that each daughter cell receives a copy of the two segregated materials.

Nutrient availability could potentially affect generation time and any of the above mentioned steps (Wang and Levin 2009). Our understandings of the bacterial cell cycle under different growth conditions derive largely from early physiological studies. These studies indicated that, at constant temperature, mass doubling time decreases in response to increase in nutrient availability, however, both the C period and the D period remain essentially constant. Consequently, under nutriend-rich conditions, *E. coli* reaches growth rates at which the period required for chromosome replication and cell division is greater than the mass doubling time, C+D > τ. To resolve this paradox, rapidly growing cells initiate new rounds of chromosome replication before completing the previous round, a situation that results in two, three or even four rounds of replication proceeding simultaneously. This phenomenon, termed "multifork replication" (Yoshikawa *et al.* 1964), distinguishes bacteria from eukaryotic cells (Fig. 2B).

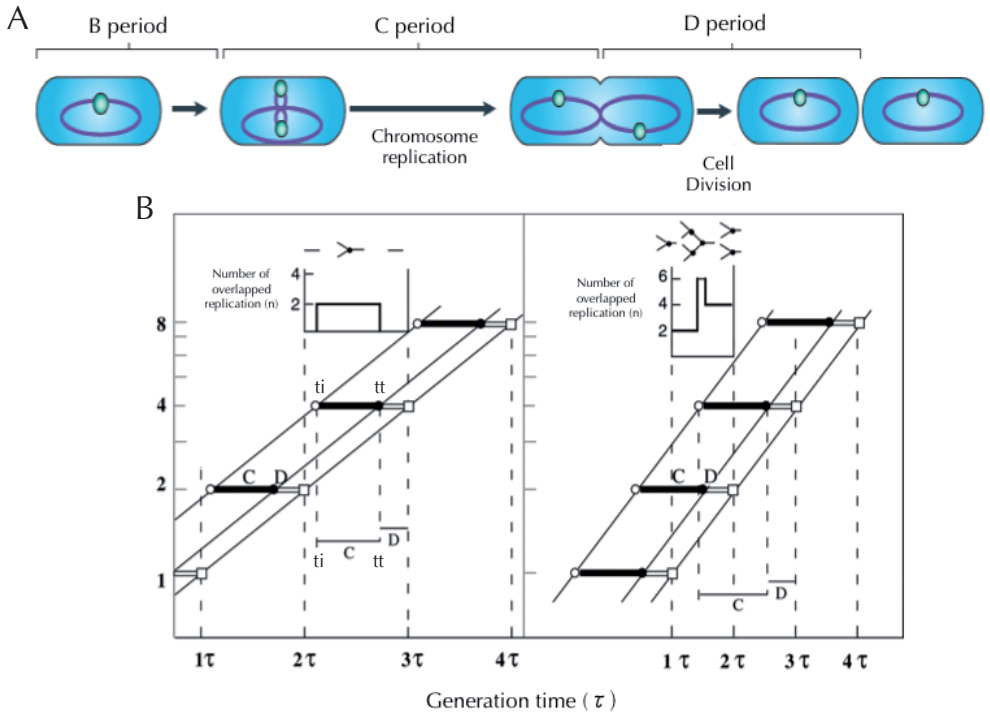

Figure 2. *E. coli* cell cycle. (A) Divided in period B, C and D. Schematic representation of the replicating chromosome (purple oval), and *oriC* region (green region). (B) *E. coli* cell cycle of slow and fast growing cultures. Cell cycle of slow (τ > *C+D*) and fast (τ < *C+D*) growing cultures (A and B, respectively). Time of initiation of the replication round, named *ti* (O) and termination, named *tt* (●) and the time of cell division (□) are indicated. C, is C period. D, is D period. Upper scheme represents the number of replication forks per cell and the structure of the bacterial chromosome (half) running one or two rounds of replication on ti. (This figure has been adapted from Jiménez-Sánchez, 1998).

1.2. *E. coli* DNA replication

The replication of genomic DNA is a key proccess for the life cycle of any organism. It is a well-regulated process with the purpose of making two exact copies of the DNA to be passed on to the two daughter cells.

E. coli K-12 chromosome is made by a circular double stranded DNA molecules of 4.6 millions base pairs (Blattner *et al*. 1997). It has been studied intensively during the last 50 years and is the best described model for bacterial chromosome replication.

The unique initiation site for DNA replication, *oriC,* is located at 84.1 minutes on the genetic map. One clockwise and one counterclockwise replication fork are formed to move in oposite directions from *oriC* towards the terminus, *terC,* located on the opposite side of the chromosome (Bird *et al*. 1972). After this, the two new fully replicated chromosomes are decatenated to be segregate into the two daughter cells (Kornberg and Baker 1992). Thus, DNA replication can be divided into three specific events: initiation, elongation and termination.

The replication proccess is coordinated with cell growth to ensure that the initiation step was undertaken once and only once every cell cycle in all origins present in the cell (Cooper and Helmstetter 1968, Skarstad and Boye 1994, Weigel and Messer 1996). In this way, only one round of replication is completed every cell cycle and the nucleoids segregated before the completion of cell division. Not surprisingly, both the initiation of replication and the replication process itself (referred to here as elongation) are subject to metabolic controls (Donachie 1968, Wang and Levin 2009).

1.2.1 Initiation of replication

1.2.1.1 The *oriC* region

In *E. coli* the replication starts at one specific site, called *oriC*. A replication origin is the DNA sequence capable of initiating autonomous replication. The minimal *oriC* region consists of 258 base pairs and is composed of highly conserved regions proposed to be recognition sites for proteins interacting with the origin (Messer *et al*. 1978,1979, Oka *et al*. 1980, Zyskind and Smith 1986, Asai *et al*. 1990, Simonetti *et al*. 2009), which makes the processes of initiation to require the interaction between origin sequencies and initiation factors.

Initial strand separation takes place in a region of helical instability containing three repeats of an AT-rich 13-mer sequence, 5´-GATCTNTTNTTTT-3, each starting with a GATC sequences (Bramhill and Kornberg 1988, Kowalski and Eddy 1989). The minimal *oriC* region contains eleven GATC sequences (Zyskind *et al*. 1977) that are recognition sites for Dam methylation. The SeqA protein, required for inactivating newly replicated origins, also interacts with the GATC sites in *oriC* (von Freiesleben *et al*. 1994, Lu *et al*. 1994, Slater *et al*. 1995, Brendler *et al*. 1995, Brendler and Austin 1999). The AT-rich region of *oriC* contains three 6-mer binding sites (5´-AGATCT-3´) for the DnaA initiator protein (Fuller *et al*. 1984, Speck *et al*. 1999, Speck and Messer 2001, Ozaki *et al*. 2012 a,b). DnaA also binds to five copies of the 9-mer consensus DnaA recognition sequence, 5´-TGTGNA$^{T}/_{A}$AA (Schaper and Messer 1995), termed R boxes, (R1-R4) (Fuller *et al*. 1984) and R5(M) (Matsui *et al*. 1985) and to 9-mer I-sites (5´T$^{G}/_{T}$GGATCA$^{G}/_{A}$)(Fuller *et al*. 1984, Matsui et al.1985, McGarry *et al*. 2004). The I-sites and DnaA binding sites in the AT-rich region are low affinity sites and discriminate between the active form of DnaA, ATP-DnaA, and the inactive ADP-DnaA (Castuma et al. 1993, Speck *et al*. 1999, 2001, McGarry *et al*. 2004, Regev et al. 2012, McGarry *et al*. 2004, Kawakami *et al*. 2005, Leonard and Grimwade 2011)

The *oriC* region also contains binding sites for the architectural proteins IHF (Integration Host Factor) and Fis (Factor for inversion stimulation) (Leonard and Grimwade 2005).

Figure 3. *oriC* sequence. The sceme shows the most characteristic regions (has been adapted from Jiménez-Sánchez, 1998).

1.2.1.2. Initiation events at *oriC*

Initiation of chromosome replication in *E. coli* is a very complex process, not yet fully understood. Many different proteins, protein complexes, and processes act sequential during the initiation process. (Mott and Berger 2007). The DnaA initiator protein, associated with either ATP or ADP, occupies the high affinity binding sites (R1, R2 and R4) in *oriC* throughout most of the cell cycle (Samitt *et al*. 1989, Cassler *et al*.1995, Nievera *et al*. 2006). Immediately prior to initiating replication, binding of ATP-DnaA to the low affinity binding sites leads to the unwinding of the AT-rich region (Speck *et al*. 2001, Leonard *et al*. 2005). Several factors assist DnaA in DNA strand opening. The presence of negatively supercoiled molecule, transcriptional activation and the architectural proteins IHF or HU have a positive effect, whereas Fis affects duplex opening negatively (Dixon and Kornberg 1984, Skarstad *et al*. 1990, Hwang and Kornberg 1992, Asai *et al*. 1992, Wold *et al*. 1996).

The *oriC* bound DnaA directs a hexameric ring of the DnaB helicase in complex with six DnaC monomers, each binding one ATP (Baker *et al*. 1987, Funnell *et al*. 1987, Sekimizu *et al*. 1988, Fang *et al*. 1999). In complex with DnaC, the DnaB helicase activity is blocked. Release of DnaC is associated with ATP hydrolysis and activates bidireccional movement of DnaB helicase to open the DNA template (Wahle *et al*. 1989). In the last stage of the initiation process, a physical interaction of DnaB with DnaG primase attracts the primase to the replication fork (Tougu *et al*. 19994). DnaG synthesizes RNA primers to which DNA polymerase III holoenzyme binds. DNA polymerase III holoenzyme extends the primer end and, assisted by DNA gyrase that releases topological stress, performs the replication of the whole template (Kornberg *et al*. 1992).

Newly replicated origins are prevented from reinitiation in the same cell cycle through a process called sequestration (Campbell and Kleckner 1990). The GATC sites are targets for DNA adenine metyltransferase (Dam methylase). At the time of replication initiation, all *oriC* GATC sites are fully methylated and sequestered by the binding to SeqA protein (von Freiesleben *et al*. 1994, Lu *et al*. 1994, Slater *et al*. 1995, Boye *et al* 1996, Skarstad *et al*.2000).

1.2.2. Elongation of replication

A DNA replication fork is formed by the coordinated action of many proteins required to ensure an error-free and continuous DNA synthesis. The replication fork move through the chromosome at a rate of about 1,000 nucleotides per seconds to meet with its brother fork at the *ter* region about 40 min after their initiation (Kornberg and Baker 1992).

The replication fork includes two DNA polymerase III holoenzyme molecules (DNA pol III), DnaB helicase and DnaG primase. The activity of DnaB generates single-stranded DNA that is protected by the single-stranded DNA binding protein (SSB) and DNA gyrase relaxes the positive supercoils made by the helicase. DNA pol III moves in the 5' to 3' direction on one strand and in the 3´to 5' direction on the other stand. Because DNA pol III acts by adding deoxyribonucleotide triphosphates to the 3'-OH primer, only the strand complementary to the old 3' to 5' strand is synthesized continuously (leading strand (Hamdam *et al.* 2009, Fijalkowska *et al.* 2011).

The new strand complementary to the old 5' to 3' strand is synthesized by series of relatively short pieces called Okazaki fragments (lagging strand). (Lia *et al.* 2012) Each fragment begins with a RNA primer made by Primase that, after being used, they are replaced by DNA by the action of DNA Pol I 5'-exonuclease and the polymerase activity and followed by the coupling of the Okazaki fragments by the DNA ligase (Kornberg and Baker 1992). Each molecule of the DNA pol III is associated with a ring-shaped sliding clamp, a dimer of the β-subunit of DNA pol III (β-clamp). The β-clamp encircles the DNA strand (Kong *et al.*1992 Kelman and O´Donnell 1995) and stabilizes the DNA pol III-DNA association. The β-clamp is loaded onto DNA by the clamp loader, the γ-complex of DNA pol III (Stukenberg *et al.* 1991, Katayama 2001).

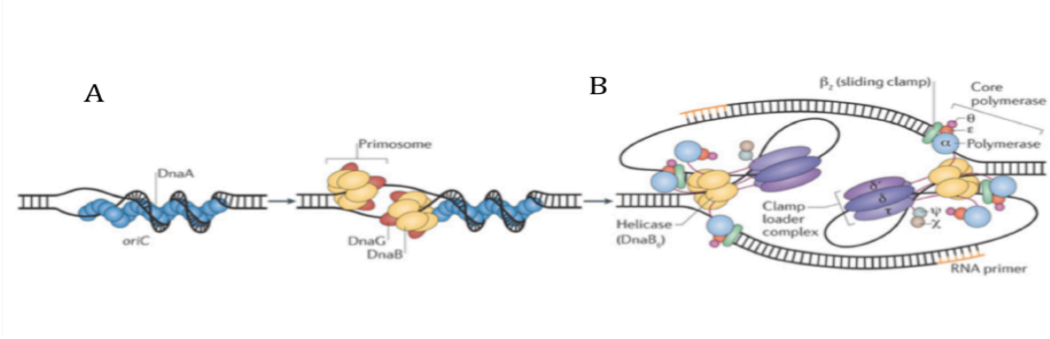

Figure 4. Initiation and elongation replication chromosome *in E. coli*. (A) DnaA and several factors in DNA strand opening and (B) replisome complex.

1.2.3. Termination of replication

A cycle of DNA replication in *E. coli* ends when the replication forks converge at the opposite side of the chromosome in a region called the terminus (generically called *terC*). The *ter* region is composed of two sets of inverted repeats that allow replication forks to enter the terminus but not to exit (Hill *et al*. 1987,1988). These sequences are binding sites for the Tus protein which, when bound to *ter,* allows replication to proceed in only one direction (Kaplan and Batia 2009) by blocking the action of the replicative helicase DnaB, resulting in the termination of replication (Neylon *et al*. 2005).

1.3. Chromosome segregation and cell division

The products of circular replication are topologically linked catenated. Homologous recombination between sister chromosomes also often causes a knotted dimer. Such structures must be resolved into monomeric chromosomes prior to segregation. The replication products are decatenated by topoisomerase IV (Kato *et al*. 1990), which is composed of a heterotetramer formed by a ParE dimer (with an ATPase domain) and a ParC dimer (with a DNA binding and cleavage domain) (Peng and Marians 1993). The XerC/ XerD recombinase, in conjugation with FtsK protein, is responsible for resolving knotted dimers at the *dif* site in the terminus (Steiner and Keumpel 1998, Steiner *et al*. 1999).

Cell division involves partitioning of the cytoplasm into two compartments, each containing one copy of the cell genetic information. The site of cell division is placed with high fidelity at the mid-cell position prior to cell division and is accomplished by the action of the MinCDE system. The MinCDE system undergoes an oscillation cycle, in which the structures disassemble at one pole and then undergoes another assembly and disassembly at the opposite side of the cell. As a result, division is prevented at the pole of the cell and only allowed at sites near mid-cell (Rothfield *et al*. 2005). FtsZ is a tubulin-like GTPase that is essential in cell division. FtsZ forms a circumferential ring (called the Z ring) on the inner side of the cytoplasmic membrane at the division site . The Z ring is probably placed at mid-cell by a combination of nucleoid occlusion that prevents septum formation at positions occupied by the nuleoids (Mulder and Woldringh 1989, Yu and Margolin 1999) and prevention of septum formation at the cell poles by the MinCDE system. The presence of the Z ring is a prerequisite for assembly of at least ten other division proteins into a multiprotein complex to perform cell division (Rothfield *et al*. 2005).

1.4. Coordination between chromosomal replication and cell division

During balanced growth of *E. coli*, chromosome replication is coordinated with the processes leading to cell division (Thanbichler 2010). Cell division is tightly linked to DNA replication and segregation because the cell-division machinery, termed the *divisome*, can begin to be assembled at mid cell only when DNA has been replicated and segregated away from that region. It is only at this moment when the septal region is free of most chromosomal DNA that FtsZ protein and its partners can begin to make a stable FtsZ-ring. Placement of FtsZ-rings mid-cell depends on negative activities of the Min proteins and nucleoid occlusion. The MinC protein of *E. coli* binds to FtsZ, preventing FtsZ-ring assembly at all positions except at mid-cell, where its time-averaged concentration is lowest (Hu *et al*. 1999, Raskin and de Boer 1999). It is only at this moment when the septal region is free of most chromosomal DNA that FtsZ protein and its partners can begin to make a stable FtsZ-ring. Before that, oligomerizing cytoskeletal polymers would be excluded from the nucleoid due to their size and by the nucleoid occlusion proteins, SlmA of *E. coli*.

Timing of Fts-Z-ring formation in relation to replication events has been studied with different approaches leading to different models: replication and division are regulated by independent control systems, with an SOS checkpoint to block division when replication/segregation fails (Bernander and Nordstrom 1990), early stages of replication initiation and replisome assembly potentate the mid-cell FtsZ-ring assembly site but that FtsZ-rings cannot form until replication is almost complete (Moriya *et al*. 2010, Rodrigues and Harry 2012), the replication and FtsZ-ring assembly stages are coordinated exactly throughout the *E. coli* division cycle; both replication initiation and Fts-Z-ring assembly began at the same cell size, Fts-Z-rings changed from a faint band to a well-defined band when replication terminated, and nucleoid separation and Fts-Z-ring constriction occurred at the same cell size (Inoue *et al*. 2009), that mid-cell Fts-Z-rings began forming midway through replication, i.e., shortly after nucleoid splitting became visible (Wang *et al*. 2005) or that initiation of FtsZ-ring formation occurred slightly before replication was completed and concluded that termination, or near termination, of replication could signal division(den Blaauwen *et al*. 1999)

Coordination of cell division with DNA replication under "replication stress" conditions is accomplished through the use of *checkpoints*. Two checkpoints have been shown to play a role in regulating cell division in response to DNA damage or other forms of replication stress. The nucleoid occlusion protein, SlmA, acts as a spatial checkpoint by preventing cell division

at midcell until chromosomes have segregated and reduced the concentration of DNA at that location. A second checkpoint pathway prevents cells from dividing if the chromosome has been damaged activating the transcription of a diverse set of genes that attempt to repair the lesions. This regulating system is called the SOS response that is initiated when the recombination protein RecA binds to single-stranded DNA (Walker 1996). DNA-bound RecA protein undergoes a conformational change that allows it to interact with the LexA transcriptional repressor. The LexA/SOS regulon includes genes involved in various modes of DNA repair and replication restart (Au *et al.* 2005) and the overexpression of a cell division inhibitor (SulA) that prevents cells from dividing until the damage has been repaired (D'Ari and Huisman1983, Kawai *et al.* 2003).

Recently Cambridge and collaborators proposed that the presence of a compact, incompletely replicated nucleoid or unsegregated chromosome masses at the normal mid-cell division site inhibits FtsZ-ring formation without the requirement of SlmA, MinC or SOS system (Cambridge *et al.* 2014).

2. Thymineless death (TLD)

Replication of the genetic material is an essential process in life. Problems with DNA replication can result in mutagenesis and DNA damage that can lead to consequences such as decreased cell fitness or even death of the lineage. Cells employ both avoidance and repair mechanisms to maintain genetic and chromosomal stability. Replication fidelity is ensured in cells via both proofreading of misincorporations during replication and maintaining a correct balance of nucleotides in the DNA precursor pools. Various DNA repair pathways, such as mismatch repair for misincorporations that escaped proofreading and recombinational repair for double strand breaks, can correct mistakes and lesions created during and following replication (Hanawalt 2003, 2007,2008)

DNA synthesis is required for three processes in the cell: replication, recombination and repair. DNA synthesis is carried out by DNA polymerases using the dNTPs (dNTP + DNAn → PPi + DNAn+1). Five different DNA polymerases are known in *E. coli*, each serving a different function with varying polymerization rates and fidelity. The controlled production of dNTPs depends on an enzyme termed ribonucleotide reductase (RNR), which catalyzes the reduction of ribonucleotides to the corresponding 2´deoxynucleotides (Nordlund and Reichard 2006). This reduction is a chemically difficult reaction (Stubbe *et al.* 2003, Chang *et al.* 2004) and requires the presence of a stable organic radical (Nordlund *et al.* 1990, Eklund *et al.* 2001, Stubbe *et al.* 2003). In

light of the observed mutant phenotype *nrdA101*, RNR has been proposed to be a component of the replication hyperstructure (Guzmán *et al*., 2002, Guzmán *et al*., 2003, Guarino *et al*., 2007a, Guarino *et al*., 2007b, Riola *et al*., 2007, Sánchez Romero *et al*. 2010, 2011). The presence of RNR as a structural element of this hyperstructure would facilitate the provision of the four dNTP at the rate required for replication (Mathews, 1993).

The four dNTPs used in DNA synthesis are critically important for the efficiency and fidelity of DNA replication and the DNA repair processes needed for genomic stability (Kunz 1985). Genetically and pharmacologically-induced dNTP pool changes have long been recognized to have genotoxic consequences that can lead to mutagenesis and cell death (Kunz *et al*. 1994, Wheeler *et al*. 2005, Mathews 2006, Schaaper and Mathews 2013) being also implicated in disease (Bourdon *et al*. 2007, Bornstein *et al*. 2008).

A dramatic example of how detrimental nucleotide pool imbalance can affect to the cell viability is the starvation for thymine in strains *thyA* defective that are unable to symthesize thymine. The lack of TTP undergoes to the cell to loss viability, known as *thymineless death* (TLD). TLD is the phenomenon in which cells rapidly lose viability during thymine starvation, ultimately meaning lack of dTTP. During the period of thymine starvation cells accumulate irreversible damages that disable then to form viable cells.

TLD was first discovered in the laboratory of Seymour Cohen in 1953 during a study to find the origin of 5-hydroxymethylcytosine, an unusual pyrimidine in T-even bacteriophages DNAs, that required the use of a thymine requiring strain of *E. coli* (Wyatt and Cohen 1953, Cohen 1971) and was initially described as death due to unbalanced growth by a specific inhibition of DNA synthesis under conditions in which RNA and protein synthesis and general cell growth continued (Cohen and Barner 1954). This is an example that monitoring metabolism and growth, as evidenced by the increasing turbidity of thymine starved cells, is a misleading indication of cells being alive. TLD has been demonstrated in many organisms, ranging from bacteriophage T4 (Bernstein *et al*. 1972) to human cells (Rueckert and Mueller 1960).

Inhibition of cell division, SOS induction, perturbations to other cellular dNTP pools, increased mutagenesis, induction of prophages, loss of transforming ability, elimination of plasmids, stable DNA replication, DNA damage and recombination events have all been observed in bacteria during TLD, and numerous studies have tried to correlate TLD with various cellular events, but a satisfactory hypothesis for the mechanism of TLD is still lacking (Ahmad *et al*. 1998).

Approaches to stop TTP synthesis inducing TLD include: (i) starving for thymine in *thyA* mutants of *E. coli*, (ii) starving auxotrophic mutants for reduced folate/folate precursors such as in *fol1* or *fol2* mutants of *Saccharomyces cerevisiae* (Little and Haynes 1979), (iii) treating cells with inhibitors of thymidylate synthetase, such as fluorodeoxyuridine (FUdR), (iv) starving cells for the reduced folate supply by drugs, such as methotrexate, trimethoprim (DHFR inhibitors) or sulfa drugs (analogues of *p*-aminobenzoic acid) that inhibit de novo folate synthesis.

2.1. Nucleotide metabolism and thymine starvation

The synthesis of TTP is unusual compared to other dNTPs. First, dATP, dGTP, dCTP and dUTP are formed at the diphosphate levels (dNDPs) from the corresponding ribonucleotides (NDPs) by the same enzyme, ribonucletotide reductase (Nrd), while TTP appears in the cell in its monophosphate form (TMP). There are three pyrimidine dNTPs, as oppose to the two purine dNTPs, because dUTP, a close analog of TTP, is an intermediate in the biosynthesis of TTP. In vitro, dUTP can be utilized by DNA polymerase at 25% efficiency compared to TTP (Kornberg and Baker 1992). The *in vivo* concentration of dUTP in *E. coli*, however, is maintained at low levels by the dUTPase (*dut*) that hydrolyses dUTP to dUMP (Greenberg and Somerville 1962, Bertani et al. 1963), the immediate precursor of deoxythymidine monophosphate (TMP). Therefore, normal levels of dUTP cannot support DNA synthesis *in vivo*. Inactivation of the dUTPase allows massive dUTP incorporation into the chromosomal DNA (Neuhard and Kelln 1996).

The other pyrimidine deoxycytidine triphosphate, dCTP, can also be converted to dUTP, then into dUMP and ultimately to TMP as described above.. Most importantly, thymidylate synthesis is coupled with folate metabolism, which serves as the methyl donor for many other important cellular processes.

De novo biosynthesis of TTP starts from the key enzyme thymidylate synthetase (TS), encoded by *thyA* in *E. coli*, using the methyl donor from N5, N10-methylene tetrahydrofolate (THF) to convert dUMP to TMP. The resulting dihydrofolate (H2folate) is converted back to THF via the enzyme dihydrofolate reductase (DHFR), while THF is then converted back to N5,N10-THF by the action of serine hydroxymethyl transferase.

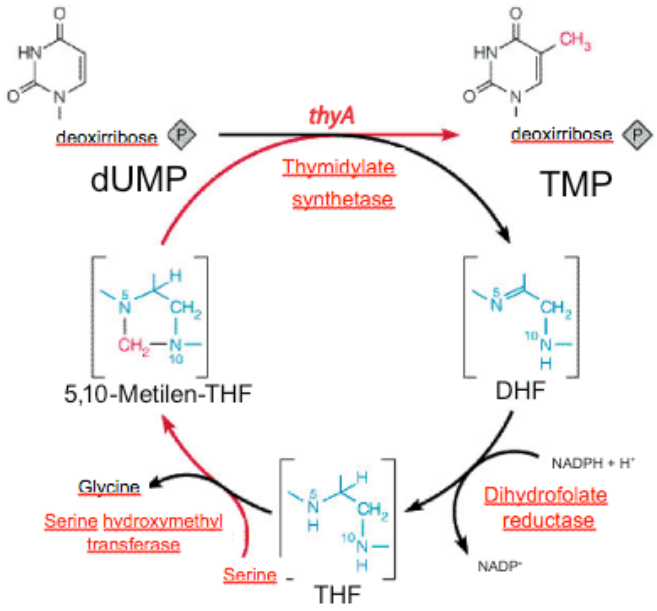

Figure 5. Thymidilate synthetase activity.

Both enzymes TS and DHFR are required for TMP synthesis and the inhibition of either will result in TMP starvation. TMP is subsequently phosphorylated by TMP kinase to the TDP form and by the more general nucleotidediphosphate kinase to TTP. The *de novo* biosynthetic pathway for TTP is the longest and the most energy-consuming of the four DNA precursor pathways. Due to the additional steps in the biosynthetic pathway compared to the other DNA precursors, TTP is the only dNTP that can be specifically starved for.

In *thyA* mutants exogenous thymine can be used via the nucleotide salvage pathway to produce TTP. Adding deoxyribose-1-phosphate (dRib-1-P) to thymine by thymidine phosphorylase (DeoA) produces thymidine (TdR) that will be subsequently phosphorylated to TMP by thymidine kinase (Tdk). Tdk can use either TdR or deoxyuridine (UdR) as substrate, but it can also degrade dUMP and TMP into UdR and TdR respectively, so DeoA can recycle the dRib-1-P pools (Ahmad and Pritchard 1969, Munch-Petersen 1970, Neuhard and Kelln 1996). Wild type *E. coli* cannot utilize thymine because they lack dRib-1-P and any produced TdR will be degraded by DeoA (Budman and Pardee 1967, Munch-Petersen 1970). In *thyA* mutants, dUMP accumulates due to the absence of the TS reaction and will be degraded by Tdk into UdR and dRib-1-P,

providing the cells with the dRib-1-P required for salvage TTP synthesis (Beacham and Pritchard 1971). Inactivation of the genes involved in the *deoCABD* operon encoding the other enzymes that catalyze catabolism of dRib-1-P can help to ensure high levels of dRib-1-P, so that exogenous TdR is all channeled into TTP synthesis.

The levels of other nucleotides are also affected during thymine starvation (Biswas *et al*. 1965, Neuhard 1966, Neuhard and Thomassen 1971, Ohkawa 1975, Fuchs 1977). In *E. coli*, dATP, dCTP, dCMP and dUMP pools increase while dGTP levels remain stable (Neuhard 1966, Neuhard and Thomassen 1971). The change of each nucleotide pool depends on the interplay between their catabolism and anabolism. In summary, the overall effect of TTP starvation on the other dNTPs observed can be very sensitive to strain and growth condition changes.

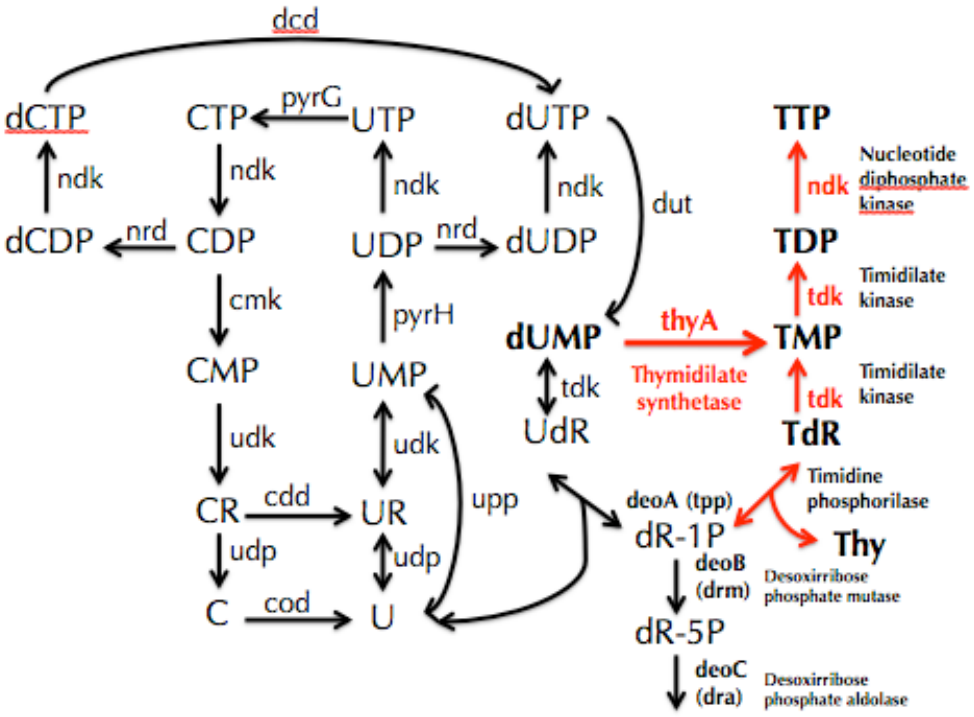

Figure 6. Nucleotides pirimidine metabolism pathways in *E. coli*

2.2. Death by thymine starvation

To understand how and why cells are killed during thymine starvation it is important to know under what conditions death occurs. It turns out that thymine starvation does not kill in all situations and there are certain cellular processes that are required for TLD because in their absence thymine starvation is not lethal. In other words, the source of the irreversible toxicity during thymine starvation that does not allow cells to recover after starvation ends may come from those required processes .

Carbon and energy source. It was shown early that active metabolism is necessary for the killing during thymine starvation (Barner and Cohen 1954, Cohen and Barner 1954, Freifelder and Maaloe 1964). When log phase cells growing in glucose media are transferred to thymine and glucose free media, no killing is observed (Cohen and Barner 1954). After thymine starvation is initiated metabolism has to be continuously active for the killing to occur and once respiration stops (as by oxygen removal), the killing stops immediately at any stage (lag or exponential). Furthermore, when oxygen/respiration comes back, killing resumes at the point where it paused during the halt in metabolism (Freifelder and Maaloe 1964). Changing the carbon source in the media from "fast" to "slow" or viceversa has only minor effects on the kinetics of TLD (Freifelder and Maaloe 1964), showing that it is the continuous supply of energy that matters rather than the rate of energy consumption. Whenever respiration is blocked, cells are not killed by thymine starvation. TLD is not observed in stationary phase cultures (Maaloe and Hanawalt 1961, Hanawalt 1963, Cummings and Kusy 1970, Nakayama and Couch 1973) or in cultures treated with cyanide (Nakayama and Hanawalt 1975, Kuong and Kuzminov 2009), nitric oxide (Kuong and Kuzminov 2009), or in the absence of glucose (Barner and Cohen 1954, Cohen and Barner 1954, Nakayama and Hanawalt 1975). A cytochrome oxidase deficient *cydA* mutation is also reported to protect against TLD, but only in a rich medium (Strauss 2005, Kelly *et al.* 2005). Thus, the mechanism of killing during TLD requires energy (ATP production). Absence of nitrogen source (Cohen and Barner 1954) or lack of inorganic phosphate (Gallant and Suskind 1962, Nakayama and Hanawalt 1975) also saves cells from TLD.

RNA synthesis. RNA synthesis is required for TLD. Uracil starvation inhibits RNA synthesis completely while having no effect on protein synthesis, and relieves TLD in *E. coli* (Barner and Cohen 1958, Deutch and Pauling 1974). Rifampicin (Rif) binds to the RNA polymerase-DNA complex sterically blocking the extension of the nascent RNA chain after the first or second condensation step and inhibits transcription (Campbell *et al.* 2001, Floss and Yu 2005). Cells treated with rifampin do not undergo TLD (Hanawalt 1963, Nakayama and Hanawalt 1975, Pauling *et al.* 1976). Since the RNA synthesized during TLD has the same base composition as the RNA synthesized in the presence of thymine, it was proposed that it is the unwinding of DNA during transcription that kills, rather than the production of a toxic form of RNA (Gallant and Suskind 1962, Sicard *et al.* 1967). With transcription and DNA replication sharing the same road (template DNA), and the recent understanding of how collision of the two processes are handled in the cell and the detrimental consequences, such a replication fork stalling and chromosomal breakage, that can result from the collision in bacteria (Mirkin and Mirkin 2007, Wang *et al.* 2007, Boubakri *et al.* 2010, Pomerantz and O'Donnell 2010, 2011, Merrikh *et al.* 2011), the suggestion that transcription is a required process in TLD due to its adverse effects on DNA replication should be seriously considered.

Protein synthesis. The requirement for RNA synthesis during TLD could be due to the subsequent inhibition of translation, hence attempts to separate and unlink these two processes were made in the studies of TLD. During thymine starvation, protein synthesis eventually falls after 2 h while RNA synthesis continues (Medoff 1972). Various antibiotics were used to examine the role of protein synthesis in TLD. Chloramphenicol (Cm) binds to the 50S subunit of the bacterial ribosome by blocking the tRNA-accepting site of the mRNA-ribosome complex and effectively stopping transpeptidation. Puromycin is an aminoacyl-tRNA analog that inhibits peptidyl transfer by ribosomes and causing premature release of unfinished protein chains (Azzam and Algranati 1973). 5-methyltryptophan, a structural analogue of L-tryptophan, acts as a false feedback inhibitor of anthranilate synthetase (Moyed 1960) and also inhibits protein synthesis in *E. coli* (Turnock and Wild 1966). In cultures treated with high concentrations of Cm (Cummings and Kusy 1969, Bouvier and Sicard 1975, Nakayama and Hanawalt 1975) or puromycin (Nakayama and Hanawalt 1975) or 5-methyltryptophan (Gallant and Suskind 1962, Nakayama and Hanawalt 1975) TLD is relieved but low concentrations of Cm have little or no effect on TLD (Gallant and Suskind 1962, Hanawalt 1963, Pauling *et al.* 1976).

The requirements for protein synthesis in TLD are thus not settled, as Gallant *et al* proposed that higher concentrations of Cm also block RNA synthesis, while at low concentrations only protein synthesis is inhibited (Gallant

and Suskind 1962). Others also attribute the effect of Cm to inhibition of RNA synthesis (Hanawalt 1963). In addition, when protein synthesis is inhibited by Cm, but RNA synthesis is stimulated in the same cells by 5-methyltryptophan, TLD is still observed (Gallant and Suskind 1962). As the effect of theses antibiotics on cellular processes is multi-faceted, prevention of TLD by inhibiting one or both transcription and/or translation remains a controversial tusk until more careful studies were done.

DNA replication. The role of DNA replication in TLD has long been a subject of interest in the field because DNA replication is the only cellular process that specifically requires thymine. This is the main point of the goal of this work.

Although DNA synthesis is the main process inhibited during TLD, there is still a detectable increase in the DNA content (15 to 20 per cent of the pre-starvation DNA level) (Kuong and Kuzminov 2009). This lead to interesting debates concerning whether this residual ongoing DNA synthesis, in addition to unbalanced dNTP pools, causes the damage that leads to TLD. In conditions where there is no residual DNA synthesis, such as when respiration is inhibited, cells are not killed during thymine starvation, consistent with the idea that some DNA synthesis is required for the killing (Freifelder and Maaloe 1964).

The initiation of DNA replication is a highly-controlled process. There are studies reporting that preventing DnaA initiation of replication by incubating *dnaA46*(Ts) mutant at the non-permissive temperature relieves TLD (Bouvier and Sicard 1975). A similar conclusion was reached in studies using *Bacillus subtilus dnaB*(Ts) initiation mutants (Sargent 1975). Moreover, there are also studies reporting that TLD is relieved only when initiation was already inhibited before the onset of thymine starvation by pre-incubation at the non-permissive temperature before removal of thymine from the media (Nakayama *et al.* 1994) which may explain why these studies found no supression of TLD if the *dnaA*(Ts) or *dnaC*(Ts) mutants are shifted to the non-permissive temperature at the time of thymine removal (Nakayama *et al.* 1982). What is lacking in these studies, however, is the physical evidence that replication initiation is inhibited completely in these *dnaA* or *dnaC* mutants at the non-permissive temperatures hence further investigation is needed before we can conclude that replication initiation is required for TLD.

2.3. Defective mutants

DnaE encodes the alpha subunit of the replicative DNA polymerase III, DnaB encodes the replicative DNA helicase, which unwinds DNA at the replication forks, and DnaG encodes the DNA primase that synthesizes RNA primers on single-stranded template DNA for the subsequent Pol III-catalyzed DNA synthesis. Temperature-sensitive mutants in *dnaB, dnaE* and *dnaG* are known as fast stop *dna* mutants, because they stop DNA elongation immediately after the culture is shifted to the non-permissive temperature. An example to support the argument that TLD is a direct consequence of the replication inhibition comes from the killing observed in mutants with impaired replication. DNA Pol III polymerase catalytic subunit mutants *dnaE74* and *dnaE486* undergo rapid loss of viability during incubation at the non permissive temperature (Bouvier and Sicard 1975, Strauss *et al*. 2004, Davies *et al*. 2009). The same is true for the replicative helicase mutants *dnaB70* and *dnaB466*, and the primase *dnaG*(Ts) mutants (Bouvier and Sicard 1975).

On the other hand, initiation defects by *dnaA*, *dnaC* or *dnaD* (an allele of *dnaC*) mutations have a bacteriostatic effect at the non permissive temperature (Bouvier and Sicard 1975). It was suggested that the killing mechanisms in the elongation-defective mutants were similar to those in TLD (Strauss *et al*. 2004, Strauss *et al*. 2005). Physical and genetic analysis of the killing mechanism in these replication mutants and TLD indeed share similarities such as that both killing conditions are partially mediated by recombinational repair activities.

If DNA replication is a requirement for the killing during thymine starvation, stopping DNA replication elongation should stop TLD. The effect on TLD of the fast-stop *dna* mutants differs from study to study: mutations were reported to show no effect on TLD, or in some cases were even shown to accelerate the killing (Bouvier and Sicard 1975), while other mutations offered protection against TLD (Bouvier and Sicard 1975).

It has been proposed that the DnaB helicase does not directly participate in the killing; rather, *dnaB* mutants at the non-permissive temperature may be in a state that is no longer subject to the toxic effect of thymine starvation, implying that DNA elongation is required for TLD. However, there is also a report that failed to confirm the reliev effect of the *dnaB* mutants (Nakayama *et al*. 1982). Hence, the requirement for ongoing (rather than newly-initiated) replication forks during TLD is yet to be confirmed. An additional complication to the interpretation of these data is that the elongation defect will also interfere with new initiations. Therefore dNTP starvation is a better overall comparison to thymine starvation, which in both cases should not affect the ability for new

replication forks to be established right after the initiation process while the replisome mutants may.

There are two more important players in both replication and TLD: the DNA polymerase I (PolA) and the NAD+ dependent DNA ligase (LigA). The role of PolA and LigA in joining Okazaki fragments during lagging strand synthesis is well known, although they are also suspected or shown to participate in other important cellular pathways, for example during DNA repair (Zimmerman *et al.* 1967, Conley and Saunders 1984, Friedberg *et al.* 2006). How Pol I and LigA contribute to survival during TLD in *Escherichia coli* is still unclear, but studies on the effect of PolA and LigA on TLD are more coherente than other mutants previously studied, both mutants are always found to be hypersensitive to TLD. There are also many studies on characterization of the effects of the *polA* and *ligA* defects on the profile of DNA strands during TLD.

In conclusion, both the ligase-defect and thymine starvation induce DNA strand breaks and rapid killing, but it is unlikely that they do it by the same mechanism.

3. Major hypotheses on the cause of TLD

3.1. DNA damage

DNA strand breaks and fragmentation are observed during TLD in bacteria, including *E. coli*, *Bacillus subtilis* and *Diplococcus pneumoniae*, (Mennigmann and Szybalski 1962, Freifelder 1969, Reichenbach *et al.* 1971, Breitman et al. 1972, Bhattacharjee and Das 1973, Hill and Fangman 1973, Nakayama and Hanawalt 1975, Bousque and Sicard 1976, Pauling *et al.* 1976). All these studies used methods such as alkaline sucrose gradient that do not distinguish between double strand breaks (DSBs) and single stranded breaks (SSBs). Conditions that suppress killing, such as treatment with Cm, Rif or cyanide, or starvation for glucose or inorganic phosphate, block break formation (Nakayama and Hanawalt 1975, Pauling *et al.* 1976). Two studies have reported that DSBs occur during TLD, by using neutral sucrose gradients (Yoshinaga 1973) or by Pulse Field Gel Electrophoresis (PFGE) (Guarino *et al.* 2007), while another study using PFGE did not find any DSB in *E. coli* (Nakayama *et al.* 1994).

Uracil incorporation is the most frequently mentioned hypothesis is that the increased levels of dUTP, its incorporation into the DNA, and the subsequent excision by uracil-DNA-glycosylase (UDG in humans or Ung in *Escherichia coli*) generates futile cycles in the absence of TTP (Goulian *et al.* 1980, Ahmad *et al.* 1998, Webley *et al.* 2000, Ladner 2001, Longley *et al.* 2003) leading to cell death via hyper-recombination and DNA fragmentation.

Extensive incorporation of uracil into DNA is lethal in bacteria (el-Hajj *et al*. 1988) and yeast (Gadsden *et al*. 1993, Kouzminova and Kuzminov 2008), and uracil incorporation into DNA is indeed observed in human cells during thymine starvation (Goulian *et al*. 1980). The prediction from this model is that death depends on the activity of a single enzyme, uracil DNA glycosylase. However, this enzyme does not seem to play a major role in the killing mechanism: *ung* mutations in *E. coli* show no effect on TLD (Kunz and Glickman 1985, Kuong and Kuzminov 2010), and expression levels of UDG do not affect viability or sensitivity to TS-inhibition in human cell lines (Welsh *et al*. 2003). Therefore, it is possible that wild type levels of dUTPase (*dut*) are enough to hydrolyze any increased levels of dUTP (Neuhard and Kelln 1996) during thymine starvation. As a result, uracil incorporation into DNA is not significantly elevated. dUTPase was shown to be an essential enzyme in wild type and also in the *ung*– strains in *E. coli* (el-Hajj *et al*. 1988) even in the presence of exogenous thymidine, and despite the fact that uracil incorporation is unlikely to be the major cause of killing during TLD, excessive levels of dUTP are indeed toxic in *E. coli*.

Mutagenesis Increased mutagenesis during thymine starvation was demonstrated in *E. coli* by screening for histidine auxotroph revertants (Adelberg and Coughlin 1956), streptomycin resistance (Latham and Weinberg 1956, Bresler *et al*. 1973), uracil auxotroph revertants (Kanazir 1958), tryptophan auxotroph revertants (Bridges *et al*. 1968, Mennigmann and Pons 1979), arginine auxotroph revertants (Deutch and Pauling 1974), *lacI* mutations and reversion of the *trpE9777* frameshift mutation (Kunz and Glickman 1985), as well as in other organisms including bacteriophage T4 (Smith *et al*. 1973; Ripley 1975), *Salmonella enterica typhi* (Holmes and Eisenstark 1968) and *Bacillus subtilis* (Bresler *et al*. 1973). Analysis of the revertants reveals that the induced mutations are predominantly AT → GC transitions, as well as all possible transversions, frameshifts and deletions. The *ung* mutation inactivating uracil-DNA-glycosylase does not affect the frequencies of mutations during TLD (Kunz and Glickman 1985) while the *lexA*(ind-) mutations were reported to abolish mutagenesis during TLD (Bridges *et al*. 1968). The increase of mutagenesis during thymine starvation reported ranged from approximately 1 to 2 orders of magnitude depending on the methods and time of starvation.

3.2. DNA recombination intermediaries

Several *Rec* proteins, which function in DNA repair by homologous recombination (HR) and other aspects of DNA metabolism, promote *thymineless death*. These include RecA (Fonville *et al*. 2010, Kuong *et al*. 2010), RecF (Nakayama *et al*. 1982, Courcelle 2005, Fonville *et al*. 2010, Kuong *et al*. 2010), RecO (Nakayama *et al*. 1988), RecJ (Nakayama *et al*. 1988). Conversely RecBCD prevents TLD and RecQ, was discovered in a screen for mutants resistant to TLD (Nakayama *et al*. 1994, Nakayama 2005).

During thymine starvation complex DNA structures are generated by attempts to repair gaps, including recombination processes. It has been observed DNA breaks that could be repaired after thymine addition (Nakayama and Hanawalt 1975). In cells starved for thymine, Nakayama *et al*. observed special DNA structures, referred to as non-migrating DNA (nmDNA), including X-structures and Y-structures, dependent on presynaptic HR proteins RecA, RecF, RecJ, RecO, and RecQ, leading the authors to hypothesize that the nmDNA might be HR intermediates promoting TLD (Nakayama *et al*. 1994). In addition to DNA breaks, DNA degradation occurs during TLD and is most severe near the origin of replication (Sangurdekar *et al*. 2010, Kuong and Kuzminov 2012), then later occurs at the replication terminus (Fonville *et al*. 2010). The origin-specific degradation requires RecF, RecJ, RecO (Sangurdekar *et al*. 2010) RecA and RecBCD (Kuong and Kuzminov 2012) and shows a slight reduction in mutant lacking RecQ (Sangurdekar *et al*. 2010) suggesting that either recombination or the SOS DNA-damage/replication-stress response, or both, promote origin-specific DNA degradation.

3.3. The SOS response and *Rec* proteins

At least three independent pathways of TLD related to *Rec* proteins and SOS response occur in *E. coli*. (i) One implies **the SOS response in TLD.** RecA plays a central role in recombinational repair but also in the SOS response. RecA bound to ssDNA binds LexA, the repressor of the SOS regulon, to promote its self-cleavage and inactivation. This removes the repression and leads to a rapid production of the corresponding proteins, including RecA and LexA themselves. During TLD, SOS is induced continuously (O'Reilly and Kreuzer 2004, Kuong and Kuzminov 2010). The *lexA3* mutants, with a noncleavable LexA repressor that cannot induce the SOS response, were reported to have either no effect (Howe and Mount 1975; Morganroth and Hanawalt 2006), or a relieving effect (Fonville *et al*. 2010), or a mild relieving effect (Kuong and Kuzminov 2010) on TLD. The *recA430* allele, which is proficient in

recombinational repair but not in SOS induction, was reported to also confer resistance to TLD (Fonville *et al.* 2010) suggesting that the role of SOS induction is not through increased DNA damage repair proficiency. Confusingly, in *lexA71* mutants, where SOS is induced constitutively, the effect is the same as in *lexA3* mutants (Kuong and Kuzminov 2010), whereas using a *recA* SOS-induction-constitutive mutant causes no effect on TLD (Fonville *et al.* 2010), making it difficult to draw any conclusion about the role of the SOS induction during thymine starvation. (ii) Cell division inhibition by **SulA** is a unique aspect of the SOS induction. The *lon-* mutants are defective in the protease that degrades SulA and are TLD-hypersensitive (Cummings and Mondale 1967, Walker and Smith 1970, Huisman *et al.* 1980) and the *sulA* (*sfiA*) mutation can suppress this hypersensitivity (Huisman *et al.* 1980) that suggests a toxic role of SulA in TLD. *sulA* mutations, however, were reported to have no effect on TLD (Huisman *et al.* 1980) or to bring a relief to TLD (Fonville *et al.* 2010). The relief in the latter study is sometimes stronger and sometimes minimal, suggesting that the effect of *sulA* may be influenced by minor changes in growth conditions. (iii) An independent pathway of TLD in *E. coli* requires **RecQ and RecJ** recombinational DNA repair proteins, such that cells that lack RecQ or RecJ and RecA have even less TLD than cells blocked for SOS or RecQ/J singly (Fonville *et al.* 2010). These authors proposed that, although both RecA and RecQ/J function in HR, at least part of their roles in TLD were independent and additive indicating separate pathways, implying that the RecQ/J route is not solely recombinational.

3.4. MazEF toxin-antitoxin

A different model attributes TLD to the *mazEF* system (Sat *et al.* 2001), a suicide addiction module in the *E. coli* chromosome that consists of a pair of genes that encode a stable toxin and an unstable antitoxin that neutralizes the lethal action of the toxin). MazF is a sequence-specific mRNA endoribonuclease that acts as a stable toxin, while MazE codes for a labile antitoxin. Stressful conditions can affect continuous expression of MazE by preventing either its transcription and/or its translation. Without MazE, MazF cleaves mRNAs at ACA sequences to inhibit protein synthesis (Engelberg-Kulka *et al.* 2005).

It was reported that *ΔmazEF* mutants are completely resistant to TLD, induced by trimethoprim, sulfonamides or in Δ*thyA* mutants in *E. coli* (Sat *et al.* 2001, 2003). Sat *et al.* reported that the *mazEF* suicide system is activated by thymine starvation-induced transcriptional changes and have suggested that this built-in cell death system is responsible for TLD. Although the *mazEF* theory of TLD has attracted significant attention over the past few years, it cannot account for the phenomenon of TLD.

The more significant problem with the *mazEF* theory is that it is

inconsistent with the well-documented resistance to TLD when transcription is inhibited (Hanawalt 1963, Cummings and Kusy 1969, Nakayama 1975). In contrast, the *mazEF* system causes cell death whenever transcription is inhibited, because the labile MazE is degraded but not replaced by new synthesis, allowing the stable MazF toxin to kill the cell. Sat et al. demonstrated that antibiotics inhibiting transcription and translation, including Rif and Cm, cause cell death through the *mazEF* system (Sat *et al*. 2001). If the *mazEF* system were the cause of TLD, inhibition of transcription with Rif during thymine starvation would be expected to enhance killing. Conversely, as has been demonstrated in numerous other studies (Hanawalt 1963, Cummings and Kusy 1969, Bouvier and Sicard 1975, Nakayama and Hanawalt 1975, Pauling *et al*. 1976, Martin and Guzman 2011 and this work) inhibition of transcription with such agents prevents TLD. TLD is a complex, multifaceted process, and the *mazEF* system may account for one pathway of death triggered by thymine starvation, but the suicide module is not sufficient to account for TLD.

3.5. Loss of replication origins during thymine starvation

A well-documented loss of *oriC* region (Fonville *et al*. 2010, Sangurdekar *et al*. 2010, Kuong and Kuzminov 2012) and a critical role of the initiation of replication in TLD (Martín and Guzmán 2011, Martin and Guzmán 2014) has been emerged over the past five years while this work has been developed.

Back in 1970, Ramareddy and Reiter reported sequential loss of chromosomal markers in a *Bacillus subtilis thyA* mutant synchronized by germinating spores and switched to a medium without thymine. The loss of the markers started at the position of replication forks going towards the replication origin, although these authors did not specifically looked at the disappearance of markers around the replication origin (Ramareddy and Reiter 1970, Reiter and Ramareddy 1970).

Nakayama and colleagues reported in *E. coli* that, after 1 h of thymine starvation, the chromosomal fragments unable to enter pulsed-field gels were enriched in replication origin (Nakayama *et al*. 1994). Recently, Rosenberg and colleagues reported that thymine starvation causes a shift from cells containing mostly two origins and one terminus to cells containing one origin and one terminus which suggests a loss of the origin region (Fonville *et al*. 2010). Khodursky and colleagues reported the dramatic decrease of a broad zone centered at replication origin, detected by marker-frequency profiling of the entire chromosome (Sangurdekar *et al*. 2010). Finally, these studies were expanded showing the RecA dependence of this selective degradation of the *oriC* region (Kuong and Kuzminov 2012). By the time these effects were described, part of the work presented here was published showing the key role

of DNA initiation in TLD (Martín and Guzmán 2011) and the presence of replication intermediates at *oriC* region accumulated under thymine starvation (Martín and Guzmán 2014).

By using different approaches, all these works display a new element in the complex scenario of TLD, contributing to elucidate the molecular mechanism undertaken this phenomenon.

4. Clinical significance of TLD.

The mechanism of TLD is closely tied to the mode of action of many anticancer, antibacterial and antiviral drugs. TS inhibitors, such as fluorodeoxyuridine (FUdR), and DHFR inhibitors, such as methotrexate and aminopterin, have been used for decades as anticancer, antibacterial and antiviral agents. Sulfa drugs that inhibit de novo folate synthesis also induce thymine starvation. Due to the excellent chemotherapeutic efficacy of folic acid antagonists and TS inhibitors, they and their derivatives remain the most popular anti-cancer drugs for certain cancer types, for example for gastrointestinal cancers, to this date (Ladner 2001, Bertino 2009). Drug resistance has been observed nevertheless (Mader *et al*. 1998, Bertino 2009) and understanding the molecular mechanisms of their toxicity will be useful in combating drug resistance and predicting anti-drug responses.

Outside the context of chemical inhibition, folate deficiency is the most common vitamin deficiency, affecting 10% of the general adult population, up to 60% of juveniles or the elderly in certain social groups (Senti and Pilch 1985). Folic acid acts as cofactor in numerous biochemical reactions, including TMP synthesis, through its ability to donate or accept one-carbon units. Mammals are unable to synthesize folic acid de novo and so must obtain it from food or from microbial breakdown in the gut. Severe folate deficiency causes megaloblastic anaemia in humans, while inadequate folate intake during pregnancy is related to neural tube defects in the newborns, development of certain cancer and heart diseases (Duthie 1999). It is proposed that some of the consequences of folate deficiency, such as DNA instability in human cells, are due to thymidylate limitation/starvation (Duthie 1999).

Lastly, there is an understanding now that mitochondrial dysfunction is a hallmark of cancer cells, as mutations in the mitochondrial genome have been reported in every cancer type examined to date (Modica-Napolitano and Singh 2004; Singh et al. 2005). It was reported that cellular dNTP pools are lowered in mitochondrial-dysfunctional human cells and the TTP pool is the most severely diminished of the dNTPs (Desler *et al*. 2007), which may be the cause of the chromosomal translocations and rearrangements observed in these cells.

5. Evolutionary significance of thymidylate synthesis and the discovery of ThyX.

DNA differs from RNA not only in the sugar deoxyribose (lacking the 2'-OH ribose), but also that it has thymine instead of uracil.
DNA is viewed as the most stable form of the two nucleic acids and is the carrier of genetic material in all free-living life forms on Earth today. It is conceivable that the genetic information carrier in the primitive life forms was RNA, but due to the significant instability of the RNA backbone, life switched to the DNA backbone. However, why uracil had to be replaced with thymine in "modern" DNA still remains a mystery (Jiménez-Sánchez 1995).

Because of this uracil-to-thymine switch, the ability to synthesize TTP by thymidylate synthase (TS) is one of the essential requirements for life to make this hypothetical transition from RNA genome to DNA genome. It was thought that the ThyA form of TS is universal across all forms of DNA based life until the realization after a large scale comparative genome analysis in 2000 that many organisms lacked ThyA (Galperin and Koonin 2000). However, sequences with no homology to ThyA that complemented a thymidine auxotroph of *Dictyostelium discoideum* during a genomic library complementation screen were identified in many of these ThyA lacking organisms (Dynes and Firtel 1989). It was demonstrated that the flavoprotein ThyX is the TS of many organisms that have neither ThyA (for de novo thymidylate synthesis), nor Tdk (thymidine kinase, for thymidine-scavenging) homologs.

Currently, about 20 bacterial species are known to have both *thyA* and *thyX* genes, again including potent pathogens. Though having both *thyA* and *thyX* or two *thyA* genes in the same genome may give a certain selective advantage for the organisms, such as increased virulence or resistance to inhibitors of one of the two thymidylate synthesis routes, it is also proposed that the primary driving force is selective advantage to the viruses.

On the other hand, a recent study in *Corynebacterium glutamicum* that has both ThyA and ThyX shows that although *thyX* is not essential in *C. glutamicum*, *thyX* mutants lose viability significantly during stationary phase.

This suggests differential expression of the two TS enzymes during various growth phases, ThyA is used preferentially during the exponential growth phase, while ThyX is used preferentially during the stationary growth phase (Park *et al.* 2010). *thyX* is also shown in the same study to be sufficient to confer resistance to a DHFR inhibitor. The potential growth advantage to the organism of carrying two structurally distinct (ThyA and ThyX) or similar (2 ThyA) TS genes could be that organisms can synthesize thymidylate under more diverse environments.

More studies that examine the physiological regulation and performance of ThyX will be useful for our understanding of the requirements of thymidylate and its regulation during different growth phases, especially in organisms that have two TS in which some form of regulation between the two may be found. Because of the late discovery of ThyX, TLD is not yet characterized in ThyX only organisms. With TS being such a fundamental enzyme in life, along with ThyA and ThyX being so distinct, this gives us an opportunity to understand what factors are driving the development of DNA as the genetic carrier if we can understand why certain organisms carry ThyX, ThyA or both. New insights into evolutionary phylogenetics of organisms may be generated too. Perhaps then we may be able to hypothesize about what makes life addicted to thymine, which may be directly linked to the basis of TLD. Starvation for TTP is the more potent acute killing of current life forms compared to starvation for other essential building blocks, such as RNA precursors. In addition, with complex and highly energy consuming pathways to synthesize TTP that arose at least twice in evolutionary history, there must be very strong reasons for do not use dUTP for replication, which is, nevertheless, the precursor for TTP synthesis and is readily available in the cell.

Materials

1. Strains

Escherichia coli K12 MG1693 (*F⁻, λ⁻, thyA715, rph-1, deo?)* is a spontaneous Thy- derivate of the MG1655 strain (selected with trimethoprim) obtained from the Genetics Stock Center. We founded this strain to be sensitive to high concentrations (100μg ml^{-1}) of thymidine (TdR). Our guess is that strain MG1693 could be defective in the deo operon.
All strains used in this work are presented in table 2.1 All strains were derived from the parental strain MG1693 (*thyA175, deo, λ⁻, rph-1*) using standard P1 transduction, and transformation with plasmid presented in table 2.2

Table 1 Bacteria strains

Strain	Genotype	Source or construction
MG1655	wt (*thy, rph*)	Judith Zyskind´s lab.
MG1693	*thyA175, deo, λ⁻, rph-1*	Coli Genetic Stock Collection (CGSC6411)
# 976	MG1693 *recB258*::Tn10	This lab
# 989	MG1693 *dnaA46* tnA::Tn10 ts	This lab
# 1052	MG1693 *asnA101*::Cm	This lab
# 1053	MG1693 *asnA101*::Cm *Pgid103*::Cm	This lab
# 1054	MG1693 *asnA101*::Cm *PmioC112*::Cm	This lab
# 1055	MG1693 *asnA101*::Cm *Pgid103 PmioC112*::Cm	This lab
# 1056	MG1693 *mioCp9:*::Tn5	This lab
# 1061	MG1693 *Rif^{R} (rpoB)*	This lab

Strain	Genotype	Source or construction
# 1097	MG1693 /pBAD::Tc10	This lab
# 1098	MG1693 pRW901::Tc10	This lab
# 1099	MG1693 /pRWSR1:: Tc10	This lab
# 1100	MG1693 /pRWSR2:: Tc10	This lab
# 1101	MG1693 /pRWSR3:: Tc10	This lab
# 1160	MG1693 *oriC201*::Cm	This lab
# 1161	MG1693 *oriC227*::Cm	This lab
# 1162	MG1693 *oriC228*::Cm	This lab
# 1163	MG1693 *oriC229*::Cm	This lab
# 1164	MG1693 *oriC230*::Cm	This lab
# 1166	MG1693 *oriC233*::Cm	This lab
MG1693/HU-GFP	pBAD24-hup-gfp (MW-162)	Fishov´s Lab, (Reshes *et al*. 2008).
CR34	*thy-, drm-, dra-, thy-, leu-, thr-*	Zaritsky´s Lab (Zaritsky and Woldringh 1978)
TAU-bar of 15T-	*arg met pro trp ura thyA deoB*	Zaritsky´s Lab (Hanawalt and Wax 1964)

2. Plasmids

Table 2. Plasmids

Plasmid Brief	Description	Source or construction
pBAD	Tc10, induction arabinase 0.2%	(Mitchell *et al* 2003)
pRW901	Tc10	(Mitchell *et al* 2003) (Butala *et al.* 2009).
pRWSR1	Tc10	(Mitchell *et al* 2003)
pRWSR2	Tc10	(Mitchell *et al* 2003) (Butala *et al.* 2009).
pRWSR3	Tc10	(Mitchell *et al* 2003)
HU-GFP	Amp100, induction arabinose 0.2%	(Reshes *et al.* 2002)

3. Media and growth conditions

All media were stored at room temperature. Unless stated otherwise, chemicals were dissolved in double distilled, sterile water (Milli-Q®). Medium was autoclaved at 121°C, or filtered for sterilized prior to use. Melted agar was stored at 55°C and allowed to cool prior to the addition of antibiotics and inducers (when required).

Table 3 Bacteria and P1 growth media

MEDIA	Composition (per liter)
Bacteria	
LB (Luria Bertani)	10g Bactotriptone (Oxoid), 5g yeast extract, 10g NaCl; pH adjunted to 7.2-7.5 with NaOH.
NA (Nutrient Agar)	8g Nutrient-Broth, 20g agar.
M9-Minimal Media	100ml M9x10 salts solution + 10ml $CaCl_2$ 0.01M solution + 10ml $MgSO_4$ 0.1M + 10ml Cassaa20% in 857,5 ml H_2O with 4g of glucose.
M9-Minimal Media Agar	857,5 ml H_2O with 4g of glucose. + 20g agar in stock. Add other component previously to use.
M9-Minimal Media Agar	$PO_4H_2Na_2$ without H_20 60g + PO_4H_2K 30g + ClNa 5g + $ClNH_4$ 10g + H_2O up to 1litre. pH adjusted to 7.2.
P1	
Coberture Agar	6g of agar in 1000ml of LB. After sterilization add $CaCl_2$ final concentration 2,5mM and glucose 0,1% final concentration.
LB10	10g of agar in 1000ml of LB. After sterilization add $CaCl_2$ final concentration 5mM and glucose 0,1% final concentration.

4. Antibiotics

Table 4 Antibiotics

Antibiotic	Abbrev.	Solvent	Stock Concrn	Final Concrn	Conservation
Ampiciline	Amp	H_2O	100 mg ml^{-1}	100µg ml^{-1}	-20°C
Cephalexin	Cfx	H_2O	10 mg ml^{-1}	50µg ml^{-1}	preparate in moment use
Chloramphenicol	Cm	Ethanol 50%	30 mg ml^{-1}	200µg ml^{-1}	-20°C
Hydroxiurea	Hu	growth medium	1M	75mM	preparate in moment use
Rifampicin	Rif	Methanol 100%	25 mg ml^{-1}	150µg ml^{-1}	preparate in moment use
Tetracycline	Tc	Ethanol 50%	10 mg ml^{-1}	10µg ml^{-1}	-20°C
Nalidixic Acid	NAL	H_2O	10 mg ml^{-1}	10µg ml^{-1}	preparate in moment use

5. Radioactive products

Table 5 Radioactive products

Product	Isotope	Specific. activity	Solvent	Stock Concn	Final Concn	Conservation	USE
Thymine	^{3}H	20Ci $mmol^{-1}$	Ethanol 50%	1mCi ml^{-1}	1µCi ml^{-1}	4°C	DNA synthesis DNA breaks by PFGE
Uridine	^{3}H	36.5Ci $mmol^{-1}$	Ethanol 50%	1mCi ml^{-1}	1µCi ml^{-1}	4°C	RNA synthesis
dATP	^{32}P	3000Ci $mmol^{-1}$	10mM tricine buffer (pH 7.6)	10mCi ml^{-1}	50µCi ml^{-1}	-20°C	2Dgel. Southern

6. Enzymes

All enzymes were used according to the manufacturer´s guidelines

Table 6 Enzymes

Enzyme	Solvent	Use concentration or activity	Conservation
Lisozyme	H_2O	2mg ml^{-1}	-20°C
Proteinase K	TEE	1mg ml^{-1}	-20°C
Pronase	H_2O	50µg ml^{-1}	-20°C
RNaseA	TE	50µg ml^{-1} flow cytometry 100µg ml^{-1} DNA extraction	-20°C
Restriction			
XbaI	Buffer SH 1X (B3657. Sigma Chemicals Co.)	500U 100µl-1	0°C
PfuII	Buffer G 1X (ER0631. Fermentas)	10U 1µl-1	preparate in moment use

7. Solutions and Buffers

All buffers and solutions were stored at room temperature. Chemicals were dissolved in double distilled, sterile water (Milli-Q®). Unless stated otherwise.

Table 7. Solutions and buffers

Storage	
Bacteria Strains -80ºC	in 40% glycerol
TE for plasmid	Tris HCl 20mM, EDTA 50mM in H_2O
Viability	
Washing solution (minimal medium)	100ml M9x10 salts solution + 10ml $CaCl_2$ 0.01M solution + 10ml $MgSO_4$ 0.1M + 10ml Cassaa20%
TM9	100ml M9x10 salts solution + 10ml $CaCl_2$ 0.01M solution + 10ml $MgSO_4$ 0.1M in 880 ml H_2O
Flow cytometry LIVE/DEAD	
PBS (1M pH 7.4)	NaCl 140mM+KCl 2mM+Na_2HPO_4 8mM+KH_2PO 1.5mM
Plasmid purification	
Solution I	Sacarose or Glucose 50mM, Tris 20mM pH8, EDTA 10mM
Solution II	NaOH 0,2N, 1%SDS
Solution III	AcNa 3M pH4
TE	Tris 10mM pH8, EDTA 1mM pH8

DNA and RNA synthesis in vivo

Liquid scintillation	4g of PPO and 100mg POPOP in 1000ml of toluene. Keep on darkness. 4°C
TCA trichloroacetic 5%	100g TCA in 1000ml distillated H_2O. 4°C

Gel electrophoresis

BBF solution (x6)	Blue of bromophenol 0.25% and sacarose 40% in distillated H_2O. 4°C
Ethidium bromure	5mg ml^{-1} en TES. Keep on darkness
TAE	Tris HCl 4mM and EDTA 1mM in distillated H_2O. pH 8

Flow cytometry

Bacterial Fix solution	400µl of sample in 7ml of Ethanol. 74%
TE	Tris HCl 10mM pH8 + EDTA 1mM pH8 in H_2O
TE+RNaseA	Tris HCl 10mM pH8 + EDTA 1mM pH8 in H_2O +50 µg ml^{-1} RNasa

Pulsed gel electrophoresis (PFGE)

Agarose for blocks	50mg of low melting agarose in 2,5 ml of TEE (final concentration 2%)
Agarose for gel	1g of PFGE agarose in 100ml TAE 1X (final concentration 1%)
Liquid scintillation	7.5g of PPO and 0.15g POPOP in 1000ml of toluene. Keep on darkness. 4°C with agitation 2h. After add 500ml of triton 100X and conservation with agitation in darkness to 4°C 12 h before use.

Ethidium bromure	5mg ml^{-1} en TES. Keep on darkness
TEE solution	Tris-Cl 10mM pH9 + 20ml EDTA 100mM pH8 + EGTA 10mM pH7.5 in H_2O
Solution I	sarcosyl 0.05% + 5mg ml^{-1} lisozyme in TEE
Solution II	1mg ml^{-1} proteinase K + SDS 1% in TEE
TM9	10ml M9 10x + 1ml $CaCl_2$ 0.01M + 1ml $MgSO_4$ 0.1mM in H_2O
TE washing	Tris HCl 10mM + EDTA 1mM in H_2O
TE conservation	Tris HCl 20mM + EDTA 50mM in H_2O
TAE	Tris HCl 4mM and EDTA 1mM in distillated H_2O pH8

2D gel electrophoresis

M9x1	100ml M9X10 + 900ml H_2O
TEE	Tris-Cl 10mM pH9 + 20ml EDTA 100mM pH8 + EGTA 10mM pH7.5 in H_2O
Solution I	sarcosyl 0.05% + 5mg ml^{-1} lisozyme in TEE
Solution II	1mg ml^{-1} proteinase K + SDS 1% in TEE
Agarose for blocks	50mg of low melting agarose in 2.5 ml of TEE (final concentration 2%)
TE washing	Tris-Cl 100mM pH9 + 10ml EDTA 100mM pH 8 in sterile H_2Omili Q
TE conservation	Tris-Cl 20mM + 10ml EDTA 1mM in sterile H_2Omili Q
Agarose for 1D gel	0.4g of PFGE agarose in 100ml TAE 1X (final concentration 0.4%)
Agarose for 2D gel	2.5g of PFGE agarose in 250ml TAE 1X (final concentration 1%)
TBE	Tris HCl 89mM pH8 + boric acid pH8 + EDTA 2mM in distillated H_2O

Southern blotting

Denaturation solution	NaOH 0.5M + NaCl 1.5M in H_2O
Neutralization solution	Tris HCl 1M + NaCl 1.5M in H_2O. pH7.4
SSCx10	NaCl 150mM + Na_3Citrate 15mM in H_2O. pH 7
Buffer pre-hibridization	5X SSC + 0.1%N-lauroysarcosine + 0.02% SDS +1% Blocking reagent + 20µg ml^{-1} sonicate and denatured salmon sperm.
Buffer hibridization	5X SSC + 0.1%N-lauroysarcosine + 0.02% SDS +1% Blocking reagent + 20µg ml^{-1} sonicate and denatured salmon sperm + probe
2X wash solution	2X SSC + 0.1% SDS in H_2O
0.1X wash solution	0.1X SSC + 0.1% SDS in H_2O

Extraction chrDNA

HTE	Tris HCl 50mM pH8 + EDTA 20mM pH8 in H_2O

β-galactosidase assay

Z-buffer	0.75g KCl + 0.25g $MgSO_47H_2O$ + 8.53g Na_2PO_4 + 4.87g $Na_2PO_42H_2O$+ 2.70ml β-mercaptoethanol
ONPG	0.8g l^{-1} o-nitrophenyl-β-D-galactopyranoside in Z buffer
Sodium Carbonate	1M Na_2CO_3

8. Oligonucleotides

Table 8 Oligonucleotides. Conservation -20ºC

Name	Sequence	Summary of use
Ori1	5´TTTGTCGGCTTGAGAAAGACC	PCR. probe Southern
Ori2	5´TGCAAAACAGACAGGCGAAAC	PCR. probe Southern

9. Markers for gels

Table 9. Markers for gels

Name	Type of gels	Size range
CHEF DNA size marker *H.wingei*	PFGE gels	3.1 – 1 Mb
Gene Ruler 1kb DNA ladder	Agarose gel/2D gel DNA	0.25 – 1 Kb
Lambda DNA HindIII	Agarose gel	0.5 – 2.5 Kb

2.10. Dyes

Table 10. Dyes

Name	Stock	Use	Conservation
LIVE/DEAD	solution 2X (6uM SYTO9, 3uM PI) in 5ml H_2O	solution 1X	-20ºC
DAPI	1mg ml^{-1}	1µg ml^{-1}	-20ºC
SYBR Green	1:100 in H_2O	1:100000	-20ºC

Methods

1. Storage of bacteria, P1 and plasmid

E. coli strains were grown by shaking at 37°C in rich media (LBT) in the presence of the appropriate antibiotic for selection, were stored in the long-term at -80°C in 40% glycerol (v/v) by mixing volumes of overnight (ON) liquid cell culture (stationary phase) and glycerol 100%, in eppendorf.

By maintained bacteria sample from storage -80°C were checked onto the appropriate selective media to verify the genotype of the strain. After one colony were streaked in individual colonies and were stored in the short-term (up to 2 weeks) at 4°C on inverted nutrient agar plates in selective conditions (in presence of the appropriate antibiotic) sealed with Parafilm®.

P1 phage should be stored at 4°C. It cannot be frozen

Plasmids can be maintained for a short period (up to 1 month) in bacterial strains simply by growing on selective plates and storing at 4°C. For permanent storage, plasmid DNA can be stored in TE buffer or H_2OmiliQ at 4°C for several weeks or preserved for several years by storing at -20°C or -80°C.

2. Culture growth conditions

For ON culture, single bacteria colony was picked (2-3mm in diameter) from a nutrient agar (NAT) plate, was incubated for 16-24 h by shaking at optimal temperature and optimal medium growth for strain.

The ON inoculum was diluted 1:200 in fresh medium and incubated at 37°C by shaking water bath and maintained in exponential phase for at least six doublings. Growth was monitored by assessing the absorbance at 550nm (OD_{550}) in M9 minimal medium or 600nm (OD_{600}) for LBT rich medium.

For viable counting, or checking after construction of mutants strains, the cell were growth in solid, nutrient agar plate, and incubated at 37°C for 24h. For selection of mutants were added antibiotic in NAT plate. In this work, normal temperature of growth was 37°C, but for sensitive-temperature strain was 30°C.

3. Changes of growth conditions

All change in growth conditions were done to OD_{550} or OD_{600} between 0.15-0.2, unless otherwise indicated. All materials used here, were pre-heated to incubation temperature avoiding abrupt change of temperature.

3.1. Thymine starvation

Media shifts for thymine starvation were done by filtration of the culture growing exponentially reaching OD_{550} 0.2. Cells were collected on a nitrocelulose Millipore HA WP04700 filter (47mm of diameter and 0.45µm of pore diameter). The filtration was done by vacuum pump Millipore allowing minimal time to get the new condition.

Then the cells were washed with 5x filtered-volume of the same medium without thymine then, cells were resuspended for 1min with vortex in the same volume of growth medium pre-heated and without thymine. Later incubation was performed in the same conditions of shaking and temperature. At this moment additional treatments such addition of the drugs or change of incubation temperature were performed.

3.2 Addition of thymine, antibiotic or change of temperature

Performing recovery experiments thymine was added at different times after thymine starvation. Thymine (20 µg ml^{-1}) was added to an aliquot that was separated and incubated in other flask in the same conditions of shaking and temperature.

Antibiotics were added to a separated aliquot of the cultures growing in exponential phase OD_{550} 0.2 or under thymine starvation conditions.

The change of temperature was done by separating an aliquot of the cultures growing in exponential phase OD_{550} 0.2of to a flask pre-incubated to new temperature. If needed addition antibiotic and change of temperature at the same time, the change of temperature was first and then addition of antibiotic.

4. Viability analysis by counting of CFU in plate

Traditionally, the number of viable microorganisms is evaluated by plate counting. Plate counting refers to the technique by which a diluted sample is spread over a solid agar followed by case-specific incubation at an appropriate temperature. Under given cultural conditions each microorganism develops a distinct colony on the plate, and the initial number of viable organisms in the sample can be calculated from the number of colonies formed multiplied by dilution factor (Li *et al.* 1996, Madigan *et al.* 1997).

In order to improve the reliability of plate counting, the number of colonies formed on a plate (CFU) should be settled approximately between 50 and 250 CFUs, multiple dilution and numerous plates are needed to confirm the desired CFU, and the results are mostly read after a long incubation, typically over night, which denotes that plate counting is a labour-intensive and cumbersome method.

In this work viability analysis was performed to analyze survival under thymine starvation conditions. Aliquots at initial time (0 time after filtration) and at various intervals of time under thymine starvation were withdrawn, then serially diluted in TM9, and plated in duplicate on NAT plates. Plates were incubated overnight at 37°C and CFU were counted. The numbers of colony at different times were normalized to the number CFU at 0 time. All experiments were repeated at least three times, and the patterns were reproducible.

5. Bacteria viability by using the LIVE/DEAD BacLight Kit.

LIVE/DEAD BacLight Bacterial Viability Kits (Gant *et al.* 1993) now allow researchers to easily, reliably and quantitatively distinguish live and dead bacteria in minutes. Utilize mixture of SYTO 9 green-fluorescent nucleic acid stain and the red-fluorescent nucleic acid stain, propidium iodide (PI). SYTO 9 stain generally labels all bacteria in a population, those with intact membranes and those with damaged membranes. In contrast, PI penetrates only bacteria with damaged membranes, the emission properties of the stain mixture bound to DNA change due to the displacement of one stain by the other and quenching by fluorescence resonance energy transfer (Stocks 2004). Although this kit enables differentiation only between bacteria with intact and damaged cytoplasmatic membrane, it is often used to differentiate between active and dead cells (Gasol *et al.* 1999, Sachidanandham *et al.* 2005). While it seems accurate to assume that membrane- compromised bacterial cells can be considered dead (Nebe-von-Caron *et al.* 2000, Berney *et al.* 2006), the reverse (that intact cells are active cells) is not necessarily true (Joux and Lebaron 2000).

5.1. By flow cytometry

The flow cytometry assessment of bacterial viability is usually based on fluorescent staining to distinguish between viable, dead and dormant cells (Davey and Kell 1996, Alvarez-Barrientos *et al.* 2000, Veal *et al.* 2000). The dyes applied in flow cytometric analyses posses an affinity to specific cell compounds, and most dyes are actually the same as used in microscopic detection. Using divergent staining applications it has been measured both the metabolic activity (Nebe-von-Caron *et al.* 2000) and the membrane integrity (Nebe-von-Caron *et al.* 2000, Gregori *et al.* 2001).

For analysis of relative viability by flow cytometry, bacteria culture was grown up to 0.2 OD_{550} then, an aliquot was filtered and resuspendeded in thymine free medium. At 0 time and different times under thymine starvation samples of 1ml of culture were taken. The samples were concentrated by centrifugation at 5,000 rpm for 5 min at 4°C. Then were removed the

supernatant and the pellet resuspended in 1ml of PBS (these processes repeated twice). Then cells were resupended in 100µl PBS and 50µl LIVE/DEAD 1X and incubated at room temperature with rotating and darkness for 15min. After incubation 350µl PBS were added and the samples maintained at 4°C in darkness. Flow cytometric measurements were performed on a Beckman Flow cytometer, using 488 nm excitation from a blue solid-state laser at 50 mW. Optical filters were set up such that red fluorescence was measured above 630nm (FL3) and green fluorescence was measured at 520nm (FL1). The trigger was set for the green fluorescence channel FL1.

5.2. By microscopy

Microscopic detection has traditionally been applied for direct enumeration of microbial cells in a sample, and a normal light microscope represents probably the most conventional instrument to be used for that purpose. On the other hand, more sophisticated instruments such as fluorescence microscopes share the same optical principles of common microscopy but differs in sample handling and in the operation of instrument. Fluorochrome is a fluorescent dye, which holds affinity to the certain cell compounds such as nucleic acids, lipids and proteins, and is therefore suitable for staining microorganisms. Fluorescent staining has notably improved both the direct counting of bacteria (Maruyama *et al.* 2004) and the quantification of bacterial viability (Lopez-Amoros *et al.* 1997, Auty *et al.* 2001, Burnett and Beuchat 2002, Gatti *et al.* 2006). Typically separates bacteria according to the differences in membrane integrity, which allows the dye(s) to be bound to the different cell component as a function of membrane integrity.

For analysis of relative viability by flow cytometry, bacteria culture was grown up to 0.2 OD_{550} then, an aliquot was Filtered and resuspendeded in thymine free medium. At 0 time and different times under thymine starvation samples of 1ml of culture were taken. The samples were concentrated by centrifugation at 10,000 rpm for 5 min at 4°C. The pellet was resuspended in 1ml of NaOH 5% and incubated at room temperature for 1h. After incubation samples were centrifugated at 10,000rpm for 5 min, pellet resuspended in 50µl NaOH 5% and 50µl LIVE/DEAD 1X and incubated at room temperature for 15 min previously to analysis with microscope.

Images were acquired using a Nikon, model Eclipse E600, with has a high precision Mercury lamp, HB-10104AF model, by UV light, and has coupled digital camera Hamamatsu, C4742-95-10NR model, and the acquisition software NIS-Elements F2.30 by image capture. To obtain immobilization and spreading of the cells in one focal plane, a smooth agarose surface was prepared, 2% agarose (gelling point 35 ºC, SERVA-A1U04,

Heidelberg) in growth medium, that was keet it liquid at 60ºC-70°C in a dry bath.

A aliquot of 100 µl agarose was taken and put on an object slide, then was covered with a siliconized coverslip of 22 x 50 mm. After solidification of the agarose, the coverslip was carefully removed sideways resulting in an agarose "microslab". A drop of 5 µl was added, and was covered with a clean coverslip 22x22.

6. Cells counting by Neubauer chamber

There are some treatment in which it was necessary to determine the number of cells independently whether they are live or death. The number of cells per unit of the volume is often determined by the device called a counting chamber. Despite the fact of the recent technical development of scientific laboratories, the Neubauer chamber or hemocytometer remains the most common method used for cell counting around the world.

A counting chamber is a precision measuring instrument that is used to count cells or other particles in suspensions under a microscope. The Neubauer chamber is a thick glass slide with the size of a glass slide (30x70 mm and 4mm thickness). In a simple counting chamber, the central area is where cell counts are performed. The chamber has three parts, the central part, where the counting grid has been set on the glass and two independents counting areas.

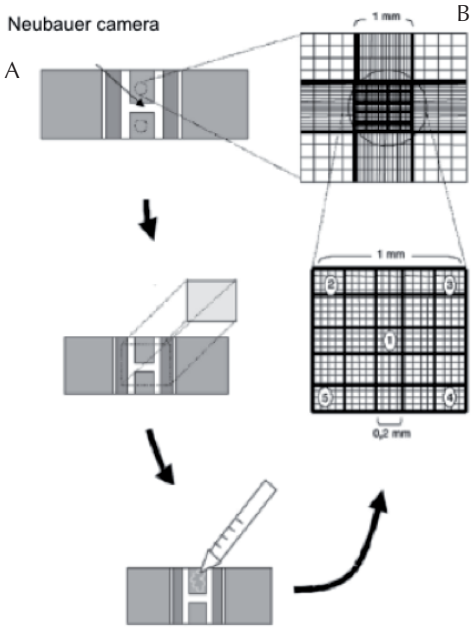

Figure 7. Neubauer counting chamber. A) Protocol for adding sample. B) Counting area.

Bacteria culture was grown up to 0.2 OD_{550}, an aliquot was filtered and resuspended in thymine free medium. At 0 time and different times under thymine starvation samples of 100 µl of culture were taken and deposited on the central area of the Neubauer chamber as follow: put the glass cover in the central area of the chamber, place pipette tip close to the glass cover edge. Release the plunger slowly watching how the liquid enters the chamber uniformly, being absorbed by capillarity. Leaved for 15 min. A flat surface to place the chamber, like a table or a workbench is recommended for the process.

In order to avoid overlap of the particles dilution of the sample has to be enough to distinguish single cells. Once set, the cells are counted in the selected squares being the total count around 150-250 cells to obtain a statistically significant number.

The Neubauer chamber was placed on the microscope stage. The microscope was focused until a sharp image of the cells was observed. The cells contained into the five big squares from the Neubauer were counted as described in figure 7. Images were acquired using a Nikon, model Eclipse E600, with has a high precision Mercury lamp, HB-10104AF model, and has coupled digital camera Hamamatsu, C4742-95-10NR model, and the acquisition software IpLab (Scanalytics) by image capture.

To determine the number of cell per ml the next formula was applied:
[Number of cell in the squares counted/ number of squared counted] x 10,000

7. P1 transduction

Transduction was discovered by Zinder and Lederberg (Zinder 1992, Zinder and Lederberg 1952) and in 1955 Lennox (Lennox 1955) reported that bacteriophage P1 could carry out generalized transduction in *E. coli*. Transduction is the heritable transfer of bacterial DNA from one cell (the donor) to another (the recipient) by a bacteriophage. Transducing bacteriophage particles are formed in donor bacterial cells during phage development. Generalized transducing phage particles carry a random fragment of host chromosomal DNA approximately the same length as the P1 DNA, completely lack DNA originating from the phage genome and contain instead only bacterial DNA sequences. They arise when P1 genome-sized fragments of donor DNA are packaged into phage heads in place of phage DNA. The process is called generalized transduction because any part of the bacterial genome can be packaged and transferred in this way. Host cells that are infected by transducing phage particles receive only non-viral sequences, so they are not killed. New genotypes in the recipient cells result from RecA-dependent (homologous) recombination, which can lead to the replacement of a recipient gene by an allele acquired from the donor genome via the transducing phage.

About 30% of the phage particles in a lysate contain host DNA rather than phage DNA. Given the relative sizes of the *E. coli* and P1 genomes, approximately 1 in 1,500 phage particles in a lysate will carry a given gene from the donor. Only about 2% of the transduced DNA is recombined into the recipient genome. Up to 90% of the transduced DNA remains trapped in the cytoplasm of the recipient cell as a stable form that does not replicate or recombine.

7.1. Preparing a P1 Lysate

A culture of the *E. coli* strain carrying the gene or mutation of interest was grown up to exponential-stationary growth phase (0.8 OD_{600}) at 37°C in LBT, then were added 2.5 mM $CaCl_2$ and 100mM $MgSO_4$. 1ml of this culture was then mixed with 1-10 µl of P1 lysate (10^7-10^8 phage ml^{-1}), then incubated for 17 min without agitation at the optimal temperature. 3ml of molten LB soft agar supplemented with 2.5mM $CaCl_2$ was added to the cell-phage mix and then poured onto and LB10 agar plate containing 5 mM $CaCl_2$ and incubated upright at 37°C overnight. Next, phages were then harvested from the plate that gave confluent lysis by removing the top agar and adding 100µl chloroform to kill any *E. coli* cells present and then left at 4°C for 10-20 min. Finally, following centrifugation (10,000 rpm for 10min at 4°C), the supernatant was transferred to a fresh bottle and were added 200µl of chloroform and stored at 4°C in dark.

7.2 Transduction

A culture of the *E. coli* strain was grown up to exponential-stationary phase (0.8 OD_{600}) at 37°C in LBT, and 2.5 mM $CaCl_2$ and 1mM $MgSO_4$ were added. 1ml of cells was mixed with 0,1,10 and 100 µl of P1 lysate from the bacterial donor and incubated for 30 min without agitation at optimal temperature for bacteria growth. Next, 5ml of LBT supplemented with 5 mM sodium citrate was added to the culture to avoid further infection by the phage and cells incubated at optimal temperature with gentle agitation for a 1 h to allow time for the selection marker to be expressed. Finally, the cultures were centrifugated at 10,000 rpm for 10 min at 4ºC and pellet was resuspended in 100 µl of LBT, plated onto NAT selective media and incubated at growth temperature. Transductants were tested phenotypically to ensure the successful co-transduction of the desired mutation with the selection marker.

8. Bacterial transformation with plasmids

The purpose of this technique is to introduce a foreign plasmid into a bacteria and to use that bacteria to amplify the plasmid in order to make large quantities of it. This is based on the natural function of a plasmid to transfer genetic information vital to the survival of the bacteria. Transformation of bacteria with plasmids is important not only for studies in bacteria but also because bacteria are used as the means for both storing and replicating plasmids.

A plasmid is a small circular piece of DNA (about 2000 to 10000 bp) that contains important genetic information for the growth of bacteria. Were discovered in the late sixties, and it was quickly realized that they could be used to amplify a gene of interest. A plasmid containing resistance to an antibiotic is used as a vector. The gene of interest is inserted into the vector plasmid and this newly constructed plasmid is then put into *E. coli* that is sensitive to antibiotic. The bacteria are then spread over a plate that contains antibiotic. The antibiotic provides a selective pressure because only bacteria that have acquired the plasmid can grow on the plate. Therefore, as long as you grow the bacteria in antibiotic, it will need the plasmid to survive and it will continually replicate it, along with your gene of interest that has been inserted to the plasmid.

The method for the preparation of competent cells depends on the transformation method used and transformation efficiency required. For a high transformation efficiency, we use electroporation. Below is a protocol for preparing electro-competent *E. coli*. If a lower efficiency is sufficient, we use heat shock transformation and chemically competent cells. Here is a protocol for preparing heat shock competent *E. coli*.

8.1. Plasmid purification

To isolate plasmid DNA from the *E. coli* strain harboring the desired plasmid an ON was grown in the presence of appropriate antibiotic selection in LBT at 37°C. 1.5ml of culture were centrifuged at 10,000rpm 4°C 3 min. Then cells resuspended in 200µl *solution I* and incubated during 5 min on ice. 200µl *solution II* were added and mixed by inversion, 300µl *solution III* were added and mixed with vortex in invert position for 10 sec following incubation during 3-5 min at 0°C. Mix was centrifuged at 4°C 12,000rpm for 10-15 min. Phenol-chloroform extraction was performed by transferring the supernatant to special eppendorf for phenol extraction adding 400µl of phenol-chloroform and mix by inversion for homogenation (take care, no pick up gel!). Then it was centrifuged at 4°C 12,000rpm for 5 min. (The supernatant is in top zone, gel in middle zone, and phenol in lower zone). The supernatant was mixed with 200 µl of isoamil-

chloroform, centrifuged at 4°C 12,000 rpm for 5 min. Supernatant was then transferred to clean eppendorf.

DNA was precipitated by adding 2x volume of ethanol 100% and incubated for at least 1h at -70°C. The solution was centrifuged4°C 10,000rpm for 15min and the pellet was resuspended in 800µl of ethanol 70%, centrifuged at 4°C 10,000rpm for 10 min and pellet resuspended on 25µl of TE (with RNasa 50µg ml^{-1}). Plasmid DNA was verified by electrophoresis and was stored in double distilled sterile water at -20°C.

8.2. Preparation of competent cells and transformation

As DNA is a very hydrophilic molecule, it won't normally pass through a bacterial cell's membrane. In order to force bacteria to incorporate the plasmid DNA they must first be made "competent" by creating small “holes” in the bacterial membrane.

The procedure to prepare competent cells can sometimes be tricky. Bacteria are not very stable when they have holes put in them, and they die easily. A poorly performed procedure can result in cells that are not very competent to take up DNA. A well-performed procedure will result in very competent cells. The competency of a stock of competent cells is determined by calculating how many *E. coli* colonies are produced per µg of DNA added. An excellent preparation of competent cells will give ~10^8 colonies per µg. A poor preparation will be about 10^4 µg or less. Our preps should be in the range of 10^5 to 10^6.

The principal procedure to prepare competent cells and we have used, are: incubating the cells and the DNA in a high concentration of calcium or electric charges that produce holes in membrane.

8.2.1. By calcium chloride method

A culture of the strain to be transformed, first, it was grown in ON at optimall temperature in LBT up to 0.3-0.4 OD_{600} with vigorously shaking. Samples of 5ml were incubated for 10 min on ice, then centrifuged at 4ºC 13,000rpm during 8 min, resuspended in 1/5 initial volume of 0.1M cool $CaCl_2$ and incubated during 10 min on ice, centrifuged and the pellets were resuspended in 1/25 initial volume of 0.1M cool $CaCl_2$ and keep on ice.

Plasmid DNA (no more than 50ng in a 10µl final volume) was mixed with 100µl of competent cells on cool sterile tube, incubated for 30 min on ice and transferred 90 sec at 42°C then returned to ice during 1-2 min. The change of temperature causes the entrance of plasmid by causes of holes in bacteria membrane. 400µl of LBT was added and cells were incubated with slow shaking

for at least 2 h. Then the cell suspension was plated onto selective media and incubated ON at the appropriate temperature.

8.2.2. By electroporation method

Electroporation is a laboratory method used to introducing foreign DNA into cells. An apparatus is used to generate a very short electrical charge which passes through the cell suspension. The electric charge causes very small, and short-lived, holes to form in the cell membrane. Vectors containing the foreign DNA, which are suspended in the solution, move into the cells through these holes.

The electroporation protocol is based on the physics of electrical conductivity and permeability of cell membranes. A pulsed charge of about 10,000 to 100,000 V/cm, depending on cell size, is used to alter the transmembrane voltage along the cell membrane. The pulse lasts a few microseconds to one millisecond. Beyond a certain voltage threshold, too much charge will destroy the cells. However, short bursts of the right charge will temporarily interrupt the phospholipid bilayer, allowing macromolecules like plasmids to pass through.

A sample of 1 ml of cells was centrifuged at 4ºC 10,000 rpm during 1 min. Pellet was resuspended with pipette in 1ml of H_2OmiliQ to 0ºC and this protocol repeated twice. Pellet was resuspended with pipette in 200µl glycerol 10% to 0ºC. Finally the supernatant was resuspended with pipette in 40 µl glycerol 10% to 0ºC. Each electroporation consume 40µl of competent cells. The competent cells were stored at 0°C in individual eppendorf. No more than 50 ng plasmid DNA was added to 40µl of competent cells, mix incubated to 0°C during 1 min and eventually transferred to a cooled electroporation cuvette. Electroporation was performed buy using the following conditions: 25µF, 2,5 Kv and 200V. After electroporation, 1 ml of LBT was added and cells incubated during 2-3 h at optimal temperature. Cells were plated onto selective media and incubated ON at the appropriate temperature.

9. Measuring DNA synthesis

The progression of the DNA synthesis was determined by quantification of incorporated ^{3}H-Thy in DNA as TCA-precipitated material. ON cultures were diluted 1:200 -1:500 in fresh M9 minimal medium containing ^{3}H-Thy at 1 µCi ml^{-1}. In order to get uniform labeling, samples were withdrawn at least four generations after the dilution.

Each sample consisted in 200µl aliquots of culture that processed as follows: precipitated on 3ml of ice-cold 5% TCA where the cells lysed and the DNA precipitated. The precipitate was filtered by using Wathman GF/C 24 mm

diameter glass fiber filters previously soaked in distilled water. Each tube was washed with 1ml of TCA 5%, filtered this volume and washed twice with distilled water. The filters were dried to 50°C and soaked in scintillation liquid. Disintegrations per min (dpm) of each sample were determined in Beckman LS3801 liquid scintillation counter.

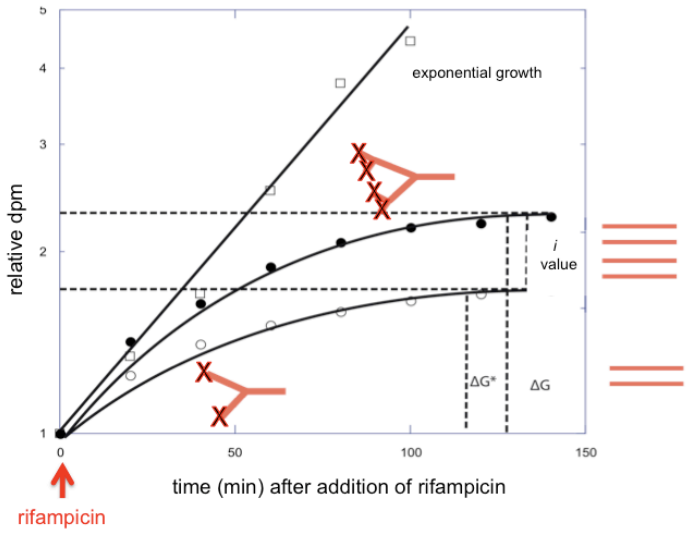

Figure 8A. Schematic representation of DNA synthesis after inhibition of new initiations by addition of Rif to obtain ΔG and ΔG′ values. Fully replicated chromosomes are represented by a line. Only half of the replicating chromosome is drawn.

9.1 In exponential growth

The amount of ^{3}H-Thy incorporated in DNA was determined at different times of exponential growth in M9 minimal medium containing ^{3}H-Thy at 1 µCi ml^{-1}at 37ºC. From these data the time required to duplicate DNA content was determined and checked to be similar to generation time τ determined by OD_{500}.

9.2 Determining the number of replication rounds per chromosome, *n* and the chromosomal replication period, *C period*

In *E. coli* the time required to replicate a single chromosome (C period) can be longer than the generation time (τ), and in these conditions the number of replication rounds overlap in the chromosome. The number of replication rounds per chromosome, defined as *n* (Sueoka and Yoshikawa 1965, Cooper

and Helmstetter 1968). *n* was determined by using the ΔG value, which is defined as the relative runout DNA synthesized after inhibiting new initiation events, while ongoing forks are allowed to finish (Pritchard and Zaritsky 1970). This experimental condition can be achieved by addition of Rif 150μg ml^{-1} or Cm 200μg ml^{-1} to exponentially growing the cells in M9 minimal medium containing 1 μCi ml^{-1} of ^{3}H-Thy and assaying the radioactive TCA-precipitated material. Rif inhibits RNA polymerase and Cm inhibits protein synthesis, both activities are known to be required for the initiation step. From the amount of runout DNA synthesis (ΔG), the value of *n* was obtained from $\Delta G=[2^n n \ln 2/(2^n-1)]-1$ by using computer software implemented in our lab (Jiménez-Sánchez and Guzmán 1988, *Ciclon* http://genuex.unex.es/alf/c/ciclon.html) (Fig. 8A). To apply for this analysis additional flow cytometry profiles have to be performed to verify the completion of chromosome replication rounds. Flow cytometry analysis was performed in the presence of 150μg ml^{-1} Rif and 50μg ml^{-1} Cfx to inhibit cell division. The C period value in steady state cultures was determined to be $C=n\tau$. τ is the generation time, defined as the mass doubling time in minutes as measured by OD_{550}.

9.3. Determining the percentage of initiated origins (*i*)

In a mid-log phase culture Δ*G* is related to the number of replication rounds per chromosome, *n*, being 2^n the number of origins per chromosome (Sueoka and Yoshikawa 1965). If after a period of time under thymine starvation additional initiations occurred in a fraction of *i* origins and further new initiations are prevented by adding Rif; then the runout of DNA synthesis accumulated under this treatment will be denoted as $\Delta G' = [2^n(i+1)n\ln 2/(2^n-1)]-1$, being the final number of origins $2^n(i+1)$.

The fraction of additional replication origins initiated during thymine starvation treatment, *i*, was calculated from the ratio $\Delta G'/\Delta G=[2^n(i+1)]/2^n= i+1$ From here, the *i* value for a given treated culture can then be calculated (Jiménez-Sánchez and Guzmán 1988, http://genuex.unex.es/alf/c/ciclon.html).

9.4. Determination of the optimal concentration of thymine for MG1693

Changes of thymine concentration in the growth medium affect the chromosome replication time of Thy- strains without causing a detectable difference in the growth rate (Pritchard and Zaritsky 1970). Consequently, is essential determining the optimall concentration of thymine to achieve a replication rate not limited by the amount of exogenous thymine added in growth medium (Molina *et al.* 1998).

In this work we estimated the optimall thymine concentration for MG1693 by determining runout of DNA synthesis at 37°C in M9 minimal medium containing different thymine concentrations (μg ml^{-1}) and [methyl-^{3}H]-thymine (20Ci $mmol^{-1}$) at 1 μCi ml^{-1} to label DNA. The cultures were grow up to 0.2 OD_{550} , a portion of the culture was treated with Rif (150 μg ml^{-1}) in order to inhibit initiation of chromosome replication allowing the elongation of chromosome replication, and runout DNA synthesis was measured as TCA acid-precipitated material. From the amount of runout synthesis, ΔG, the number of replication rounds per chromosome equivalent, n, was obtained by the algorithm $\Delta G = [2^n \times n \times \ln 2/(2^n - 1)] - 1$ (Pritchard and Zaritsky 1970; Sueoka and Yoshikana 1965). From this we obtained the length of the C period by the equation $C = n\tau$, where τ is the time for mass doubling and DNA duplication.

Mass doubling and doubling of DNA content took 39 min for MG1693 at all thymine concentrations. Otherwise, runout synthesis and hence the length of the C period increased with decreasing thymine concentrations in the growth medium, as expected for a Thy- phenotype, where the thymine concentration limits the replication velocity. From figure 8B the optimall thymine concentration can be determined, as the minimal thymine concentration giving the minimal C period. Thus, in the case of MG1693, this concentration is 20 μg ml^{-1}

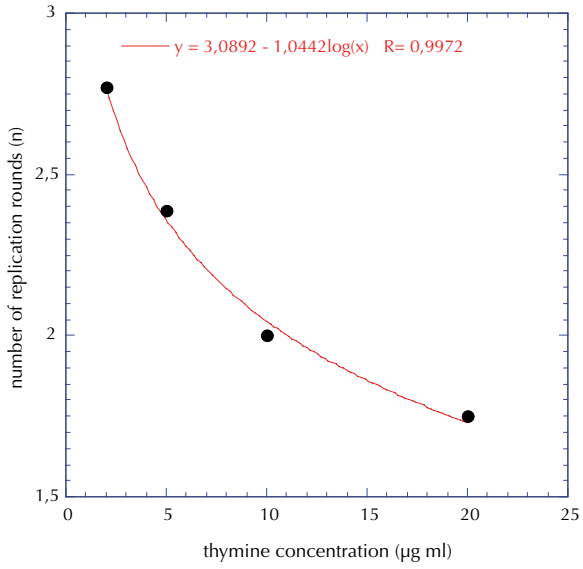

Figure 8B. Determining the optimal thymine concentration for MG1693.

According to these results all experiments of this work were performed in M9 minimal medium supplemented with 20 μg ml^{-1} of thymine (unless otherwise indicated).

10. Measuring DNA degradation

The quantification of degradation of DNA *in vivo* was undertaken as follow: ON culture was diluted 1:200 in fresh medium with presence of 1 µCi ml^{-1} ^{3}H-Thy, the cells were grown up to 0.2 $OD_{550n,}$ a aliquot was filtered and resuspended in the same medium of growth without thymine neither ^{3}H-Thy. Samples were withdrawn at different times and dpm were estimated as TCA acid-precipitated material.

11. Measuring RNA synthesis *in vivo*

RNA synthesis *in vivo* was estimated as RNA transcription rate. Samples of 500µl were separated at various time intervals in exponential growth and during the different treatments. The samples were incubated in pre-heating tubes with shaking in presence of 1 µCi ml^{-1} of [*methyl*-^{3}H]-uridine (20 Ci/mmol) during 2 min. The reaction was stopped by addition of 2ml of TCA and incubated on ice. The precipitate was then filtered by using Wathman GF/C 24 mm diameter glass fiber filters previously soaked in distilled water. Each tube was washed with 1ml of TCA 5%, filtered this volume and washed twice with distilled water. The filters were dried to 50°C and soaked in scintillation liquid. Disintegrations per min (dpm) of each sample were determined in Beckman LS3801 liquid scintillation counter.

12. Flow cytometry

Flow cytometry (FCM) is an analytical instrument that allows a rapid analysis of thousands of cells per second, highly resembles microscopy with advantages of automation, objectivity and speed (Veal *et al.* 2000). Is based on the optical analysis of individual cells, cells suspended in a buffer are introduced in to the flow cell with flowing sheet fluid. The suitable light source (mostly an assortment of lasers) is used to illuminate cells as they pass individually through a beam of light focused on flow cell. Light scattered by cells, consisting of forward scatter (FS) and side scatter (SS), is collected by suitable filter units, amplified by photomultiplier tubes and send to data processing unit. Forward scattered light, collected in the same direction as the illuminating light, is related mainly to cell size, and side scattered light, collected at an angle of 90°, gives an information of surface properties and internal structure of cells. Additional information is obtained through fluorescence emission by staining cells with different dyes exhibiting bright fluorescence when illuminated with suitable excitation light. The detectors produce electronic signals proportional to the optical signals striking them. List mode data are collected on each particle or

event. The characteristics or parameters of each event are based on its light scattering and fluorescent properties. The data are collected and stored in the computer.

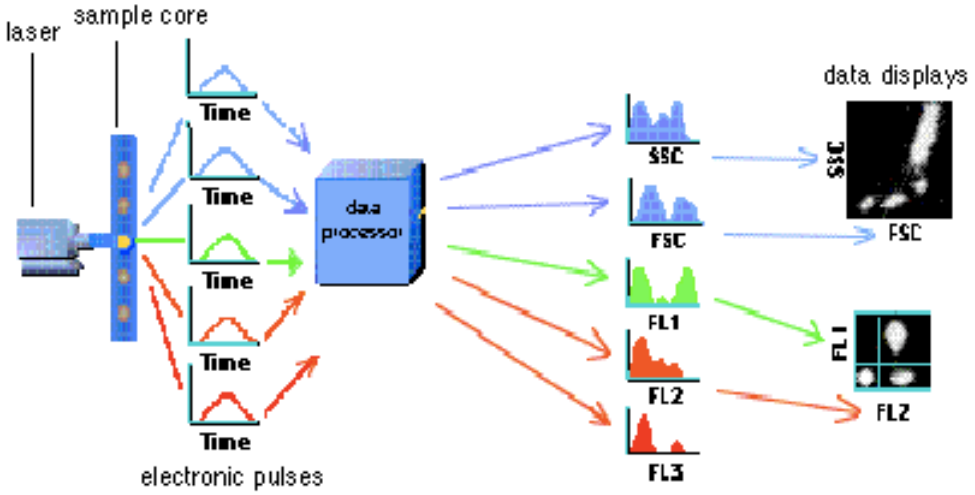

Figure 3M . Flow cytometry. Scattered and emitted light are converted to electronic pulses that can processed by the computer

The DNA content per cell was measured by flow cytometry as previously described (Skarstad *et al*. 1985). When the cultures reached an OD_{550} of 0.2, a portion was transferred into another flask, and 150µg ml^{-1} Rif and 150µg ml^{-1} Cfx were added. Rif is an antibiotic that inhibits the β-subunit of RNA polymerase (Lark 1972, Messer 1972, Zyskind and Smith 1977). Initiation of replication, but not elongation, requires transcription by the RNA polymerase. Thus, treatment with Rif allows ongoing rounds of replication to finish and no new initiations of replication take place. Cfx inhibits cell septum formation and therefore cell division. Cells simultaneously treated with Rif and Cfx will end up with an integral number of chromosomes that reflect the number of origins at the time of drug treatment 2^n (Sueoka and Yosikawa 1965). In a culture with cells that initiate replication synchronously cell will end with 2^n (n = 1, 2, 3…) chromosomes, i.e., 2, 4 or 8 chromosomes, while cells that initiate asynchronously will also contain 3, 5, 6 or 7 chromosomes (Skarstad *et al.* 1986).

Cultures were treated with Rif + Cfx for 4 h with continuous shaking, 400 µll of sample were fixed by 7 ml of 74% ethanol. Approximately 1.5 ml of each fixed sample was centrifuged, cells washed in 1 ml of ice-cold staining buffer, then centrifuged and resuspended in 1 ml of the same buffer. Cells were incubated on ice in darkness for at least 30 min in the presence of 1µl of SYBR. Samples were analyzed by using a Beckman coulter cytomics FC500. For data analysis WinMDI version 2.9 software used.

13. Agarose Gel Electrophoresis

In this work agarose gel electrophoresis was used to analyze PCR DNA fragments and plasmid DNA preparations.

Gel conditions were 0.8% agarose prepared in TAE buffer (w/v) and TAE (1x) buffer for running. In order to visualize the DNA, ethidium bromure (EthBr) 0.5µg ml^{-1}, was added directly to the agarose gel and buffer. The samples was mixed with BBFx1 solution before leaved into the well. This solution increases sample density facilitating the entering in the wells, also the blue of bromophenol is a component that gives color to sample. Gels were run between 80-130V for up to 1h and viewed under a UV lamp (GD55000 of UVP) and the densitometry study with Quantity One program of Photodyne. Lambda DNA Hind III Digest of Sigma and GeneRuler 1Kb DNA of Fermentas were used to estimate the size of DNA samples.

14. Pulsed field gel electrophoresis

Separation of DNA fragments by standard agarose gel electrophoresis is based on the capacity of the molecules to pass through the pore generated inside the matrix gel. Using this feature as the only separation mechanism, the large DNA molecules cannot be discriminated from each other. The practical range of resolution is up to approximately 50Kb; making impossible the direct genomic analysis of large DNA molecules as those generated by the presence of complex DNA structures, or the DSBs involved in stalled replication forks in the *E. coli* chromosome.

In 1983, PFGE was developed as a method to circumvent this limitation, allowing fractionation of very large DNA molecules up to a million base pairs in size (Schwartz and Cantor 1984, Herschleb *et al*. 2007). PFGE allows the separation of these large DNA molecules through abrupt electrical perturbations to the paths crawling molecules take trough the gel. In PFGE, the direction of the electrical field is periodically changed (usually 120º), requiring electrophoresing molecules to reorientate. The time required to complete the orientation process scales with the size of the DNA, so that increasing the size of the DNA molecules, it takes more time between changes in the direction of the electric field. These intervals vary depending on the size of the fragments that have to be resolved, a few seconds for small fragments to hours for fragments larger than 5Mb. The principle of PFGE is that large DNA fragments require more time to reverse the direction in an electric field than small DNA fragments. Alternating current direction during gel electrophoresis can resolve DNA fragments of 100 to 1,000 kb.

The equipment required to perform PFGE is also different from that used in tradicional electrophoresis. The tank contains a set of electrodes (6-8), instead of a couple of them, being thicker and disposed to allow the different orientations of the electric field. Maintaining a constant the temperature (14ºC) during the process is important to avoid temperature variations through the gel, which could affect the resolution of DNA fragments. Accordingly, the system should include a cooling device. Due to the fragility of the very large DNA fragments to be separated, preparing the sample is the most critical step for PFGE. To avoid breakage of genomic DNA during manipulation, the DNA is not extracted, but the cells are embebed in agarose plugs and then fixed into the wells.

14.1. Determination of percentage of lineal DNA

We used this approach to quantify lineal DNA in different strains. The amount of linear DNA resulting from DSBs can be estimated by using PFGE combined with cell lysis in agarose plugs (Michel *et al.* 1997).

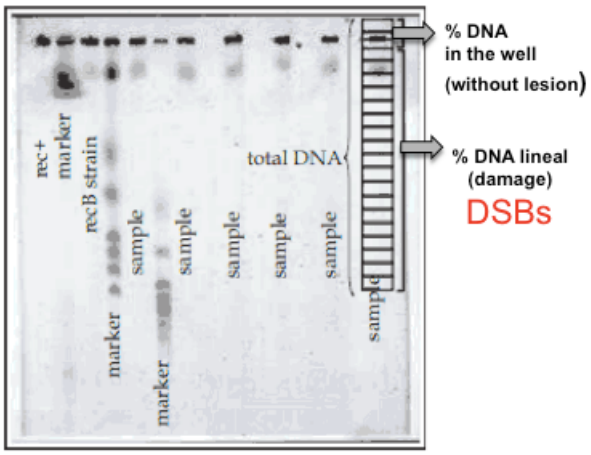

Figure 9 . PFGE. Visualization of a representative PFGE gel stained with EthBr.

Cultures of *recB* was grown in M9 minimal medium labeled by addition of 5 µCi ml^{-1} [*methyl*-3H] thymine (100 Ci $mmol^{-1}$). When cultures reached 0.2 OD_{550} nm, 1ml of cells were centrifuged at 4°C 5,500rpm at 10 min, pellet was washed with 2 ml of TM9 (using pipette, no vortex). This process was repeated twice and pellet was resuspended in 120 µl of *solution I* (using pipette, no vortex) and it was incubated at 37°C for 10-15 min. Sample of 120 µl was mixed with 140 µl of low melting agarose 2% in TEE and 7 µl of SDS 20% at 55ºC and poured into the mould. Once agarose had solidified, cell lysis was performed in the plugs. This ensures only linear chromosomes to enter the gels, while circular

molecules remain in the wells (Michel *et al*. 1997, Seigneur *et al*. 1998). Plugs were incubated with 1ml of solution I for 2 hours at 37°C. Then, plugs were retrieved and incubated with lysis *solution II* at 56ºC ON. The plugs can be stored during 3-4 weeks to 4ºC in 1ml of conservation TE. Each plug of DNA was put in a well of agarose 1% gel in TAE, and was sealed with agarose.

CHEF-DR III Pulsed Field Electrophoresis System of BioRad was used, and the run conditions were performed as described (Seigneur *et al*. 1998); initial run 500 sec, final run 500 sec, 3 volts/cm and 106° reorientation angle during 48 h at 4°C. TAE buffer was used for refrigerating at 7°C.

DNA was visualized by EthBr staining. Lanes were cut into slices and the proportion of migrating DNA was determined by calculating the amount of tritium present in each slice with respect to the total amount of tritium present in the corresponding lane plus the well (Fig. 9). The 3 mm width gel slice were melted with 800µl of HCl 0.1N at 100°C. The volume of each portion was put on a vial with 10 ml of scintillation liquid (see *Material* section) and the amount of radioactivity was determined using a counter of scintillation Beckman LS3811. All the PFGE linear DNA data were analyzed by the least-squares statistical approach, considering measures as highly significantly different if $p< 0.01$.

14.2. Determination of percentage of nmDNA after digestion with XbaI

Cultures of MG1693 were grown in M9 minimal medium up to 0.2 OD_{550}, 2 ml of cells were centrifuged at 4°C 5,500rpm at 10 min, pellet was washed with 2 ml of TM9 (using pipette, no vortex). This process was repeated twice and pellet was resuspended in 120 µl of *solution I* (using pipette, no vortex) and it was incubated at 37°C for 10-15 min. Sample of 120 µl was mixed with 140 µl of low melting agarose 2% in TEE and 7 µl of SDS 20% at 55ºC and poured into the mould. Once agarose had solidified, cell lysis was performed in the plugs, incubated with 2 ml of solution ESP for 2 h at 55°C. Then, plugs were transferred to a tube of 50 ml and 8-10 ml or sterile water pre-heating to 50ºC were added and during 10 min on shaker. This wash procedure was repeated 4 times. These plugs can be store during 3-4 weeks to 4ºC in 1ml of conservation TE. Each plugs of DNA was incubated in 50 µl de XbaI (50U/100µl 1x bufferSH) to 37°C during 3 h. After XbaI digestion the plugs were introduced into the wells of agarose 1% gel in TAE, and sealed with agarose.

CHEF-DR III Pulsed Field Electrophoresis System of BioRad was used, and the run conditions were: initial run 2.16 sec, final run 54.17 sec, 6 volts/cm and 120º reorientation angle during 18 h (Matuskek *et al*. 1996, Gautom 1997, Askarim 2007).

DNA was visualized by EthBr staining. The quantification of DNA concentration was estimated by the intensity of fluorescence and the densometry study was performed by *Image J* program. The wells and 3 mm up or down to well would correspond to non migrating DNA (nmDNA) (Nakayama *et al*. 1994). Data were presented relative to total DNA amount in the lane.

15. 2D DNA agarose gel electrophoresis.

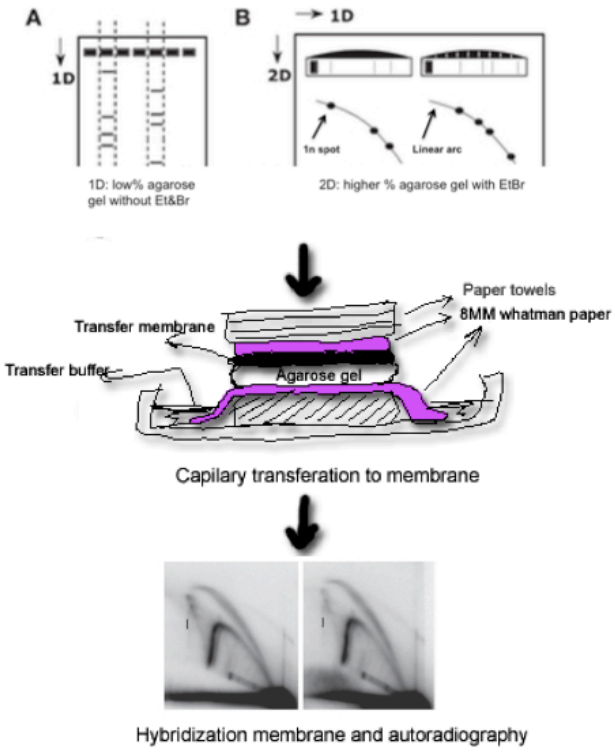

Figure 10. 2D DNA gel electrophoresis. (A and B) 1º dimension and 2º dimension run gel DNA electrophoresis. (Low) Southern blot. (lower) Result of hybridization with the labeled probe and autoradiography.

Two-dimensional (2D) agarose gel electrophoresis is one of the most powerful methods to analyze the mass and shape of replication intermediates. It is often use to map replication origins but it is also useful to characterize termination of replication, replication fork barriers and even replication fork reversal. The movement of a DNA molecule through an agarose gel is determined either by factors intrinsic to the electrophoretic conditions (agarose concentration, the strength of the electric field, the presence of intercalating agents, etc.).

2D agarose gel electrophoresis consists of two successive electrophoreses in which the second dimension occurs perpendicular to the first. Two different migration conditions are used so that the first dimension conditions (low voltage, low agarose concentration) minimize the effect of molecular shape on electrophoretic mobility, whereas this effect is maximized during the second dimension (high voltage and high agarose concentration, in the presence of an intercalating agent) (Friedman and Brewer 1995). As a consequence, a branched DNA molecule like a recombination or a replication intermediate is separated from a linear molecule of the same mass during the second dimension. The different migration patterns of a branched DNA or recombination intermediary digested with a specific restriction enzyme are revealed after southern blotting hybridization with a specific probe and it indicates the mode it has been replicated.

15.1 Isolation of DNA from bacterial cells. Digestion with PvuII

Culture of MG1693 was grown in M9 minimal medium up to 0.2 OD_{550}, 10 ml of cells was withdrawn and treated with 1 ml sodium azide 1M to stop of metabolic state of cells. The aliquot was centrifuged at 4°C 4,500rpm for 10 min, then the pellet was washed twice with 1 ml of M9x1 (using pipette, no vortex). Pellet was resuspended in 250µl of TEE (using pipette, no vortex) and incubated at 37°C for 10-15 min. 200 µl of cells was mixed with 200 µl of low melting agarose 2% in TEE at 55ºC and poured into the mould. Once agarose had solidified, cell lysis was performed in the plugs, that were incubated with 1.5 ml of *solution I* during 2 h at 37°C. Plugs were then retrieved and incubated with 1ml lysis *solution II* at 56ºC ON. The plugs can be stored during 3-4 weeks to 4ºC in 1ml of conservation TE.

Before incubation with the restriction enzyme plugs have to be washed several times in order to eliminate trace of protease from previous treatment. The plugs were washed three times with 10 ml TE 1x for 30-60 min on sterile tube at 37°C into rotating incubator. Then the plugs were washed with 1 ml buffer G 1X Fermentas during 30 min in sterile eppendorf at 37°C into rotating incubator. After this, plugs were washed with 1ml buffer G 1X Fermentas + BSA 1X during 30 min in sterile eppendorf at 37°C into rotating incubator. Finally, the plugs were incubated with 250 µl restriction buffer and 1.5 µl of PvuII restriction enzymes (see *Material* section) for 2 h, then another 1.5 µl PvuII ON. The incubation with enzymes was done into rotating incubator at 37ºC.

15.2. 1ª and 2º dimension

The optimall electrophoresis conditions differ according to the shape and size of the DNA to be analyzed.

In this work the first dimension was run at 0.9 V cm^{-1} in 0.4% agarose gel prepared in TBE buffer 1X at room temperature for 22 h. Following that, the first lane containing the Gene Ruler 1Kb DNA ladder was excised, stained with 0.3 µg ml^{-1} EthBr and photographed. During this period, the agarose lanes containing the DNA samples were kept in the dark.

Each lane of the gel was cut and was run for the second dimension at a 90º angle with respect to the first dimension (Fig. 10). It was run at 5 V cm^{-1} in 1% agarose gel prepared TBE buffer 1X at 4ºC cold room for 7.5 h and containing 0.3 µg ml^{-1} EthBr.

15.3. Southern blotting

The Southern blot (Southern 1975) is an example of a basic nucleic acid hybridization technology, DNA fragments from an agarose electrophoresis gel are first transferred onto a membrane and then detected by a labeled "probe." The transfer step starts the procedure and is problematic for at least two reasons. First, electrophoretic transfer cannot be used because excessive heat would result from the high ionic strength required for DNA to bind to most membranes (especially small fragments, < 500 bp.) Thus, a slower “capillary” transfer is required which allows high ionic strength. Second, as the transfer proceeds, the gel shrinks and retards the migration of the larger pieces of DNA, which are often the size containing a gene or a substantial portion of a gene. Thus, cleaving DNA into smaller fragments (HCl depurination followed by NaOH), along with long periods of time (18-48 h) are usually needed to effectively transfer large DNA fragments. Cellulose nitrate and Nylon are the most common membranes used and the procedure below may be used for both. After transfer to a membrane, the DNA fragments are detected by hybridization with a "probe."

After 2D electrophoresis the DNA is transferred to appropriate membranes by Southern blotting by following these steps: (i) Depurination: the was rinsed in 250 ml of ClH 250 mM solution for 15 min with gentle shaking, then gel washed with distillate H_2O. (ii) Desnaturation: the DNA was denatured with NaOH 0.5 N and ClNa 1.5M for 15 min (twice) with gentle shaking, then gel washed with distillate H_2O. (iii) Neutralization: the gel was incubated with Tris 1M and ClNa 1.5M (twice) for 15 min.

The transfer of the entire gel was built from bottom as indicated in figure 10. Hybon N+ (GE BioSciences) membrane was used being sure that: there were no bubbles between the gel and paper and it was correctly orientated marked by using pencil.

After ON transfer was completed, the membrane nylon was removed and washed with SSCx6 for 5 min and finally dried.

The gel was stained with EthBr 0.5 µg ml^{-1} for 20 min to verify the complete transfer of the DNA from the gel to the membrane.

15.4. Labeling the probe

DNA probe was radiolabeled with ^{32}P by using $^{32}P\alpha$-dATP in a random primer reaction in the Thermo Scientific DecaLabel DNA labeling Kit

Mix A

Components	
DNA template (probe)*	100 ng
Decanucleotide in 5X buffer	20 µl
Water nuclease-free	up to 84 µl

The mix was heated in boiling water for 10 min, then was placed quickly at 0° C to avoid DNA renaturalization.

Reaction (for 100 µl)

Components	
Mix A (heated)	84 µl
Mix C	6 µl
^{32}P-dATP (1.85MBq=50 µCi)	5 µl
Klenow fragment (5µl)	2 + 2 µl
Reaction was incubated at 37°C for 5 min	
dNTP mix	4 µl

Reaction was incubated 37°C ON and stopped by addition of 1µl of 0.5 M EDTA pH 8.

15.5. DNA hibridization

Membranes were pre-hybridized in "hybridization tubes" containing 20 ml pre-hybridization solution at 55ºC for 1h then, 20µl labeled DNA probe was added and incubated for 14-16 h. Hybridized membranes were sequentially washed in 30 ml of 2X washing solution at room temperature for 5 min twice; and in 30ml of 0.1X washing solution at hybridization temperature 55°C during 15 min twice.

Membranes were covered by Saran wrap, exposed and developed by Molecular Imager FX of BioRad system. Images were analyzed by Quantity One software.

16. Polymerase Chain Reaction (PCR)

Standard PCR protocols were performed for amplification of DNA fragments later used for two purposes:

(i) Sequencing *oriC* region to verify *oriC* partial deletions mutants (see *Material* section for primers sequence)

(ii) Amplification of *oriC* DNA fragment used as probe in Southem blot (see *Material* section for primers sequence).

PCR reactions were carried out in a thermo-cycler Eppendorf (Mastercycler Gradient Model).

Table 11. Components of PCR reaction for 100µl of final volume

Components	STOCK concentration	FINAL concentration
Buffer (10X)	10µl	1X
MgCl(25mM)	6µl	1.5mM
dNTP(2mM)	5µl	0.1mM
chDNA(from colony)	1µl	30ng
PrimerA (2.5µM)	10µl	0.25µM
PrimerB (2.5µM)	10µl	0.25µM
Taq (Promega)	1µl	5U
H_2O	57 µl	

Table 12. PCR protocol.

Phases	Cycles	Temperature	Time
Initial denaturation	1	95°	2min
Denaturation	2-35	95°	1min
Annealing		55°	1min
Extension		72°	2min
Final extension	1	72°	10 min
Hold	1	4°	∞

Amplification was verified by DNA agarose gel electrophoresis

17. Extraction and Purification of chromosomal DNA

17.1. Extraction

Cells from ON cultures were diluted 1:200 into fresh medium, grown at 37ºC up to stationary phase, 0.8 OD_{600}, , quickly chilled and centrifuged 8,000 rpm for 1min. Cells were washed with 310 µl of HTE buffer and 310 µl N-lauril-sarcosine 2% (in HTE) then mixed by inversion. 5µl of RNaseA (10mg ml^{-1}) were added and incubated at 37°C for 15 min. After that was added 17.5 µl of Pronase (1 mg ml^{-1}) and incubated 50°C for 30-90 min. Vortex strongly during 2 min to fragment DNA. Following that, phenol-chloroform-isoamilic extraction was done by standard protocols.
DNA was ethanol-precipitated by adding the same volume of DNA solution and ethanol and keep at -80°C for 30-60 min. The sample was centrifuged at 4°C 12,000 rpm for 30 min. Pellet was washed with ethanol 70% and resuspended in 50 µl of sterile H_2O miliQ.

In the case of small fragment DNA precipitation was performed in 0.1 volume of LiCl 4M and 3 volume of cool ethanol 100% and 1µl of glicogen 20 mg ml^{-1}, mixed and incubated at -70° for 3h (better ON).

18. Microscopy

18.1. Slide preparation

To obtain immobilization and spreading of the cells in one focal plane, a smooth agarose surface was prepared, 2% agarose (gelling point 35 ºC, SERVA-A1U04, Heidelberg) in growth medium keep it liquid by incubation at 60ºC-70°C in a dry bath.

A 100 µl agarose aliquot was pored on an object slide, then covered with a siliconized coverslip of 22 x 50 mm. After solidification of the agarose, the cover slip was carefully removed sideways resulting in an agarose "microslab". A drop of 4 µl of a concentrated cell suspension was added and covered with a clean cover slip 22x22.

18.2. Fixing and Staining

For fixing cells 50µl sample from growth culture was mixed with 200µl of 1.25% formaldehide. Samples were store at room temperature.

For the study of live cells sample were directly used from the culture, without any other treatment.

For study of nucleoide we used DAPI to final concentration 1µg ml^{-1} (see *Methods* section).

18.3. Image Acquisition

Images were acquired using an inverted Olympus microscope (IX70) with a Micromax 512 camera (Princeton Instruments) and two computers controlled shutters (Uniblitz). One shutter blocks the mercury lamp and the other the halogen lamp. The appropriate shutter opens only for the duration of the exposure time. This approach reduced the photobleaching and the photodamage to cells and allowing to alternate between phase-contrast and fluorescence frames in a time-lapse experiment. The temperature of the sample was maintained at 37° by heating the objective. The focus was adjusted manually throughout the experiment.

19. β-galactosidase Assay

β-galactosidase assays were used to measure the activities of a plasmid encoded promoter:*lac*Z fusions in several genetic backgrounds (Miller 1972). 5ml of M9 minimal medium was supplemented with appropriate antibiotics and inoculated with a freshly transformed colon, and incubated overnight at 37°C. The following day, 25ml of M9 minimal medium was inoculated with the overnight culture at a 1:200 dilution for measurements of β-galactosidase activity during aerobic growth. Once cells had reached the required 0.2 OD_{550}, they were immediately placed on ice and the exact OD_{550} reading was recorded for exponential sample. An aliquot was filtered and resuspended in the same medium without thymine, to different times, were taken samples that were immediately placed on ice and the exact OD_{550} reading was recorded. To lyse cells, 2ml samples were added to 30µl toluene and 30µl 0.1% sodium deoxycholate and incubated for 30min at 37°C, with aeration. For measurements of β-galactosidase activity, 100µl of cell lysate was added to 2ml Z buffer, β-mercaptoethanol was added to Z buffer prior to use, at 0.27ml per 100 ml Z buffer. The assay was initiated by the addition of 0.5ml o-nitrophenyl-β-D-galactopyranoside (ONPG) solution, dissolved in Z buffer (3.92 g l^{-1}). Once the solution had changed from a clear color to a straw yellow color or 1h had elapsed, 1 ml of 1 M sodium carbonate was added to stop the reaction and the reaction time recorded. The absorbance at 420 nm of the resultant solution was measured and the calculation of β-galactosidase activity was calculated as follows:

β-galactosidase activity = $\frac{1000 \times 2.5 \times 3.6 \times OD_{420}}{OD_{550} \times 4.5 \times t \times v}$ nmol min^{-1} mg^{-1} bacterial mass

Where:
2.5 = factor for conversion of OD_{550} into bacterial mass, based on OD_{550} of 1.0 being equivalent to 0.4 mg ml^{-1} bacteria (dry weight).
3.6 = final assay volume (ml).
1000/4 = factor for conversion of OD_{420} into nmol o-nitrophenyl (ONP), based on 1nmol ml^{-1} ONP having an OD_{420} of 0.0045
t = incubation time (min)
v = volume of lysate added (in ml)
Each experiment was done in triplicate and the mean and standard deviation was calculated according.

Results

Chapter 1

TLD correlates with replication forks

Abstract:

TLD has been related to DNA replication. We investigated whether the number of replication forks could be related to TLD. To establish a relationship between the magnitude of the lethality under thymine starvation and the number of replication forks, both parameters were determined in the strain MG1693 grown under different conditions to achieve different numbers of replication rounds. The results show an inverse correlation between the survival of thymine-starved cells and the number of replication forks per chromosome. Cell viability was analyzed under thymine starvation in the presence of hydroxyurea (Hu). Surprisingly, ongoing DNA replication is not required.

1. Obtaining different numbers of replication forks per chromosome in MG1693 strain

TLD has been studied for decades being associated with different molecular and cellular events although the mechanism of death still remains elusive. Given that thymine starvation halts DNA synthesis, TLD has been related to active DNA replication (Maaløe and Hanawalt 1961). We investigated whether the number of replication forks could be related to TLD. To establish a relationship between the magnitude of the lethality under thymine starvation and the number of replication forks, both parameters were determined in the strain MG1693 grown under different conditions to achieve different numbers of replication rounds per chromosome, *n*, and consequently different number of replication forks, *N* (Table 13).

In contrast with eukaryotic organisms, in *E. coli* the time required to replicate the single chromosome can be longer than the generation time. This results in overlap replication cycles in the same chromosome. The larger the ratio between the generation time (τ) and the duration of the C period, the greater the number of the replication rounds per chromosome and the number of replication forks (Cooper and Helmstetter 1968). The number of replication rounds per chromosome is quantified as the ratio between the C period and the generation time and is defined as, $n=C/\tau$ from here, the number of replication forks per chromosome $N= (2^n-1).2$ can be calculated (Sueoka and Yoshikawa 1965, *Cyclon* http://genuex.unex.es/alf/c/ciclon.html)

Cultures of the strain MG1693 were grown in different media to enforce different numbers of replication cycles. As $n=C/\tau$, different *n* values can be obtained by modifying C or τ values. It is well documented that in thymine requiring strains, the replication time (C period) increases by reducing the thymine concentration without affecting τ (Zarithsky and Pritchard 1971). Furthermore C period can be altered by using different TdR concentrations in the presence of uridine 150 mM (O´Donovan 1970). To get *n* to vary in accordance with τ, the cultures were grown in glycerol instead of glucose as the carbon source.

To determine the number of replication rounds per chromosome we used the experimental approach followed by R. H. Pritchard and A. Zaritsky (Pritchard and Zaritsky 1970; Zarithsky and Pritchard 1971). Briefly, *n* has been described as a function of ΔG, which is defined as the amount of DNA synthesis that occurs following inhibition of new initiations events while ongoing forks are allowed to finish (Pritchard and Zaritsky 1970). This condition can be achieved by the addition of rifampicin (Rif), as this drug inhibits RNA polymerase and

transcription is known to be required for the initiation of replication. From the experimental value of ΔG, *n* can be calculated as follows: $\Delta G = [2^n \, n \, \ln 2/(2^n - 1)] - 1$, where *n* is the only variable value (Pritchard and Zaritsky 1970, Sueoka and Yoshikawa 1965). Thus, the amount of DNA synthesized after replication initiation is inhibited solely depends on the number of replication forks per chromosome. After Rif addition, the greater the number of replication forks running along the chromosome, the greater the accumulation of DNA.

Cultures of MG1693 grew on different media in the presence of ^{3}H-Thy up to 0.2 OD_{550} An aliquot of each was treated with Rif 150μg ml^{-1} to inhibit initiation of replication. Samples were then collected, and the relative increase in DNA content determined from radioactivity of TCA-precipitated material was determined (ΔG). From the values of ΔG, *n* was calculated and from it, the number of replication forks per chromosome *N* was determined (Table 13).

In order to determine whether those origins initiated after Rif addition were able to complete the replication of the whole chromosome, we performed flow cytometric analysis of the cells growing in the different media described above.

Figure 12 shows the profiles for MG1693 growing in different M9 minimal media. The discrete peaks corresponding to two and for or four and eight chromosomes after the runout indicate that initiation of chromosome replication is performed once in the cell cycle proceeding up to reaching the end of the chromosome.

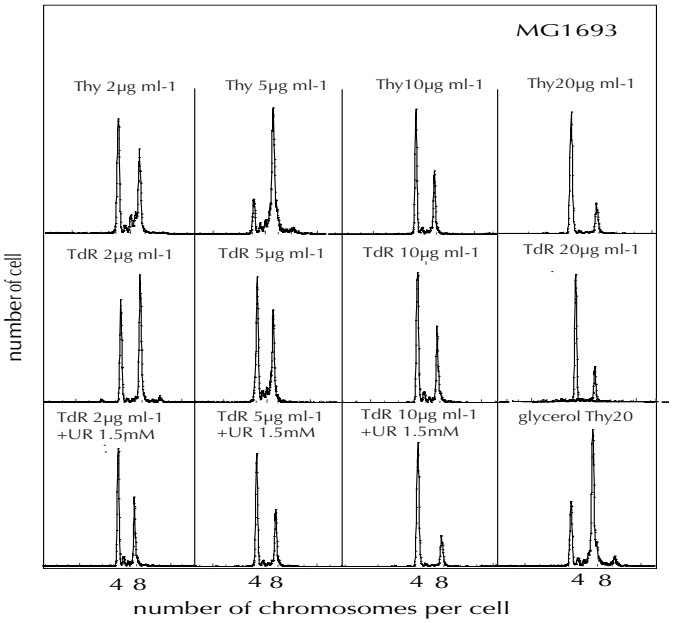

Figure 12. Flow cytometry profiles of MG1693 under different concentrations of Thy or TdR in presence or absence of UR. Samples after addition of Rif and Cfx for 3 h in M9 minimal medium.

Table 13. Cell cycle parameters and viability after 3 h of thymine starvation in MG1693 strain. The strain MG1693 growing in M9 minimal medium supplemented with different Thy and TdR concentrations in the presence or absence of UR 1.5 mM. [1]t generation time (min);[3]C period (min) [2]*n* number of replication cycles per chromosome; [4]N number of replication forks per chromosome N= (2-1).2 [5]relative number of viable cells ml^{-1} (CFU ml^{-1}) after 3 h of thymine starvation. The values of C period, *n* and CFU ml^{-1} are expressed as the mean ± standard deviation. Our guess is that the strain MG1693 could be defective in *deo* operon (O´Donovan 1970). This would explain that TdR10 at 10 µg ml^{-1} in the presence of UR is high enough to inhibit its own synthesis and therefore having a longer C period. The value of *n* and N were obtained by using the computer software *Replicon* (Jiménez-Sánchez and Guzmán 1998) implemented in our lab, and *Ciclon* http://genuex.unex.es/alf/c/ciclon.html. SD is obtained from 2-4 independent experiments.

Growth conditions	τ	n	C	N	3h TLD
TdR 5µg ml	40	3.1±0.26	123±13	15.1	8.5x10±2.0x10
TdR 10µg ml	40	2.1±0.31	84±10	6.57	1.5x10±0.7x10
TdR 2µg ml+UR 1.5mM	44	2.8±0.16	124±11	12.0	7.4x10±0.1x10
TdR 5µg ml+UR 1.5mM	38	1.84±0.12	73±4	5.16	6.4x10±1.1x10
TdR 10µg ml+UR 1.5mM	39	2.85±0.36	111±16	12.4	6.8x10±1.0x10
Thy 2µg ml	50	2.77±0.02	141±4	11.6	1.6x10±0.2x10
Thy 5µg ml	38	2.39±0.13	95.7±5.4	8.48	5.6x10±1.3x10
Thy 10µg ml	39	2±0.04	76.9±2.8	6	7.4x10±0.1x10
Thy 20µg ml	39	1.75±0.11	68±5.5	4.72	6.4x10±1.1x10
TdR 5µg ml-1+UR 1.5mM M9 glycerol	98	0.8±0.08	83±8	1.60	1.6x10±0.2x10

1.2. Correlation between TLD and the number of replication forks

To establish the relationship between the magnitude of the lethality under thymine starvation and the replication forks, both parameters were determined in the strain MG1693 growing under different conditions described above to achieve different numbers of replication rounds per chromosome. Once the growth conditions were established above, the viability under thymine starvation under these conditions was determined.

Cultures of the strain MG1693 were grown up to 0.2 OD_{550} in the media listed in Table 13. An aliquot of each culture was treated with Rif 150µg ml^{-1} to obtain ΔG values and from here, *n* and the number of replication forks per chromosome *N* (Table 13). A second aliquot of the cultures was starved for thymine for 3 h, and CFU were estimated by plating on NAT a serial dilution of the culture. The values were expressed as relative to the value obtained in time 0 of the treatment (Table 13).

We found that either the number of replication rounds per chromosome or the number of replication fork inversely correlates with the survival under thymine starvation (Fig. 13), suggesting that the replication forks or multiforked DNA are the target of TLD.

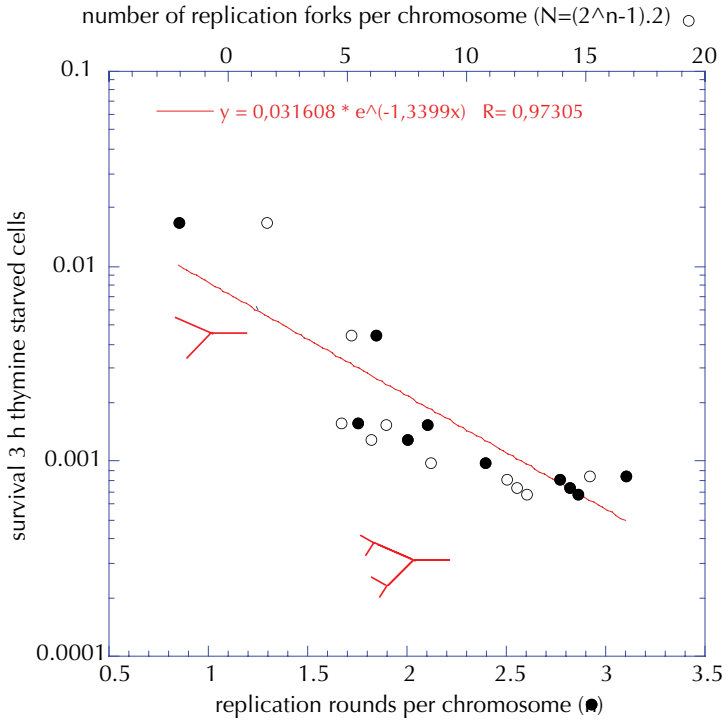

Figure 13. Relationship between *n* or *N* and the relative viability under thymine starvation. Relationship between the number of replication rounds per chromosome, *n*; or the number of replication forks per chromosome, *N* and the relative viability after 3 h thymine starvation in different growth conditions. The red draw corresponds to chromosome configuration with one or two cycles of chromosome replication.

1.3. Ongoing DNA replication is not required for TLD

TLD has been related to DNA replication. Surprisingly, TLD occurs under thymine starvation in the presence of hydroxyurea (Hu), Morganroth and Hanawalt 2006) which stops further DNA synthesis, as it is an inhibitor of the ribonucleotide reductase (RNR). Moreover the effect of Hu on DNA replication is not clear enough. Kuong and Kuzminov showed that Hu addition does not stop DNA replication at once (Kuong and Kuzminoz 2012); hence the role of active replication forks on TLD should be revised.

The inhibition of DNA replication by Hu 75 mM was verified by ^{3}H-Thy incorporation on DNA under our working conditions. Culture of MG1693 was grown in M9 minimal medium up to 0.2 OD_{550}. By this time fresh made Hu 75 mM was added, samples were undertaken and radioactivity of TCA-precipitated material was determined (Fig. 14)

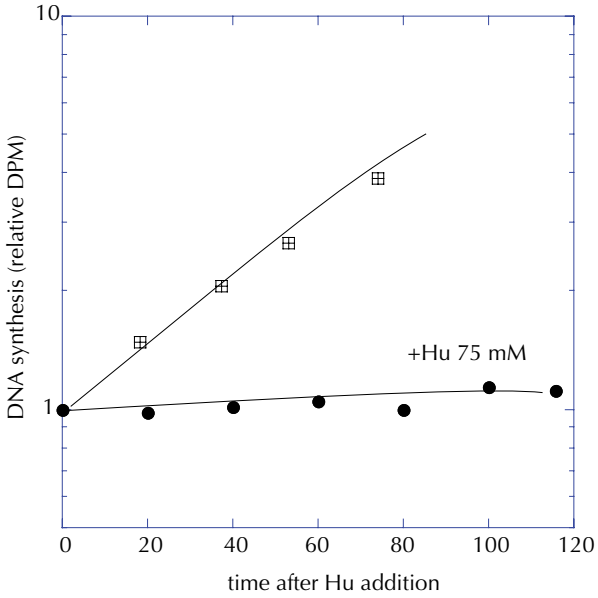

Figure 14. DNA synthesis in the presence of Hu 75 mM. Incorporation of ^{3}H-Thy was determined in culture growing without treatment (⊞) and after addition of Hu 75mM (●).

To test the role of active replication forks on TLD we examined the cell viability under thymine starvation in the presence of Hu and after addition of Hu 10 min before the thymine starvation in order to get stalled replication forks by the time thymine was removed from the growth medium.

A culture of MG1693 was grown in M9 minimal medium at 37°C up to 0.2 OD_{550}. Several aliquots were taken and treated as follow: Hu addition, thymine starved in presence or absence of Hu; Hu addition during 10 min, then thymine starvation in presence or absence of Hu. Samples were taken at different times, diluted and plated in duplicate NAT plate that were incubated at 37°C for 24 h. The number of colony on plate was referred as CFU ml^{-1} and calculated for each time. Results are given as cell number relative to the beginning of the treatment (Fig. 15).

These results show that, even though thymine starvation was undertaken under conditions where ongoing DNA replication was stalled, TLD was observed in the cultures.

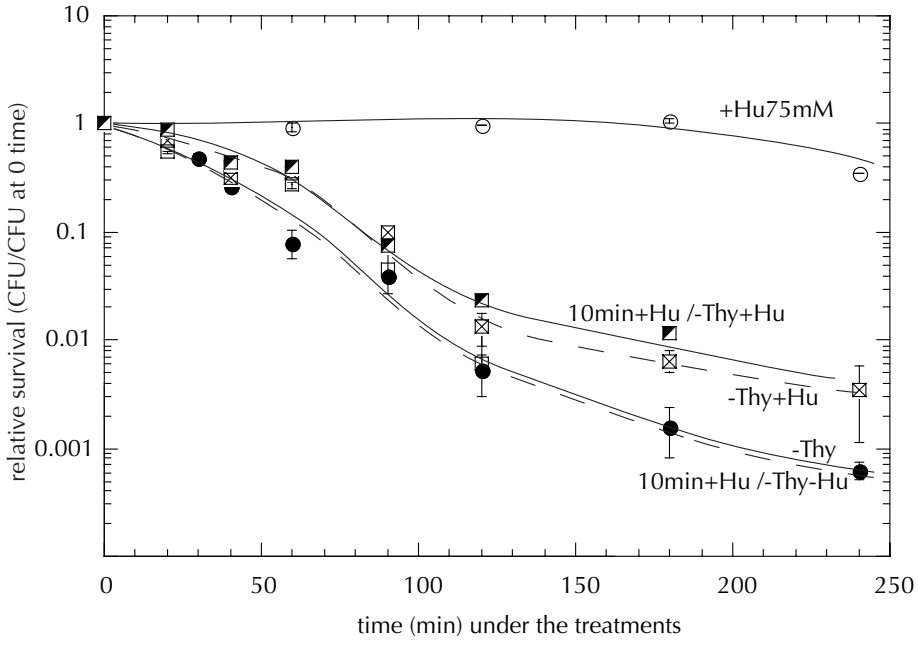

Figure 15. Viability after Hu addition and/or thymine starvation. Hu addition (○), thymine starved in the absence (●) or presence of Hu (⊞); pre-treated 10 min with Hu addition then thymine starved in the absence (□) or presence of Hu (◪

Chapter 2

Thymine starvation generates DSBs and nmDNA

Abstract:

DNA damage is associated with TLD, although the primary source of this damage is not well established. Stalled replication forks are source of DSBs and that could be cause of lethality under thymine starvation. In this chapter DSBs were quantified by the amount of linear DNA determined using PFGE combined with cell lysis in agarose plugs. DSBs levels and its relation with the lethality were analyzed under several conditions: thymine starvation, Rif, Hu or combinations of them. The results indicate that DSBs occurred under thymine starvation, but they are not sufficient to cause TLD. Some proposals postulated the formation of branched DNA as source of the toxic effect of thymine starvation. The formation of nmDNA and the correlation with TLD was studied by PFGE. The results presented here indicate that the nmDNA is generated under thymine starvation, but these structures are not necessary for lethality.

2.1. DSBs induced under thymine starvation do not correlate to TLD

Even though ongoing DNA replication is not required for TLD, the replication forks must be stalled under thymine starvation in the presence or absence of Hu. In addition, residual DNA repair or recombination events most probably occurred at replication forks (Kuong and Kuzminov 2012). Under this conditions DNA replication forks are known to be a source of double strand breaks (DSBs) (Horiuchi *et al.* 1994, Horiuchi and Fujimura 1995). DNA breakage has been observed under thymine starvation, but whether DSBs occur has been controversial (Nakayama and Hanawalt 1975). Using neutral density gradients, Yoshinaga (Yoshinaga 1973) was able to detect DSBs in thymine-starved *E. coli* cells, and using neutral filter elution, DSBs were found in thymidilate synthase-negative mutants of mouse FM3A cells (Ayusawa *et al.* 1983). In contrast, Nakayama *et al.* (Nakayama *et al.* 1994) using pulse-field gel electrophoresis (PFGE) were unable to detect linear DNA from thymine-starved cells, but the technical and genetic system used in that work differed from our experimental conditions. To determine whether DNA damage correlates with TLD we quantified the amount linear DNA and the viability of the thymine-starved cells under different conditions. It has been described that TLD was prevented by the addition of Cm at 200 μg ml^{-1} or Rif at 150μg ml^{-1}, meanwhile TLD was not affected by the addition of Hu at 75mM (Morganroth and Hanawalt 2006, Fig. 15).

To determine the amount of DSBs produced it is necessary to prevent the degradation of linear DNA and the repair of DSBs. In this work, that was achieved by using a *recB* deficient MG1693 strain in which DSBs generated by the treatments are not repaired and can therefore be evaluated by using pulse field gel electrophoresis (PFGE) combined with cell lysis in agarose plugs (Michel *et al.* 1997).

A culture of MG1693 *recB* was growth in M9 minimal medium in presence of ^{3}H-Thy up to 0.2 OD_{550}. Then, the culture was filtered and the cells were resuspended in medium without thymine neither ^{3}H-Thy during 2 h in absence or in the presence of Rif or Hu. Finally, the cells were recollected, lysed in agarose plugs, and their DNA was analyzed by PFGE, in which only linear chromosome enter the gels and circular molecules remain in the wells (Michel *et al.* 1997, Seigneur *et al.* 1998). All the PFGE linear DNA data were analyzed as described in *Methods* section (Fig. 16, Table 14).

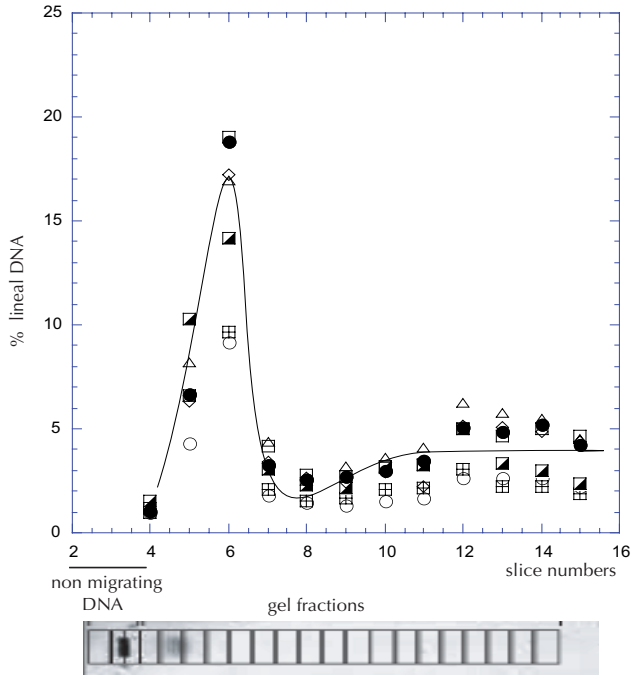

Figure 16. Representative profile of PFGE for determination of lineal DNA amount in MG1693 *recB*. Exponentially growing (⊞), after 2 h of different treatments: thymine starvation (●), thymine starvation in presence of Rif (△) or Hu (□), Rif addition (◪) and Hu addition (○). Data were analyzed by the least-squares statistical approach, with measurements considered highly significantly different if P was <0.01.

Table 14. Percentage of linear DNA in MG1693 *recB* cells after 2 h of thymine starvation in the presence or absence of Rif and Hu. [1]The values are expressed as means ± standard deviation. [2]The percentage of linear DNA relative to the exponential culture.

Treatment	%linear DNA[1]	Treatment effect[2]	Lethality
Exponential	36.13±9	1	no
-Thy	60.95±11	1.68	yes
+Hu 75mM	42.85±7	1.18	no
-Thy+Hu 75mM	57.76±8	1.59	yes
+Rif 150 μg ml^{-1}	49.85±9	1.38	no
-Thy+Rif 150 μg ml^{-1}	66.26±10	1.83	no

The viability under these conditions was verified in our genetic background MG1693 *recB* (Fig. 17).

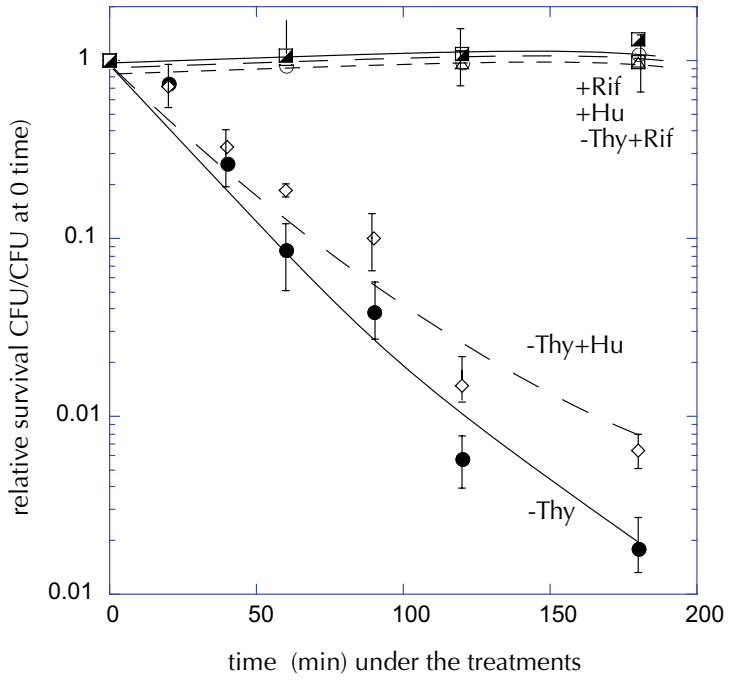

Figure 17. Viability of MG1693 *recB*. Hu addition (◯), Rif addition (◪), under thymine starvation (●) in the presence of Hu (◇) or Rif (△).

The results shown that the amount of DSBs increased under thymine starvation in the *recB* mutant, but the level do not correlated to the lethality caused by either treatment, even though they were not repaired. This indicates that the mere DNA damage wouldn't be sufficient to cause TLD, suggesting that some features of the DNA damage could be different when generated under each treatment. DSBs may be necessary but not sufficient to cause TLD. No all DSBs could have the same fate.

2.2. There is no DNA degradation under thymine starvation

The DSBs can be start point for either degradation of DNA by action of exonuclease or generation of recombination intermediates. This could explain the different impact of the DSBs caused by the different treatments.

To verify whether DNA is degraded under in thymine starvation, a culture of MG1693 exponentially growing in M9 minimal medium in the presence ^{3}H-Thy 1µCi ml^{-1} was filtered and cells were resuspended in the same medium but without Thy neither ^{3}H-Thy in the absence or presence of Rif or Hu. Samples were withdrawn at different times and the amount of ^{3}H-Thy in the DNA was determined.

Figure 18 shows that the level of radioactivity is maintained, indicating that there is no degradation under thymine starvation in the absence or presence of Rif or Hu. These results suggest that extensive degradation of DNA from the DSBs should not be cause of TLD.

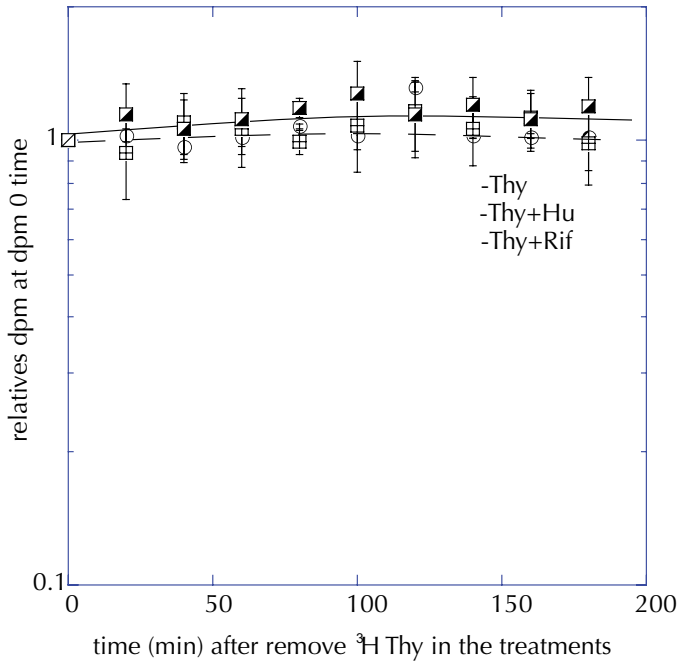

Figure 18. Degradation DNA in MG1693. Exponentially growing culture in the presence of ^{3}H-Thy was starved for thymine ((◯) in the presence of Rif (◪) or Hu (⊞).

2.3. nmDNA generated under thymine starvation is not necessary for TLD

Nakayama and co-workers demonstrated the presence of complex DNA structures in thymine-starved cells by digesting DNA of treated cultures with the restriction enzyme *XbaI* and separating it by PFGE (Nakayama *et al.* 1994). They called these structures "non-migrating DNA" (nmDNA), defined as the DNA that is unable to enter the gel and gets stuck in the well. The nmDNA was characterized as having single-stranded tails or gaps and branching with single-stranded arms. The PFGE revealed in the DNA of thymine-starved *E. coli* cells a special fraction (nmDNA) that corresponded largely of aggregates and rich in peculiar structures characterized by extended single-stranded regions or branched DNA.

To study whether the formation of nmDNA correlates with TLD under the above described replication conditions, we analyzed the DNA of cultures after thymine starvation in the presence or absence of Rif, Cm and Hu by PFGE. The amount of nmDNA was also determined after 2 h of thymine starvation of a culture of MG1693 using glycerol as the carbon source, where the number of replication cycles is lower than in glucose (Table 15).

Mid-exponentially growing culture of MG1693 was starved for thymine in the presence or absence of Rif, Cm, or Hu. Two hours after the treatment, cells were collected, washed, embedded in agarose plugs, gently lysed and plugs treated with *XbaI* (50U 100 μl^{-1}) for three hours before being used for PFGE (Matushek *et al.* 1996; Gautom 1997). The visualization of DNA bands was achieved by ethidium bromide staining (Fig. 19A). The amount of nmDNA was quantified by densitometry of the gel by using the *Image J* program. The nmDNA values were expressed as the percent of the arbitrary densitometric units corresponding to the well (see *Methods* section) (Fig. 19B, Table 15).

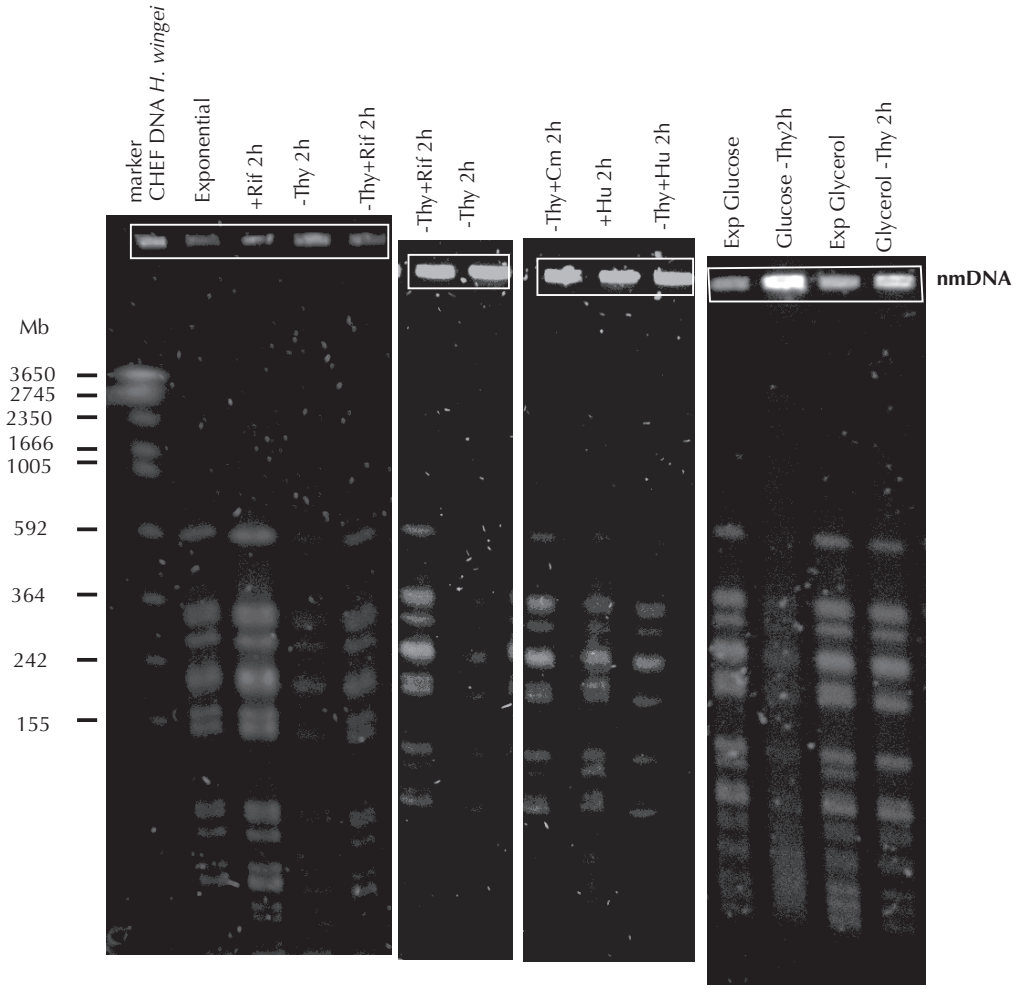

Figure 19A. Visualization of nmDNA by PFGE after treatment with *XbaI*. Visualization of the agarose gel stained by EthBr.

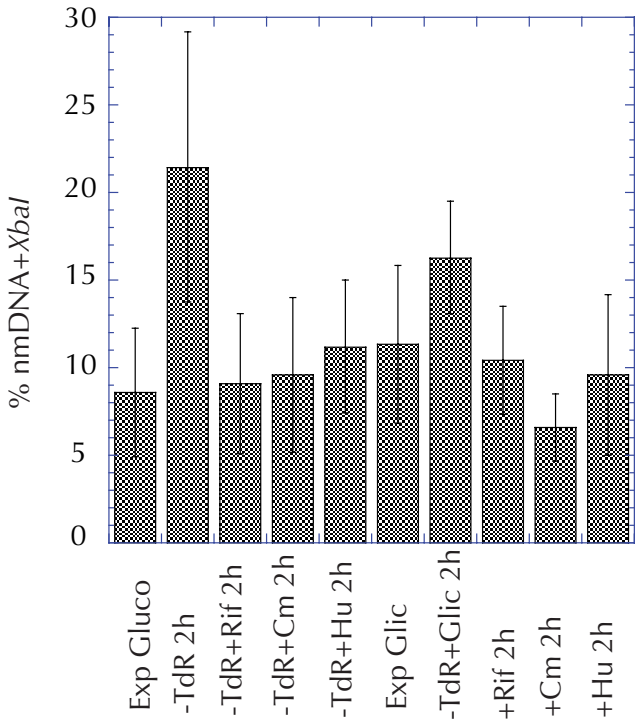

Figure 19. Quantification (%) of nmDNA obtained by PFGE after treatment with *XbaI* (Fig. 19A) by using *Image J* program

Table 15. Percentage of nmDNA in MG1693 cells after 2 h of thymine starvation in the presence or absence of Hu, Rif or Cm. [1]The percentage of nmDNA is expressed as the mean ± standard deviation. [2]The percentage of nmDNA relative to the exponential

Treatment	%nmDNA[1]	Treatment effect[2]	Lethality
Exponential	8.6±3.6	1	no
-Thy	21.4±4.7	2.48	yes
-Thy+Hu 75mM	11.2±3.8	1.30	yes
-Thy+Rif 150 µg ml^{-1}	9±3.9	1.04	no
-Thy+Cm 200 µg ml^{-1}	9.5±4.4	1.10	no
+Hu 75mM	9.8±3.3	1.14	no
+Rif 150 µg ml^{-1}	10.2±2.8	1.19	no
+Cm 200 µg ml^{-1}	6.5±2.3	0.75	no
Exponential M9 glycerol	11.3±4.5	1.31	no
-Thy M9 glycerol	16.2±3.2	1.88	yes

By using this experimental approach we show that (i) the amount of nmDNA increased under thymine starvation being higher when in glucose-grown cells than in glycerol-grown cells; thus correlating with the number of replication forks, (ii) nmDNA was not generated under thymine starvation in the presence of Rif or Cm, confirming Nakayama´s results and indicating that nmDNA could be associated to TLD. Nevertheless, (iii) we found that under thymine starvation in the presence of Hu there was no increase in nmDNA in the well. This result indicates that the generation of entangled DNA is not a requirement for TLD, even though it was generated under thymine starvation and it is absent under the conditions where TLD is suppressed.

Chapter 3

Replication initiation as a key element in TLD

Abstract:

In this chapter DNA labeling experiments and flow cytometric analyses showed that new initiations occur under thymine starvation, relating TLD to chromosomal DNA initiation. In support of this finding genetic approach showed that (i) cells carrying *dnaA46ts* mutation displayed TLD if incubated at 30ºC but suppressed TLD if incubated at 42ºC, condition that inhibits new initiation events. (ii) partial deletion of the *oriC* sequence suppressed TLD (iii) Chromosome initiation capacity (ChIC) accumulates under thymine starvation. Time-lapse experiments showed that increasing levels of ChIC during thymine starvation correlated with the accumulation of simple-Y, double-Y and bubble arc replication intermediates at the *oriC* region as visualized by two-dimensional DNA agarose gel electrophoresis (2D). None of these structures were observed following Rif addition or genetic backgrounds suppressing TLD.

3.1 New initiations occur under thymine starvation

Because TLD was related with the number of the replication forks, but cannot be explained solely by the induction of DSBs and/or nmDNA (chapter 2 this work), we speculated that the putative initiations occurred under thymine starvation (Bogan and Helmestetter 1996) could generate the target causing TLD. This relationship would be supported by the recent results showing a loss of DNA from the *oriC* region after 2-3 hours under thymine starvation (Fonville *et al*. 2010, Sangurdekar *et al*. 2010, Kuong and Kuzminov 2012). These ideas raise the possibility that new initiations events under thymine starvation could play an important role on TLD and that suppression of the lethality after addition of Rif or Cm could be consequence of the inhibition of the new initiations events under thymine starvation.

This conjectures required the verification of two points. First, to demonstrate that new initiation events occur under thymine starvation. Second, to study whether the new initiations during thymine starvation are inhibited in the presence of Rif and Cm (drugs that inhibit new initiations) or Hu (drug that inhibit elongation of replication forks).

To approach these points, we determined Δ*G* value after the addition of Rif in the presence of thymine and Δ*G'* value after 10 min of thymine starvation (see *Methods* section). Briefly, ΔG is the relative increase in the amount of DNA after the inhibition of new rounds of chromosomal replication, condition achieved by the addition of 150 μg ml^{-1} Rif to the exponentially growing culture (as explained in chapter 1). ΔG' represents the relative increase in the amount of DNA of the same culture treated during 10 min for thymine starvation (in the presence or absence of Rif, Cm or Hu), and then restoring the thymine in the presence of 150 μg ml^{-1-} Rif to inhibit new initiation allowing the initiated rounds to finished chromosome replication; $\Delta G' = [2^n(i+1)n\ln 2/(2^n-1)]-1$. If new initiation occurred in thymine-starved cells, then ΔG' is expected to be higher than ΔG.

Culture of MG1693 exponentially grown up to 0.2 OD_{550} in M9 minimal medium in the presence of ^{3}H-Thy 1 μCi ml^{-1} was starved for thymine in the presence or absence of Rif, Cm or Hu. Ten min after these treatments, Thy and ^{3}H-Thy 1 μCi ml^{-1} were restored to the aliquots in the presence of Rif (in absence of Hu if it was added in the pretreatment) to allow DNA replication of the rounds already initiated, but to inhibit the initiation of new ones. Samples were then collected, and the relative

increase in DNA content determined from the radioactivity of TCA-precipitated material, this will be Δ*G'*. Additionally the value of Δ*G* was obtained after the addition of Rif 150 μg ml^{-1} to exponentially untreated culture.

The results in Figure 20 show that Δ*G'* under thymine starvation was higher than Δ*G* indicating that new initiations are prepared during the treatment. The proportion of initiated origins, *i*, was estimated to be 19.4% of the origins present before the thymine starvation. The proportion of origins initiated under the different treatments, *i*, was estimated following the experimental approach described by Jiménez-Sánchez and Guzmán (Jiménez-Sánchez and Guzmán 1988). Briefly, *i* is calculated from the two experimental values: ΔG and ΔG'. The *i* value for a given treated culture can then be calculated from *ΔG'*/ ΔG = $[2^n(i+1)]/2^n$= i+1 (http://genuex.unex.es/alf/c/ciclon.html, see *Methods* section).

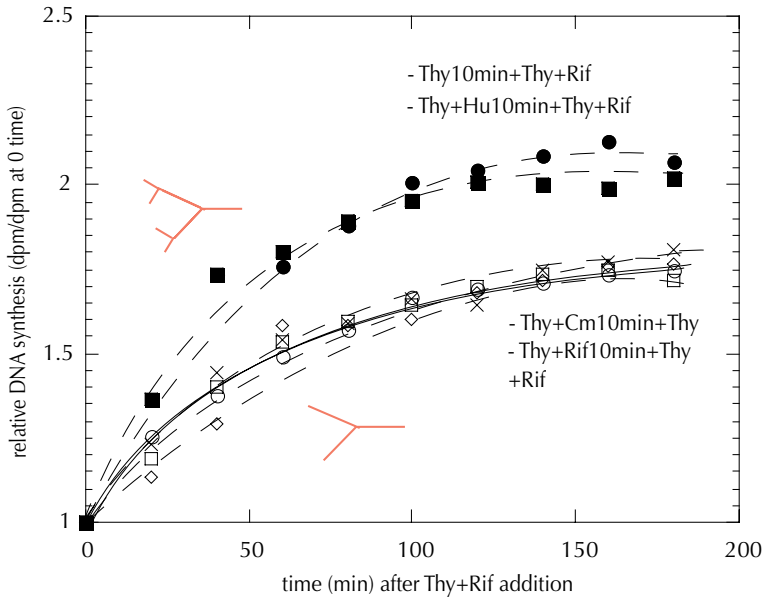

Figure 20. Runout DNA synthesis in MG1693 under thymine starvation. Exponentially growing MG1693 cultures after the addition of Rif (◗), and run-out DNA synthesis after the addition of Thy and Rif (time 0) in cultures preincubated under thymine starvation for 10 min (●), preincubated under thymine starvation in the presence of Rif for 10 min (×), Cm (□) or Hu (■). A schematic of the replication initiation events is present.

Regarding the effect of the antibiotics we found that, addition of Hu under thymine starvation allowed initiations to occur, being the number of initiated origins after 10 min of thymine starvation in the presence of Hu the same that observed under thymine starvation. By contrast, as ΔG and ΔG' were similar after 10 min of thymine starvation in the presence of Rif or Cm, this indicate that no new initiations occurred when any of these drugs were present (Fig. 20). These results indicate that the presence of Rif or Cm inhibits the new replication rounds under thymine starvation; by contrast Hu allowed new initiations events to occur in the absence of thymine.

In order to verify whether these new initiations were able to complete replication of the whole chromosome, we performed flow cytometry analysis of the cells 3 h after Rif addition and restoring thymine in all the treatments. Figure 21 reveals the DNA histogram showing discrete peaks corresponding to four and eight chromosomes after the runout that followed the 10 min of thymine starvation in the absence or presence of Rif, Cm or Hu. These results indicate that chromosomal DNA initiations occurred under the thymine starvation either in the presence or absence of the drugs, were able to complete the replication of the whole chromosome. Nevertheless it is interesting to note the relative increase of cells with 8 chromosomes if chromosomal initiation were not inhibited in thymine-starved cells, suggesting that the initiation and full replication of a number of chromosomes containing four origins.

From these labeling DNA experiments and flow cytometry analysis the relationship between TLD and chromosomal DNA initiation occurred under thymine starvation can be established. **We propose** that the *thymineless* DNA initiation would generate a fraction of DNA damage and/or nmDNA at the origins, being critical for TLD.

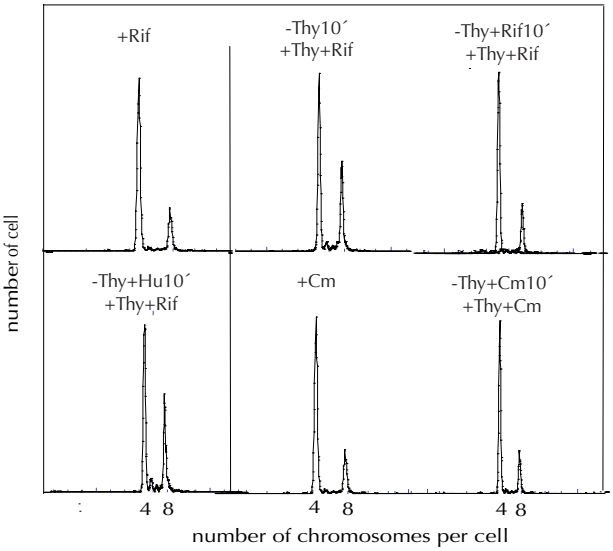

Figure 21. Flow cytometry profiles after 3 h of Rif and Cfx addition after the different pretreatments.

3.2. DnaA inactivation suppress TLD

Initiation at *oriC* requires a functional DnaA protein (von Meyenburg *et al*. 1979; Schaus *et al*. 1981, Fuller *et al*. 1981) Temperature-sensitive mutations in the *dnaA* gene allow cells to initiate at 30°C (permissive temperature) but not at 42°C (restrictive temperature) resulting in the inhibition of chromosome replication initiation while replication elongation is permitted (Hirote *et al*. 1968, Wechsler and Gross 1971). In this work we have used the *dnaA46* allele, which encodes a thermosensitive DnaA protein to achieve the inhibition of chromosomal initiation without inhibiting RNA polymerase by Rif, nor protein synthesis by Cm.

MG1693 *dnaA46* derivative was grown in M9 minimal medium at 30°C up to 0.2 OD_{550} , a portion of the culture was incubated at 42ºC. Another aliquot was filtered, and resuspended in medium without thymine either at 30°C or 42°C. Comparative study with MG1693 wild type was also performed under thymine starvation at 30ºC and 42ºC in the presence or absence of Rif 150 µg ml^{-1} to inhibit initiation events. Samples were taken at different time and plated at 30ºC in duplicate NAT after serials dilutions. Results are presented as the number of colonies relative to CFU by the time the treatments were initiated. Consistent with previous observations (Bouvier and Sicard 1975), we found that TLD occurred in thymine-starved *dnaA46* mutant cells grown at 30ºC but suppressed at 42ºC, while TLD observed in wild type MG1693 under thymine starvation at 42ºC is even greater than at 30ºC (Fig. 22A).

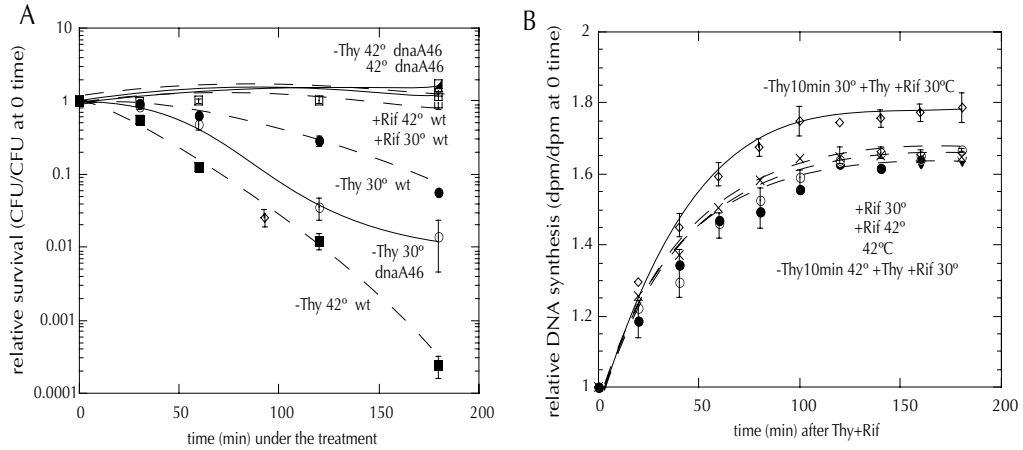

Figure 22. Relative cell survival and run-out DNA synthesis of the *dnaA46* mutant strain. (A) Relative cell survival of the *dnaA46* mutant strain at 42ºC (◇), after thymine starvation at 30ºC (ς) and at 42ºC (□), and wild type MG1693 after thymine starvation at 30ºC (●) in the presence of Rif (⊞) and at 42ºC (■) in the presence of Rif (◪). (B) Run-out DNA synthesis in exponentially growing *dnaA46* cultures after the addition of Rif at 30ºC(ς), and run-out DNA synthesis at 42ºC (●) and after the addition of Thy and Rif (time 0) and growing at 30ºC to cultures pretreated under thymine starvation for 10 min at 30ºC (◇) or 42ºC (✕).

The proportion of origin initiated under the different treatments, *i*, was estimated under 10 min of thymine starvation either at 30ºC or 42ºC. Exponentially growing culture of MG1693 *dnaA46* in M9 minimal medium with ^{3}H-Thy 1 µCi ml^{-1} was divided in different aliquots and treated as follow: Rif addition at 30°C, incubation at 42°C; thymine starved during 10 min at 30ºC or 42°C followed by thymine restoration at 30ªC in the presence of Rif to inhibit any new initiation after the treatment (Fig. 22B). These results show that, ΔG' after 10 min of thymine starvation at 42ºC was similar to ΔG, being higher if thymine starvation was performed at 30ºC. This indicate that no new initiations occurred after 10 min of thymine starvation at 42ºC. According to our proposal; suppression of TLD by inactivation of DnaA would be due to the inhibition of chromosome initiation similar to addition of Rif or Cm under thymine starvation in wild type strain.

3.3. Partial deletions of *oriC* sequence suppress TLD

Previous studies showed that *oriC*-defective mutants containing deletions in the *oriC* sequence showed decreased initiation efficiency, resulting in more detrimental effects in richer media (Stepanwick *et al.* 2009). As a different approach to verify the direct relationship between TLD and chromosomal initiation events, we determined the effect of *oriC*-defective alleles on lethality during thymine starvation.

We used the *oriC227*, *oriC228*, *oriC229*, and *oriC230* alleles that have deletions in the right half of *oriC* and the *oriC233* allele that have deletions in the left part of *oriC* (Fig. 23, Stepanwick *et al.* 2009). TLD after 3 h of thymine starvation and the proportion of initiated origins ,*i* , in 10 min thymine-starved cells were estimated for wild type and mutant strains grown in M9 minimal medium supplemented with 0.2% and 1% of casaminoacids (Fig. 24, Table 16). We found that *oriC* mutants were up to 10 times more viable than wild type after 3 h of thymine starvation in M9 minimal medium supplemented with 0.2% casaminoacids, being up to 80 times higher if M9 minimal medium was supplemented with 1% casaminoacids (Fig. 24A). The proportion of initiated origins was not greatly affected compared to wild type in MM9 medium supplemented with 0.2% casaminoacids but was increased if M9 minimal medium was supplemented with 1% casaminoacids (Fig. 24B).

Flow cytometry profiles in figure 24C revealed that the longer deletions located in the right part of *oriC* compromised the synchrony of the replication, showing peaks corresponding to cells containing number of chromosomes different to 2^n (Skarstad *et al.* 1986), nevertheless the completion of the replication was achieved in these mutants after the Rif addition to yield ΔG and ΔG' values and from here *i* values were estimated.

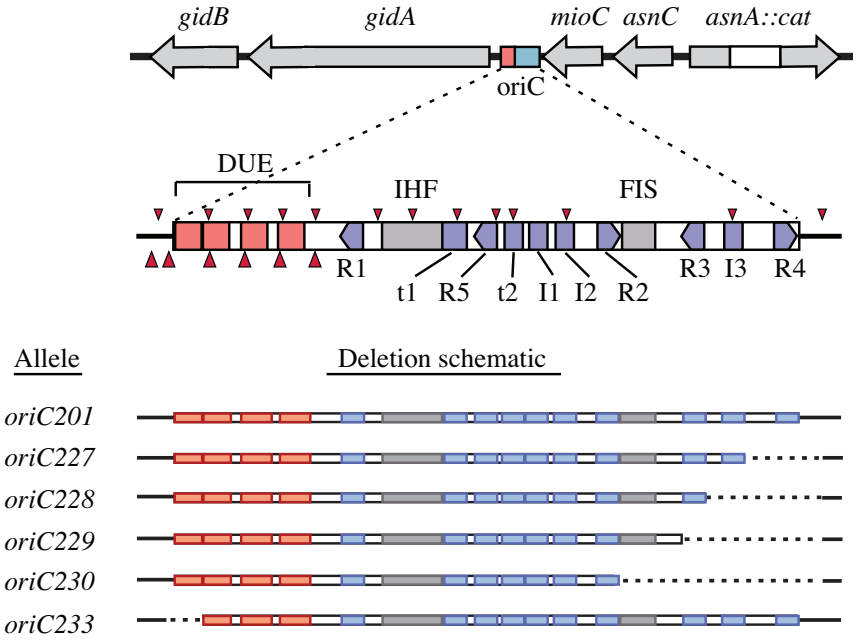

Figure 23. Scheme of the major transcription units around *oriC* and the *oriC* deletion mutations used in this work. The following regulatory elements are positioned: the 9 mer DnaA binding sites R1-R5, I1-I3 and t1-t2; the DNA unwinding elements and 13 mer repeats L, M and R (DUE); binding sites for IHF and Fis proteins. This figure has been adapted from Stepankiw *et al.* 2009

These results show that the general increasing of the survival observed in the *oriC* defective mutants under thymine starvation correlates to the decreasing in the proportion of initiated origin in the thymine starved cells, supporting that the initiation of chromosomal replication is an essential condition for TLD.

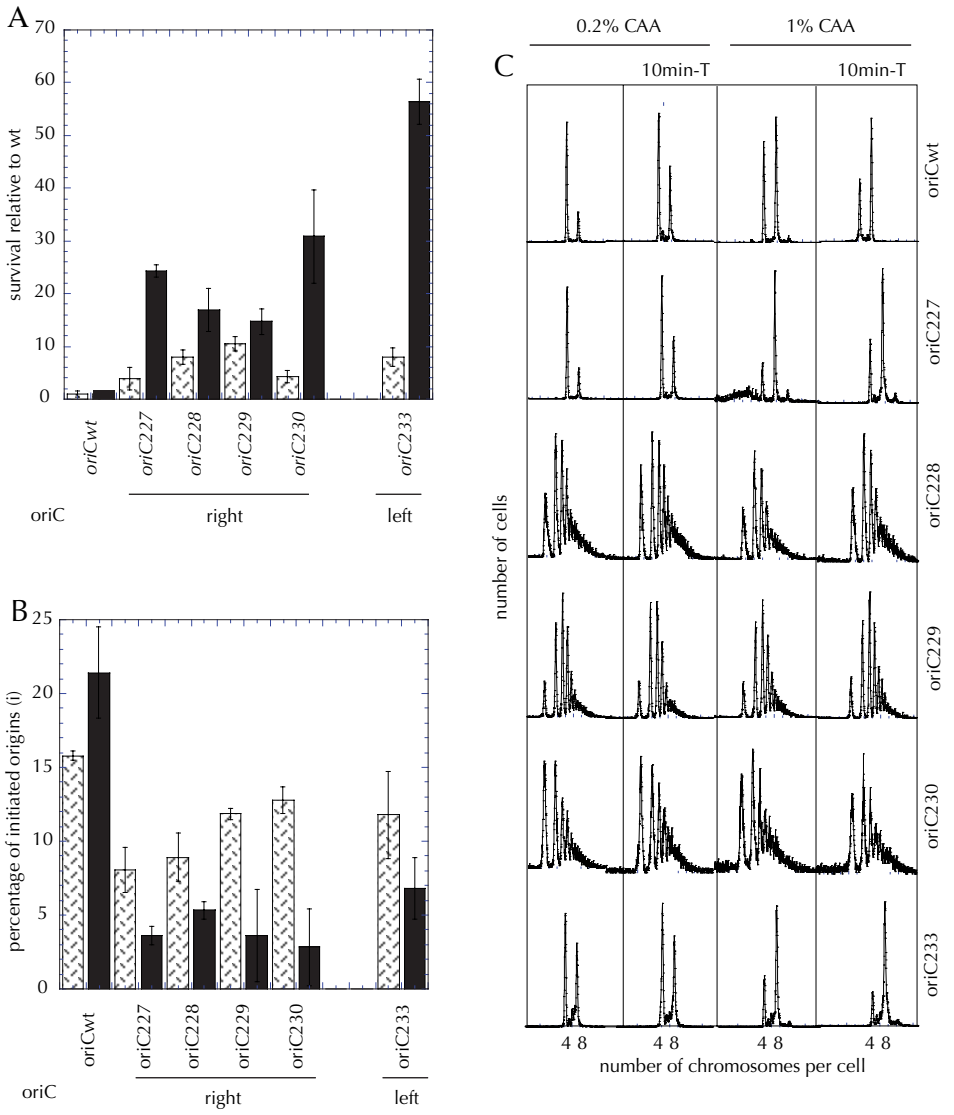

Figure 24. Effects of altering the *oriC* sequence. Being *oriC201* the wild type allele for *oriC* sequence. (A) Percent survival relative to wild type strain after 3h of thymine starvation in different mutants grown in M9 minimal medium supplement with 0.2% (▨) and 1% (■) CAA. (B). Percentage of origins initiated in 10 min in thymine starvation-starved cells of different mutant cultures grown in M9 minimal medium supplemented with 0.2% (▨)) and 1% (■) CAA for 10 min. Error bars represent the SD from 2-4 independent experiments. Values are relatives to the wild type MG1693 *oriC201.*(C) Flow cytometry profiles of the cultures growing in M9 minimal medium supplemented with 0.2% CAA and with 1% CAA, treated with Rif and Cfx for 3h (left panels) and thymine-starved for 10 min prior addition of Rif and Cfx for 3h (right panels).

3.4. Thymine starvation causes the accumulation of abortive initiations

Above results have shown that TLD correlates with new initiation events that occur during thymine starvation. To study the quantitative relationship between TLD and the initiation events, a time-lapse experiment was performed as follows. A ^{3}H-Thy-labeled MG1693 culture was grown up to 0.2 OD_{550} exponential phase, then thymine-starved for 0, 30, 60 or 120 min; followed by thymine and Rif 150 μg ml^{-1} addition (Fig. 25). Figure 25A shows that the runout values increased with the length of thymine starvation, indicating there is a Chromosomal Initiation Capacity (ChIC) that increased by the time of thymine starvation. We quantify ChIC by determining *i*; the proportion of origins that are reinitiated after the addition of thymine to thymine-starved cells, i.e. the origins initiated under thymine starvation that, eventually, will commence chromosome replication upon thymine addition (Table 16). Flow cytometry analysis detected discrete peaks following the runout of the cells thymine-starved for 10-30 min but not for those starved for 60 or 120 min (Fig. 25B). This suggests that thymine starvation for more than 30-60 min impedes the completion of chromosome replication most probably due to the collapse of newly-initiated rounds of replication from *thymineless*-origins (Sandgundekar *et al*. 2010, Kuongand Kuzminov 2012).

To verify the association of TLD with the initiation events accumulated under thymine starvation two approaches have been made. 10 min thymine starvation was carried out either in the presence of 150 μg ml^{-1} of Rif for different times in wild type strain MG1693 or incubating MG1693 *dnaA46ts* mutant at restrictive temperature (42ºC). The data in figure 25C and 25E show similar runout values following the time-lapse experiment, indicating the absence of initiation under thymine starvation in the presence of Rif or inactivated DnaA protein (Table 16). Moreover, flow cytometry revealed discrete peaks after replication runout under these conditions, indicating that chromosome replication was completed even following 2 h of thymine starvation in the presence of Rif (Fig. 25D and 25F). It has to be noticed that the flow cytometry profile of the MG1693 *dnaA46* strain showed two, three and four chromosomes per cell, indicating an asynchronous replication phenotype as previously (Skarstad *et al*. 1988). Nevertheless, the peaks were discrete indicating that most of the rounds were finished.

Overall these results of time-lapse experiments indicate that inhibition of initiation under thymine starvation would protect against TLD in two ways: by preventing the generation of *thymineless* origins, as new initiation events are inhibited by Rif or DnaA inactivation, and consequently avoiding the collapse of the ongoing replication forks when thymine is added.

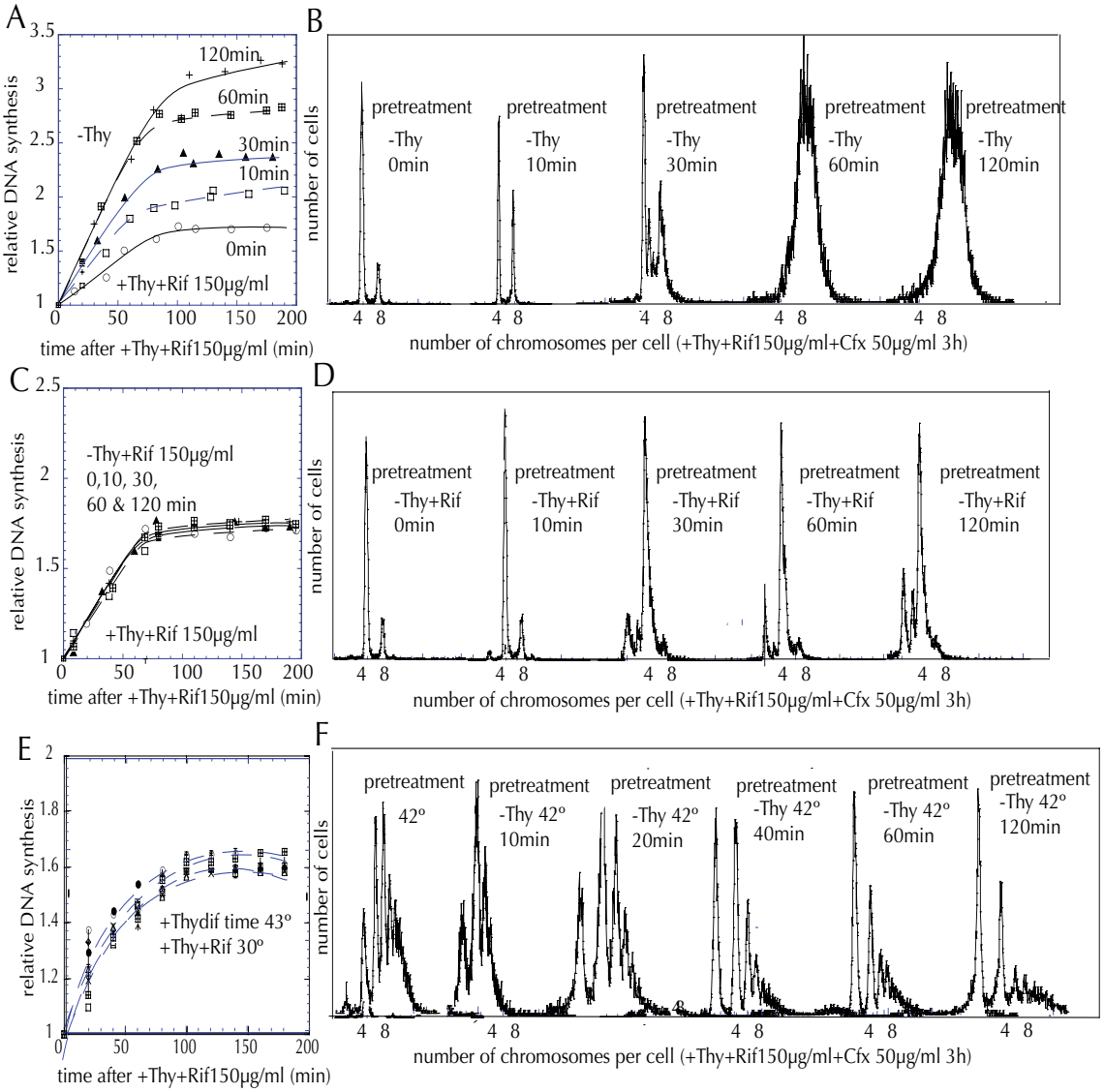

Figure 25. ChIC under thymine starvation. (A) and (C) Run-out of DNA synthesis after the addition of thymine and 150µg ml^{1} Rif to cultures that were thymine-starved for (ς)0, (□)10, (▲)30, (⊞)60 and (+)120 min in the absence (A) or the presence (C) of 150µg ml^{-1} Rif. (B) and (D) Flow cytometry profiles after adding thymine, 150µg ml^{1} Rif and Cfx to cultures starved of thymine for (ς)0, (□)10, (▲)30, (⊞)60 and (+)120 min in the absence (B) or the presence (D) of 150µg ml^{-1} Rif. (E) Run-out of DNA synthesis in *dnaA46* mutant after the addition of thymine and 150µg ml^{1} Rif at 30ºC to cultures that were thymine-starved for (⊞)0min at 30ºC, 10 min at 30ºC (●) and 42ºC (ς) and (+)20, (◇)40 , (x)60 and (□) 120 min at 42ºC. (E) Flow cytometry profiles in *dnaA46* mutant after adding thymine 150µg ml^{1} Rif and Cfx to cultures that were thymine-starved for 10 min at 30ºC (●) and 42ºC (ς) and (+)20, (◇)40 , (x)60 and (□)120 min at 42ºC

3.5. *oriC* replication intermediates accumulate during thymine starvation

The results presented above establish a direct relationship between TLD and chromosomal initiation. Hence, it is reasonable to think that abortive initiation at the *oriC* sequence could be one effect of thymine starvation. If this is true, then replication intermediates should accumulate at *oriC* during thymine starvation being prevented in the presence of Rif.

We examined the progression of replication forks at *oriC* by two-dimensional DNA agarose gel electrophoresis (2D gel) (Brewer and Fangman 1987) with different treatments and genetic backgrounds (Fig. 26).

An exponential phase MG1693 culture was starved for thymine, and samples were removed at 10, 20, 30, 40, 50, 60 and 120 min and analyzed by 2D gel (Fig. 26). Chromosomal DNA was digested with *Pvu*II enzyme and hybridized to the labeled 419 bp *Xho*I-*Sma*I *oriC*-fragment used as a probe (Fig. 26A), see *Methods* section). Because of the speed of replication forks in *E. coli*, replication intermediates are not detectable on the chromosomes of exponentially growing cells (Boubakri *et al*. 2010). Figure 26B shows several features generated during thymine starvation: a simple-Y arc, corresponding to the accumulation of Y-shaped replication intermediates, was clearly detected after 10 min of thymine starvation, indicating the arrest of replication forks within the *oriC* region. This simple-Y arc became more intense as the period of thymine starvation increased. A bubble arc was detected after 40 min of thymine starvation, indicating initiations at *oriC,* and double-Y structures were also detected, most probably produced by two forks that encounter each other at *oriC*. Schematic representations of these DNA structures identified in 2D gel are presented in Fig. 26D

These results verify two important ideas: first, the *oriC* sequence is replicated under thymine starvation, and second, the replication intermediates are accumulated progressively. Furthermore, we showed that if thymine was added after 60 min of thymine deprivation, all the structures disappeared, indicating the progression of the replication forks leaving the *oriC* region (Fig. 26C).

This 2D gel analysis was performed on samples that were thymine-starved for 60 min in *oriC228* mutant that greatly alleviate TLD by decreasing replication initiations when grown in M9 minimal medium supplemented with 1% casaminoacids (shown in Fig. 24). In agreement with our genetic results, no bubble arcs and very faint simple-Y arcs were observed after thymine starvation in these initiation-deficient mutants strains compared with MG1693 or an *oriC201* allele-containing strain grown in rich medium.

The *dnaA46* mutant strain was also analyzed by 2D gel. Supporting the critical role of chromosomal initiation on TLD, figure 27 shows a bubble and a simple-Y arc

under thymine starvation at the permissive temperature in the *dnaA46* mutant strain, indicating that replication forks accumulated at the *oriC* region at permissive temperature; but none were observed in thymine-starved cells at 42ºC. None of these structures were found in thymine-starved wild type cells in the presence of Rif for 60 min when TLD was suppressed; this observation further supports the relationship between TLD and the replication of *oriC* during thymine starvation.

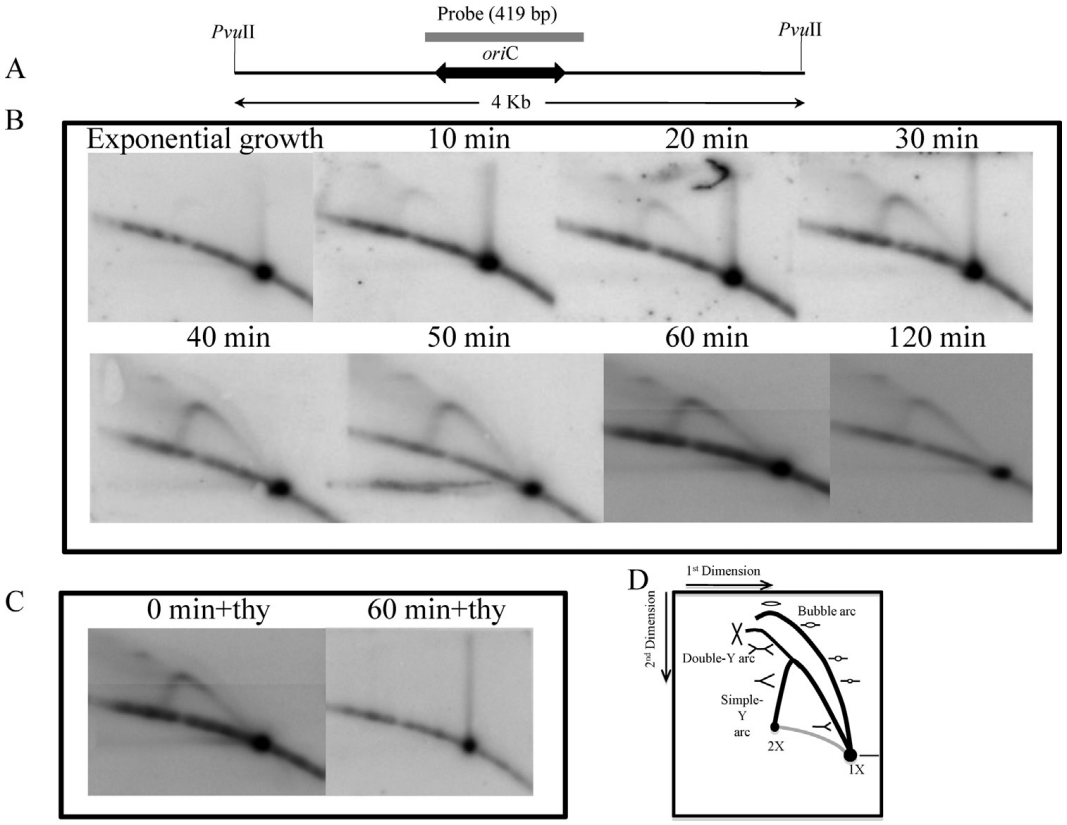

Figure 26. Analysis of chromosome replication at *oriC* during thymine starvation by 2D DNA gel electrophoresis. 2D gels were used to analyze DNA replication on a restriction fragment containing the *E. coli* replication origin *oriC*. DNA from a wild type MG1693 strain was digested with PvuII, analyzed by 2D gel electrophoresis and probed with a sequence containing the *oriC* region. (A) PvuII fragmented oriC probe used for Southern blot analysis. (B) From left to right, 2D gel DNA analysis from exponentially growing cells following 0, 10, 20, 30, 40, 50, 60 and 120 min of thymine starvation. (C) 2D gel DNA analysis from cells starved for 60 min (left) and following by thymine addition (right). (D) Schematic representation of the DNA structures identified in 2D gels.

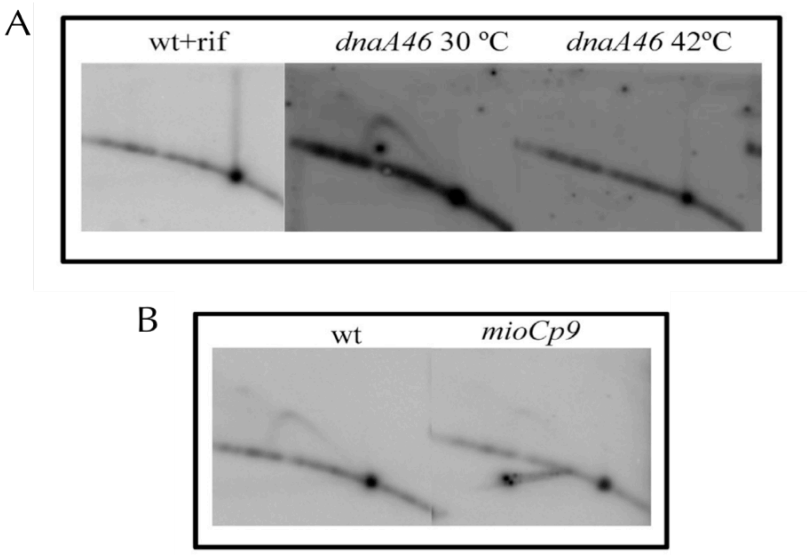

Figure 27. Analysis by 2D gel DNA electrophoresis under different conditions of inhibition of *oriC* replication. (A) From left to right, 2D gel DNA analysis of the wild type MG1693 strain in the presence of Rif at 37ºC and the *dnaA46* mutant strain after 60 min of thymine starvation at 30º C or 42ºC. (B) From left to right, 2D gel DNA analysis after 60 min of thymine starvation of wild type MG1693 and the *mioCp9* constitutive transcriptional mutant strain grown in M9 minimal medium supplemented with 1%CAA.

Table 16. Cell cycle parameters and viability after 3 h of thymine starvation in (i) MG1693 growing in 0.2% and 1% of casaminoacids, and in different mutants with alterations in *oriC* sequences, (ii) MG1693 *dnaA46ts* growing at 30ºC and 42ºC. The cells growing in M9 minimal medium supplemented with Thy 20µg $ml^{-1}$and ^{3}H-Thy 1µCi ml^{-1}. $^{1}\tau$ generation time (min); $^{2}\Delta G$ DNA accumulated in the mid-log phase culture after Rif at 150 µg ml^{-1} for 3h (run-out); ^{3}n number of replication cycles per chromosome; ^{4}C period $^{5}\Delta G'$ is the DNA accumulated after the addition of Rif at 150 µg ml^{-1} for 3h to cultures starved 10 min for thymine; ^{6}The value of *i* was obtained from the empirical formula $\Delta G'/\Delta G$ = [2 (i + 1)]/2 = i+1 (http//:genuex.unex.es/alf/c/ciclon.htm); ^{7}Cell survival after 3 h of thymine starvation relative to cero time of the treatment. SD is obtained from 2-4 independent experiments.

Strains	τ[1]	ΔG[2]	n[3]	C[4]	ΔG'[5]	i[6]	Survival 3h –Thy[7]
MG1693							
0.2% Casaa	39±1.25	72	1.75±0.11	68	108	19.46±4.55	0.0016±0.000
1% Casaa	37±1.79	100	2.38±0.09	85	157	25.30±0.91	0.0007±0.000
oriC201-wt							
0.2% Casaa	38±1.08	71	1.72±0.03	65	91	15.79±0.31	0.0036±0.019
1% Casaa	36±1.41	90	2.10±0.00	76	130	21.41±5.30	0.0074±0.019
oriC227							
0.2% Casaa	48±3.01	60	1.49±0.03	71	73	8.09±1.64	0.0140±0.008
1% Casaa	37±3.28	66	1.62±0.04	60	72	3.6±0.60	0.1800±0.009
oriC228							
0.2% Casaa	38±1.00	51	1.29±0.05	49	65	8.92±1.53	0.0280±0.005
1% Casaa	50±2.28	74	1.79±0.03	89	84	5.35±0.59	0.1260±0.022
oriC229							
0.2% Casaa	40±1.92	65	1.59±0.01	64	84	11.85±0.38	0.0380±0.005
1% Casaa	42±4.60	85	1.94±0.12	84	89	3.60±3.11	0.1090±0.018
oriC230							
0.2% Casaa	54±5.00	55	1.37±0.00	74	81	12.77±0.91	0.0160±0.004
1% Casaa	79±4.33	76	1.83±0.02	144	81	2.82±2.59	0.2280±0.066
oriC233							
0.2% Casaa	40±0.00	49	1.24±0.18	50	67	11.78±2.98	0.0290±0.006
1% Casaa	40±2.50	69	1.67±0.21	50	78	6.81±3.6	0.4170±0.032
dnaA46ts							
30ºC	72±5.66	65	1.60±0.01	114	77	7.25±2.60	0.0137±0.009
42ºC					71	3.33±1.31	0.9431±0.182

Chapter 4

Rifampicin suppresses TLD by blocking the transcription-dependent step of chromosome initiation

Abstract:

TLD can be prevented by the presence of Rif, an RNA polymerase inhibitor. In this work we have showed evidence link TLD to chromosome initiation events. This suggests that Rif-mediated TLD suppression could be due to the inhibition of RNA synthesis required for DNA chromosomal initiation at *oriC*. Here we study TLD in the presence of Rif in a *rpoB*(Rif^R) and *datA,* or different Rif concentrations in wild type strain. We show that partial inhibition of the general activity of the RNA polymerase has in thymine-starved cells specifically decreases RNA polymerase activity for the transcription-dependent step of initiation under thymine starvation cells modulating TLD and ChIC. Significantly, the introduction of *PmioC112* and *Pgid103*, mutations that alter transcription levels around *oriC*; reduces ChIC alleviating TLD. These results show that the inhibition or impairment of the transcription-dependent initiation step caused by Rif addition, is responsible for suppression or alleviation of TLD.

TLD suppression by Rif was observed early in the study of TLD (Hanawalt 1963) but its mechanism of action has not yet been elucidated. Initially, it was proposed that the DNA repair system might be unable to repair the large number of transcription-associated strand breaks generated by thymine starvation (Nakayama and Hanawalt 1975). However, in chapter 2 of this work it has been shown that Rif addition does not suppress DSBs formation associated with TLD. Hence, the mechanism underlying the suppression of TLD by the presence of Rif has remained elusive. The replication initiation events associated with TLD require transcription. Given that Rif inhibits RNA polymerase activity throughout the bacterial genome, the suppression of TLD exerted by Rif could be explained more likely by the inhibition of RNA synthesis required for chromosomal initiation at *oriC* nevertheless the inhibition of other as-yet-unidentified gene(s) or processes that could be required for TLD in some other way could not be discarded.

Here we study TLD in the presence of Rif in different genetic backgrounds such a *rpoB*(Rif^R) and *datA,* or different Rif concentrations in wild type strain. Furthermore TLD was analyzed in altered transcription pattern of *PmioC112* and *Pgid113* genes surrounding *oriC* and usually transcriptionally active.

4.1. Modulation of TLD in *rpoB* Rif^R mutants

If the abortive initiations under thymine starvation were the ultimate cause of the lethality under thymine starvation, then similar TLD would be expected in Rif^R strains in the absence or in the presence of the drug. TLD was determined by counting CFU in spontaneous *rpoB*(Rif^R) mutant in the presence of Rif. In Rif^R mutant Cm will be used as inhibitor of initiations during thymine starvation.

Exponential culture of the Rif^R mutant was grown until 0.2 OD_{550} in the presence of ^{3}H-Thy and Rif 150 μg ml^{-1} was added to a portion of the culture while a second aliquot was treated by Cm addition 200 μg ml^{-1}. Incorporation of ^{3}H-Thy to the DNA was determined to analyzed cell cycle parameters, such a generation time and number of replication rounds, *n*. Fig. 28A shows that growth features were not affected by the presence of Rif 150 μg ml^{-1} in the growth medium, as expected in Rif^R mutant strain.

To study the effect of Rif on TLD, exponential culture of the Rif^R mutant was filtered and resuspended in M9 minimal medium without thymine in the presence or absence of Rif 150 μg ml^{-1} or Cm (Fig. 28B). According to our previous results TLD was fully suppressed if new initiations were prevented, in

this Rif^R mutant by Cm addition. Unexpectedly, TLD was partially prevented in the presence of Rif. We founded similar results with different spontaneous *rpoB*(Rif^R) mutants suggesting that the partial suppression of the expected lethality of *rpoB*(Rif^R) mutants under thymine starvation in the presence of Rif could be a general effect related to *rpoB*(Rif^R) phenotype.

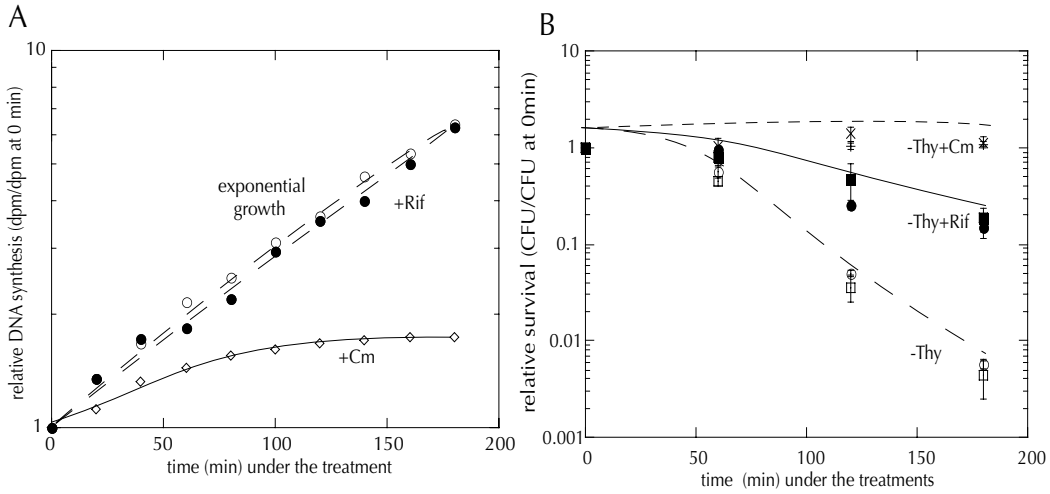

Figure 28. Effect of Rif or Cm addition on DNA synthesis and viability under thymine starvation in *rpoB(Rif^R)* mutant. (A) DNA synthesis in exponentially growing (○) *rpoB(Rif)* mutants, after the addition of Rif (●), or after the addition of Cm (◇). (B) Relative cell survival under thymine starvation of the (○ □) in the presence of Rif (● ■) or Cm (×+)

4.1.1. Initiations are partially inhibited by Rif in *rpoB*(Rif^R) under thymine starvation.

We have previously showed that TLD correlates with the occurrence of abortive initiation events under thymine starvation. Even though, in *rpoB*(Rif^R) mutant inhibition of new initiation will not be expected in thymine-starved cells in the presence of Rif, we quantified ChIC under thymine starvation in the presence or absence of Rif or Cm in this mutant. Exponential cultures of *rpoB*(Rif^R) mutant was grown until 0.2 OD_{550} and four aliquots were withdrawn and treated as follow: (i) Cm addition to inhibit initiation of chromosomal replication estimating ΔG (ii) Cm addition after 10 min under thymine starvation estimating ΔG′ (iii) Cm addition after 10 min under thymine starvation in the presence of Rif or (iv) in the presence of Cm. Following this 10 min of each

treatment, thymine was reposted to the aliquots (ii), (iii) and (iv) and runout of DNA synthesis in the presence of Cm was determined (Fig. 29). By using the value of ΔG′ after 10 min of thymine starvation in *rpoB*(Rif^R) mutant the proportion of initiated origins during 10 min thymine starvation was estimated to be 20%, similar to wild type strain; while the addition of Rif under thymine starvation decreases the initiation efficiency to 9% (Table 17). The addition of Rif in the *rpoB*(Rif^R) mutant was inhibiting a proportion of origins to be initiated under thymine starvation and hence associating the occurrence of initiation event with the alleviation of TLD.

These results show the presence of Rif 150 μg ml^{-1} in the Rif^R mutant impairs chromosome initiation under thymine starvation although was sufficient to maintain normal DNA replication in exponential growth (Fig. 28). According to our proposal, ChIC was absent if Cm was present during thymine starvation, as initiation events are inhibited.

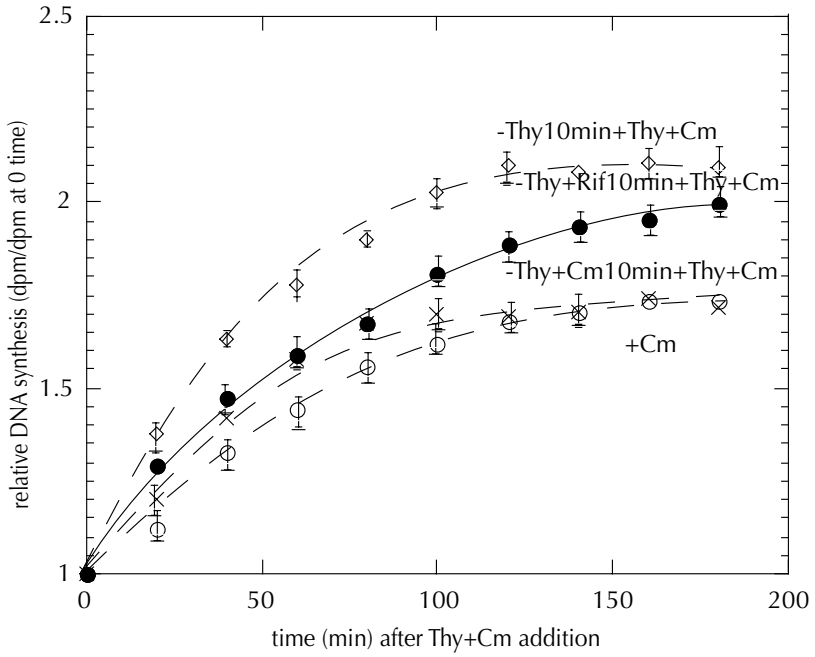

Figure 29. Runout DNA synthesis in *rpoB(Rif^R)* mutants. Exponentially growing MG1693 *rpoB(Rif^R)* cultures after the addition of Cm (○), and runout DNA synthesis after addition of Thy and Cm (zero time point) in cultures incubated under thymine starvation for 10 min (◇), incubated under thymine starvation in the presence of Rif for 10 min (●), and incubated under thymine starvation in the presence of Cm for 10 min (×).

4.1.2. *rpoB*(Rif^R) mutant is partially sensitive to Rif *in vivo*

The partial suppression of TLD in the presence of Rif observed in the Rif^R mutants could be due to partial sensitivity of RNA polymerase to Rif, and hence a partial impairment of the Rif-sensitive step for TLD.

To verify this possibility, RNA rate was determined in the *rpoB*(Rif^R) mutant and wild type MG1693 after Rif addition. As it is shown in figure 30 the addition of Rif 150 μg ml^{-1} decreased RNA rate in the Rif^R mutant, indicating that the RNA polymerase coded by *rpoB*(Rif^R) allele is partially sensitive to Rif *in vivo*.

Overall these results it can be established that *rpoB*(Rif^R) mutant displays partially suppressed TLD in the presence of Rif associated to a decreasing of ChIC. The RNA polymerase coded by the allele is partially sensitive to Rif, but none of the cell cycle parameters in exponential growth were affected. This would indicate that RNA polymerase activity is specifically decreased for the transcription-dependent step of initiation under thymine starvation.

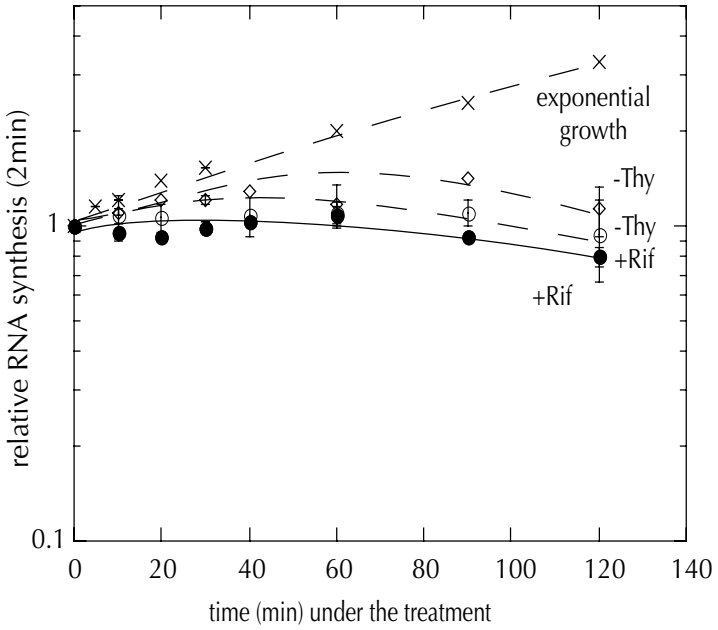

Figure 30. Relative RNA synthesis and DNA synthesis in *rpoB(Rif^R)* mutants. (A) Relative RNA synthesis as determined by ^{3}H-uridine incorporation in exponential growing MG1693 *rpoB(Rif^R)* cultures (×), after addition of Rif (●) under thymine starvation in presence of Rif (○) or absence (◇).

4.2. Survival of thymine-starved cells inversely correlates with the rate of RNA synthesis and initiation efficiency

TLD is fully suppressed by the addition of 150 μg ml^{-1} Rif (Hanawalt 1963), a concentration which also inhibits the initiation of replication. Our data support that the Rif-mediated TLD suppression step could be the inhibition of RNA synthesis required for DNA chromosomal initiation at *oriC*. According to that, we propose that RNA transcription level would affect TLD under condition where chromosomal replication was impaired for initiation under thymine starvation. To check this possibility we mimicked the partial inhibition of the RNA rate after Rif addition in the Rif^R mutant, by addition of sub-inhibitory Rif concentration in he wild type strain MG1693.

We investigated whether survival under thymine starvation was affected by Rif concentration. MG1693 culture was grown up to 0.2 OD_{550} . Several aliquots were removed and starved of thymine in the presence of 0, 5, 10, 25, 50, 100 or 150 μg ml^{-1} Rif. The viability of thymine-starved cells treated with different concentrations of Rif was estimated by determining CFU (Fig. 31A). RNA synthesis rates after Rif addition were analyzed by measuring ^{3}H-uridine incorporation during a 2 min incubation (Fig. 31B). We observed that cell survival under thymine starvation inversely correlated with the rate of RNA synthesis and showed a dose-dependent response to Rif.

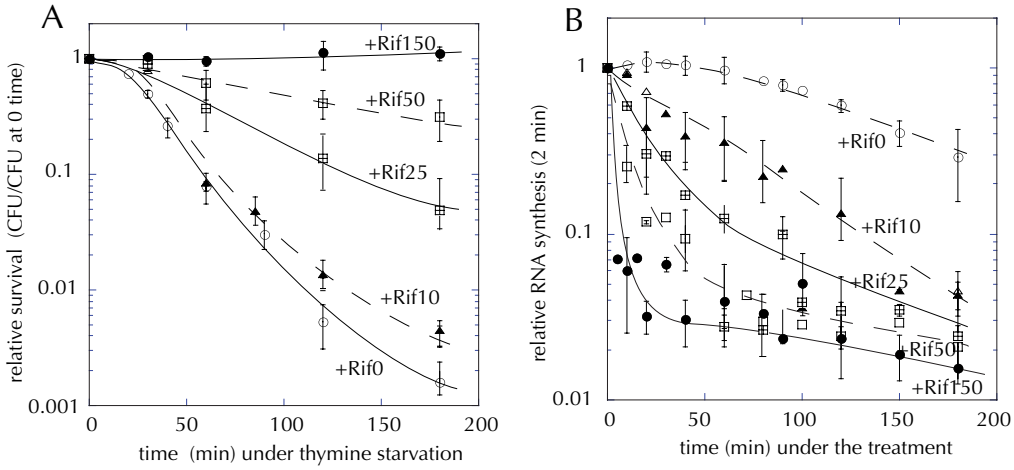

Figure 31. Effect addition of Rif at different concentrations during thymine starvation. (A) Relative cell survival after thymine starvation (TLD) in the presence of different Rif concentrations. (B) Relative RNA synthesis as determined by ^{3}H-UR incorporation. Symbols: (○) 0, (▲) 10, (⊞) 25, (□) 50 and (●)150 μg ml^{-1} Rif. Errors bars represent the SD from 2 to 4 independent experiment.

It has previously showed in this work that ChIC is reduced upon Rif-treatment in thymine-starvation conditions. Given the evidence linking TLD to initiation events, we hypothesized that there may be a causal relationship between reduced ChIC and Rif-mediated suppression of TLD. We quantify ChIC as the proportion of origins that are initiated after the addition of thymine to thymine-starved cells, denoted as *i* value. To verify the correlation between ChIC and TLD, we determined *i* values for thymine starvation in the presence of different Rif concentrations using the experimental approach detailed in the *Methods* section.

A ^{3}H-Thy-labeled MG1639 culture was grown up to 0.2 OD_{550}, aliquots removed and thymine-starved for 10 min in the presence of 0, 10, 25, 50, 100 and 150 μg ml^{-1} Rif, followed by the addition of thymine and 150 μg ml^{-1} Rif for 3 h where the initiated origins can achieve complete chromosomal replication, but new initiation events are not allowed. Samples were then collected, and the relative increase in DNA content determined from the radioactivity of TCA-precipitated material (ΔG'). The fraction of origins initiated under thymine starvation, *i*, was calculated from ΔG' and ΔG values. As shown in Figure 32A, DNA synthesis decreased after thymine and Rif addition with increasing Rif concentration in thymine-starved cells (ΔG'); from these values the fraction of origins initiated during the 10 min thymine starvation was calculated (Fig. 32B, Table 17). Significantly, sub-inhibitory Rif concentrations associated with decreased ChIC were also associated with the alleviation of TLD. This suggests that a partial inhibition of RNA polymerase might alleviate TLD by impairing a transcription-dependent step of chromosome initiation under thymine starvation.

To determine whether the origins that initiated during thymine starvation in the presence of Rif were able to complete chromosomal replication, we performed flow cytometry analysis on cells treated as described above. Figure 32C shows discrete peaks corresponding to four and eight chromosomes after runout following 10 min of thymine starvation in the presence or absence of Rif.

These profiles indicate that the rounds of chromosomal DNA replication initiated during the first 10 min of thymine starvation were able to finish following the addition of thymine in the presence of inhibitory levels of Rif, giving fully replicated chromosomes. It is interesting to note the relative decrease in the proportion of cells with eight chromosomes (Fig. 32C) corresponding to the decrease in the percentage of initiated origins (Fig. 32B).

Overall these results displays partially suppressed TLD in the presence of sub-inhibitory concentrations of Rif associated to a decreasing of ChIC. The decreases on the RNA polymerase by addition of different Rif concentrations specifically decreases ChIC under thymine starvation. This effect would be sufficient to explain the partial TLD suppression observed by the addition of subinhibitory Rif concentration, supporting the sensitive Rif step on initiation to be the best candidate for the mechanism of Rif mediated suppression of TLD.

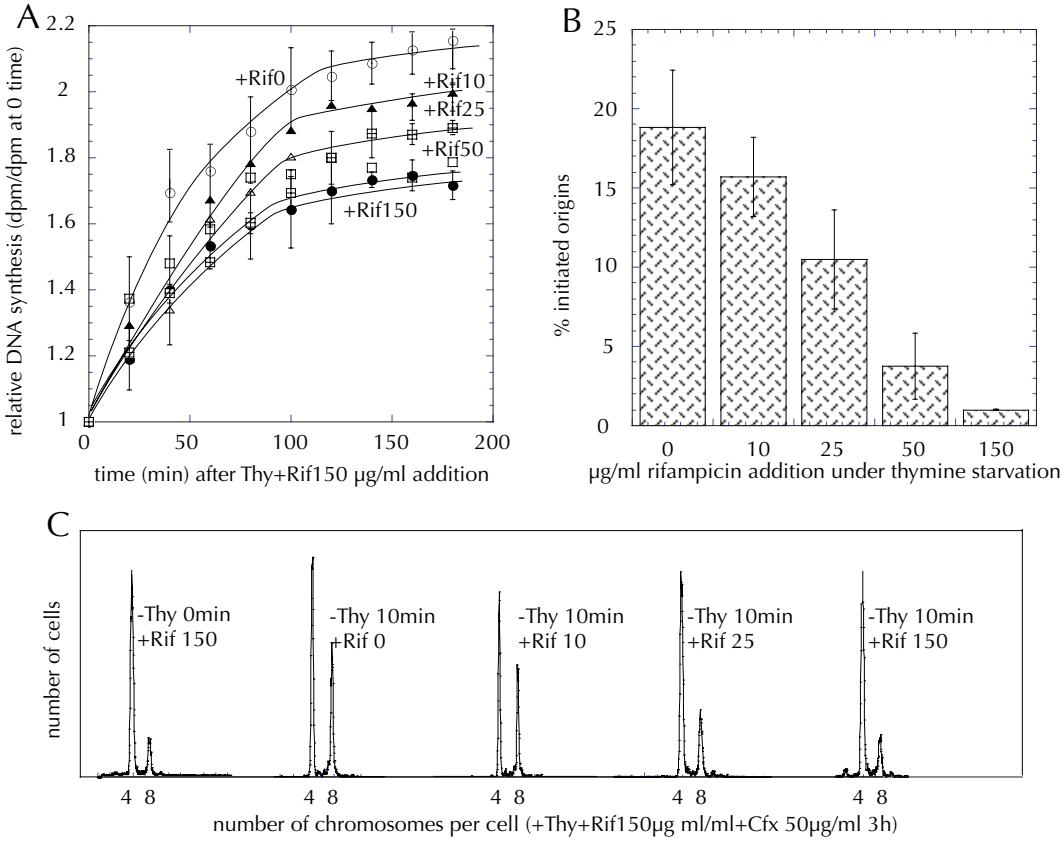

Figure 32. Addition of Rif at different concentrations in ChIC during thymine starvation. (A) Run-out of DNA synthesis after addition of thymine and 150µg ml^{-1} Rif to a culture that was thymine-starved for 10 min in the presence of different Rif concentrations. Symbols: (○) 0, (▲) 10, (⊞) 25, (□) 50 and (●)150 µg ml^{-1} Rif. (B) Quantification of ChIC, as a percentage of initiated origins after adding thymine to 10 min thymine starved cells in the presence of different Rif concentrations. Errors bars represent the SD from 2 to 4 independent experiment. (C) Flow cytometric profiles after adding thymine and 150µg ml^{-1} Rif to a culture that was thymine-starved for 10 min in the presence of different Rif concentrations.

4.3. *datA* mutant undertakes TLD in the presence of Rif

It has been described that mutant strains carrying deletion of *datA* region allowed a proportion of DNA chromosomal initiation in the presence of the standard Rif concentration (150 μg ml^{-1}). Nevertheless, in the presence of higher concentration of Rif (1,000 μg ml^{-1}) new initiation events can be inhibited (Morigen *et al.* 2005). This phenotype gives us the option to study whether TLD undertakes in the presence of Rif. The *datA* region is a 1 kb DNA sequence with five well conserved DnaA binding sites (DnaA-boxes) (Kitagawa et al., 1996) and several weak DnaA boxes (Hansen et al., 2007). Recently it was shown that the *datA* site, together with the IHF protein, stimulates the hydrolysis of the ATP bound to the DnaA protein and can therefore contribute in the inactivation of the DnaA protein in a process called DDAH *(datA*-dependent DnaA-ATP hydrolysis) (Kasho & Katayama, 2013).

We first analyzed the cell viability of Δ*datA* mutant under thymine starvation in the presence of Rif at 150μg ml^{-1}, 300μg ml^{-1} and 1,000μg ml^{-1} (Fig. 33A). These results show that Δ*datA* mutant loses viability under thymine starvation in the presence of Rif 150μg ml^{-1} and that lethality is gradually prevented in the presence of higher Rif concentrations. This is an interesting result, as it shows the first genetic condition in which TLD is observed in the presence of standard Rif concentration.

It could be possible that *datA* mutant displayed different sensitivity to the different Rif concentrations, hence explaining the partial TLD suppression observed in Fig. 33A. Checking this possibility RNA synthesis rates after 150μg ml^{-1} and 1,000μg ml^{-1} of Rif addition were analyzed by measuring ^{3}H-uridine incorporation during a 2 min incubation (Fig. 33B). Similar kinetic for diminishing the rate of RNA synthesis was observed for both Rif concentration, indicating that general inhibition of RNA synthesis could not explain the effect on TLD by the different Rif concentrations.

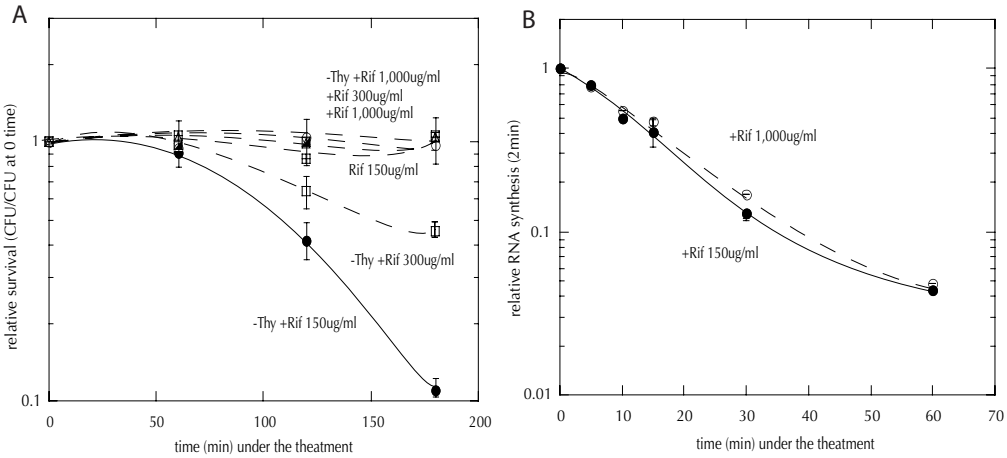

Figure 33. Effect of Rif in *datA* mutant under thymine starvation**.** (A) Relative cell survival after thymine starvation (TLD) in *datA* mutant in the presence of different Rif concentrations, (■)150, (▲) 300, and (ς) 1,000 μg ml^{-1} , and under thymine starvation in presence of different Rif concentrations (◆)150, (□) 300, and (◪) 1,000 μg ml^{-1} (B) Relative RNA synthesis as determined by ^{3}H-UR incorporation in *datA* mutant in presence of (◆) 150 and (ς) 1,000 μg ml^{-1} . Errors bars represent the SD from 2 to 4 independent experiment.

We checked for the potential of initiation inhibition for each Rif concentration 150μg ml^{-1} and 1,000μg ml^{-1} by determining runout DNA synthesis in exponential cultures after addition of the drug for 3 h. Fig. 34A shows that, even though the global RNA synthesis is inhibited displaying similar kinetic, the addition of 150μg ml^{-1} of Rif allowed higher amount of DNA accumulated than addition of 1,000μg ml^{-1} Rif; verifying the previous observation regarding the requirement of high Rif concentration to inhibit DNA initiation in *datA* mutant strain (Morigen *et al* 2005).

Regarding these results we tested the correlation between TLD and the inhibition of DNA initiation in Δ*datA* mutant by labeling experiments. This approach was performed as above but using Rif at 1,000 μg ml^{-1} instead 150 μg ml^{-1} after the pretreatments in order to get inhibition of chromosome initiation. A culture of exponentially growing MG1693 Δ*datA* strain was pretreated under thymine starvation in the presence or absence of Rif 150μg ml^{-1} or 1,000 μg ml^{-1}. 10 min after these treatments, thymine was restored together with Rif at 1,000μg ml^{-1}. Samples were then collected, and the relative increase in DNA content determined from the radioactivity of TCA-precipitated material. Figure 34B shows that in Δ*datA* strain new replication rounds (i. e., initiation events) are accumulated under thymine starvation in the presence of Rif at 150μg ml^{-1}. Addition of higher Rif concentration inhibited new initiation under thymine

starvation, as was observed in the wild type strain with the standard Rif concentration. These results show that if new initiations of replication are allowed in the presence of Rif, there is a loss of viability under thymine starvation, *i*. e.; TLD. This supports that inhibition of the required transcription for the initiation of chromosomal replication by RNA polymerase is an essential condition for TLD.

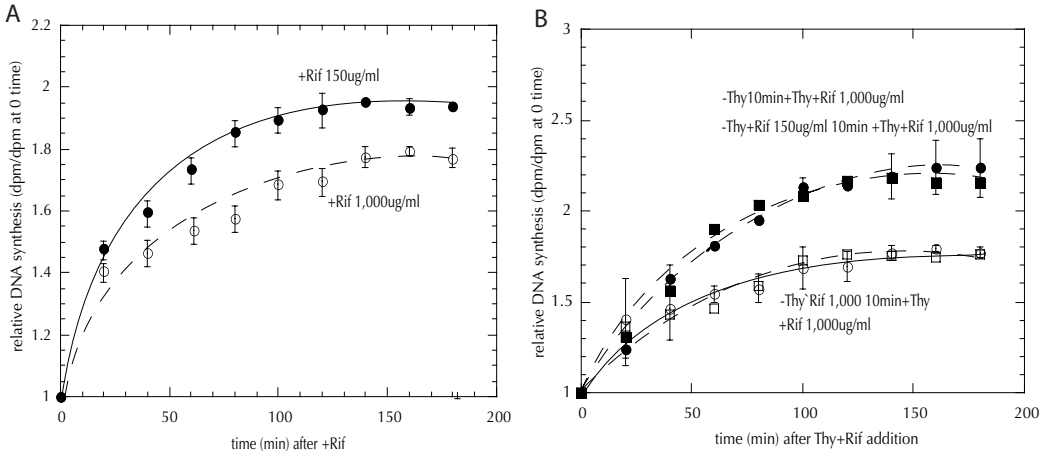

Figure 34. Effect of Rif in DNA synthesis in *datA* mutant under thymine starvation. (A) Runout DNA synthesis in *datA* mutant after Rif addition (●) 150 and (○) 1,000 μg ml^{-1}. (B) Runout DNA synthesis in exponentially growing *datA* mutant cultures after the addition of Rif 1,000 μg ml^{-1} (○), and run-out DNA synthesis after addition of Thy and Rif 1,000 μg ml^{-1} (●) (zero time point) in cultures incubated under thymine starvation for 10 min, incubated under thymine starvation in the presence of (■) Rif 150 μg ml^{-1} and (□) 1,000 μg ml^{-1} for 10 min and after addition of 1,000 μg ml^{-1}. Errors bars represent the SD from 2 to 4 independent experiment.

Overall above results presented in this chapter show that no other effect different from those observed on initiation process, seems to modulate the magnitude of TLD after Rif addition either in the *rpoB*(Rif^R) mutant, different Rif concentrations or *datA* mutant; supporting the transcription dependent step of the initiation as the strongest candidate in the mechanism for Rif-dependent suppression of TLD.

4.4. Altering transcriptional activity around *oriC* decreases ChIC alleviating TLD

The Rif-sensitive chromosomal initiation step in *E. coli* is widely accepted to be transcription of the *PmioC112* and *Pgid113* genes near *oriC* (Fig. 24R) (Lark 1972, Messer 1972, Baker and Kornberg 1988, Skarstad *et al.* 1990). Neither, deletion of the *PmioC112* or *Pgid113* promoters nor constitutive transcription from the *mioC* promoter have a large effect on the cell cycle, but some reduction in initiation efficiency has been observed in rich medium (Bogan and Helmestetter 1996, Bates *et al.* 1997, Molina *et al.* 1999, Su´etsugu *et al* 2003). This effect indicates that transcription from these genes is not absolutely necessary, but can improve the initiation efficiency when cells are grown in rich medium. Here, we take advantage of the detrimental effect on chromosomal initiation observed in these mutants to verify whether altered transcription around the *oriC* region modulates TLD.

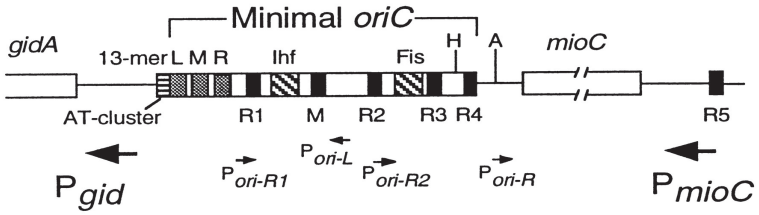

Figure 35. Scheme of the major transcription units around *oriC* used in this work. This figure was been adapted from Bates *et al.* 1997.

Isogenic MG1693 strains carrying *PmioC112, Pgid113,* and the double mutant *PmioC112 Pgid113* defective alleles in the promoter region (Bates *et al.* 1997) and a *mioCp9* allele that confers constitutive transcription from *mioC* entering into the *oriC* region (Su´etsugu *et al.* 2003) were constructed (Fig. 35). TLD was measured in wild type and mutant cultures grown in M9 minimal medium supplemented with 0.2% and 1% casaminoacids (rich medium) without thymine for 3 h. We found significant differences in bacterial viability after 3 h of thymine starvation in rich medium (Fig. 36A, Table 17). The strongest effect was observed in the *mioCp9* mutant strain, which had 60 times the viability of starved wild type cells.

Next, it was assessed the possibility that the partial suppression of TLD by altering transcriptional activity around *oriC* observed in Figure 36A might be caused by a reduction in ChIC. We determined the fraction of initiated origins, *i*, accumulated during 10 min of thymine starvation in the *oriC* transcription mutants. To this end, exponential phase MG1693 cultures carrying the *PmioC112, Pgid113, PmioC112 Pgid113* and *mioCp9* alleles were grown up to 0.2 OD_{550} in medium containing ^{3}H-Thy. Two aliquots were removed and treated either with 150 μg ml^{-1} Rif for 3 h, obtaining ΔG, or starved of thymine for 10 min and then grown with thymine and 150 μg ml^{-1} Rif for 3 h to obtain ΔG'.

The relative amount of DNA accumulated was determined by the amount of TCA-precipitated material in the samples. From this, the proportion of initiated origins that had accumulated during the 10 min of thymine starvation can be calculated (Fig. 36B, Table 17).

These results show that ChIC was reduced in the P*mioC112,* P*gid113*, double P*mioC112* P*gid113* and *mioCp9* mutants grown in M9 minimal medium supplemented with 1% casaminoacids compared to wild type cells. The reduced TLD in PmioC112 and *Pgid113* mutant strains strongly suggests that the transcriptional activity required for the initiation of chromosomal replication is a limiting factor for chromosome initiation under thymine starvation conditions. Flow cytometry profiles of the strains in M9 minimal medium supplemented with 1% of casaminoacids show that complete chromosomal replication was achieved by any of the PmioC112 or *Pgid113*mutant cells starved of thymine for 10 min after re-adding thymine (Fig. 36C).

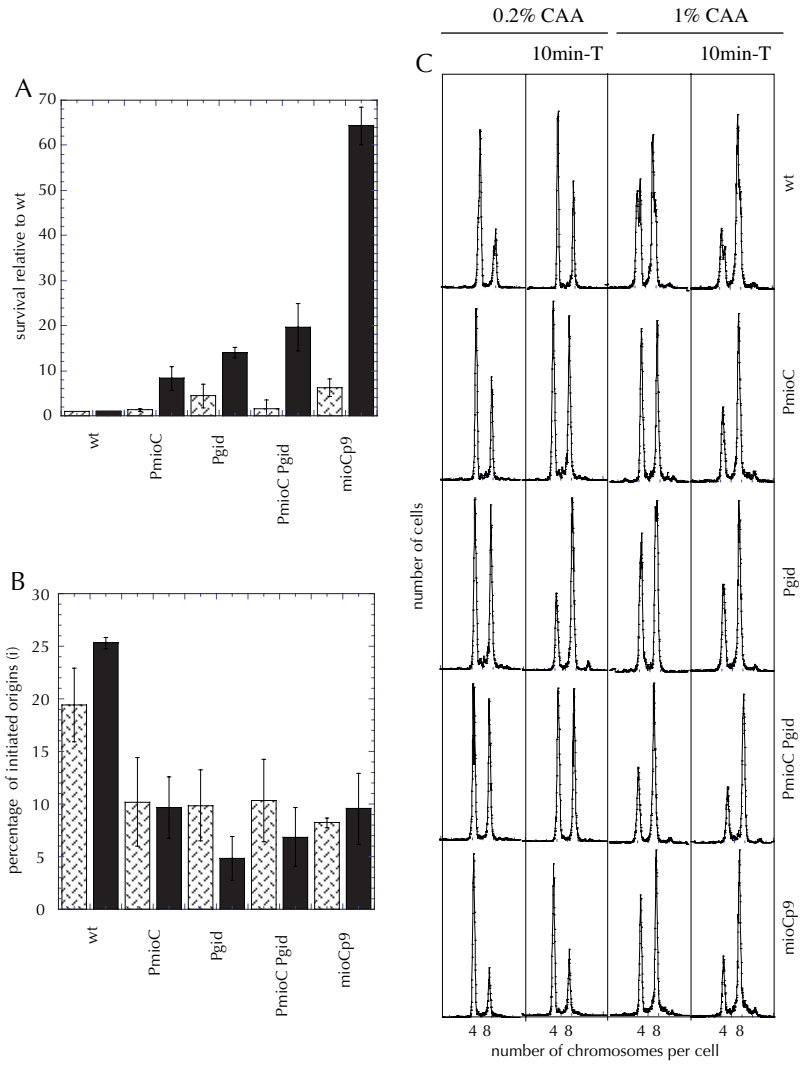

Figure 36. Alleviation of TLD by altering the transcription around the *oriC* origin. (A) Survival after 3 h of thymine starvation of different mutants grown in M9 minimal medium supplemented with 0.2% (▨) or 1% (■) CAA. Values are relative to the wild type MG1693 strain. (B) Percentage of origins initiated after adding thymine to 10 min thymine-starved cells of different mutant grown in M9 minimal medium supplemented with 0.2% (▨) or 1% (■) CAA for 10 min. Error bars represent the SD from 2-4 independent experiments (C). Flow cytometry profiles of the cultures growing in M9 minimal medium supplemented with 0.2% CAA and with 1% CAA, treated with Rif and Cfx for 3 h (left panels) and thymine-starved cultures for 10 min prior addition of thymine, Rif and Cfx for 3 h (right panels)

2D gel analysis was also performed for 1 h thymine-starved cells carrying the *mioCp9* mutation, which showed decreased replication initiation and greatly alleviated TLD when grown in MM9 medium supplemented with 1% casaminoacids. Significantly, no bubble arcs and only very faint simple-Y arcs were observed after thymine starvation of the mutant strain comparable to that observed for the wild type MG1693 strain grown in rich medium (Fig. 37).

The broad reduction of chromosome replication initiation intermediates by these mutants, strongly implicates the transcriptional-dependent step of replication initiation as being critical for TLD, even though there is not a strong quantitative correlation between both parameters.

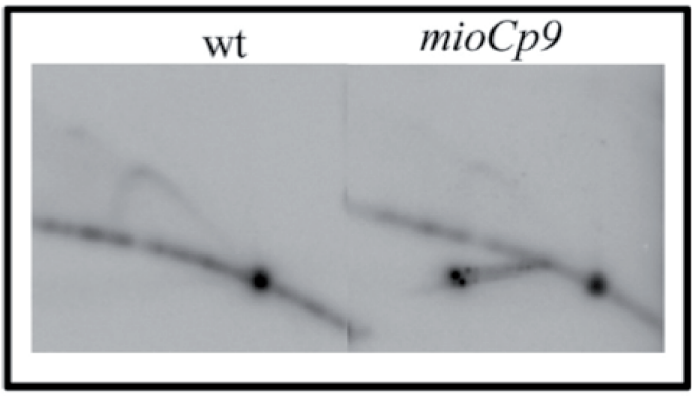

Figure 37. 2D gel DNA analysis after 60 min of thymine starvation of wild type MG1693 and the *mioCp9* constitutive transcriptional mutant strain grown in M9 minimal medium supplemented with 1% CAA.

Table 17. Cell cycle parameters and viability after 3 h of thymine starvation in (i) MG1693 growing in 0.2% and 1% of casaminoacids, and in different mutants with alterations in genes with transcriptional activity around *oriC*, (ii) *rpoB(Rif^R)* mutant, (iii) MG1693 after thymine starvation in presence of different Rif concentrations, (iv) *datA* mutant. The cells growing in M9 minimal medium supplemented with Thy 20μg ml^{-1}and ^{3}H-Thy 1μCi ml^{-1} $^1\tau$ generation time (min); $^2\Delta G$ DNA accumulated in the mid-log phase culture after Rif at 150 μg ml^{-1} for 3h (run-out); ^3n number of replication cycles per chromosome; ^{4}C period $^5\Delta G'$ is the DNA accumulated after the addition of Rif at 150 μg ml^{-1} for 3h to cultures starved 10 min for thymine; ^{6}The value of *i* was obtained from the empirical formula $\Delta G'/\Delta G = [2 (i + 1)]/2 = i+1$ (http//:genuex.unex.es/alf/c/ciclon.htm); ^{7}Cell survival after 3 h of thymine starvation relative to cero time of the treatment. SD is obtained from 2-4 independent experiments.

Strains	τ^1	ΔG^2	n^3	C^4	$\Delta G'^5$	i^6	^{7}Survival 3h –Thy
MG1693							
0.2% Casaa	39±1.25	72	1.75±0.11	68	108	19.46±4.55	0.0016±0.000
1% Casaa	37±1.79	100	2.38±0.09	85	157	25.30±0.91	0.0007±0.000
mioCp9							
0.2% Casaa	42±1.79	52	1.27±0.09	55	65	8.22±0.92	0.0100±0.003
1% Casaa	43±6.42	53	1.33±0.09	57	68	9.56±4.41	0.0450±0.030
ΔPmioC112							
0.2% Casaa	38±1.63	62	1.52±0.16	58	78	10.18±5.69	0.0020±0.000
1% Casaa	40±1.92	71	1.71±0.01	69	87	9.67±2.29	0.0058±0.002
ΔPgid113							
0.2% Casaa	40±2.00	62	1.53±0.00	61	78	9.88±4.32	0.0070±0.004
1% Casaa	43±2.31	73	1.75±0.01	76	81	4.82±4.64	0.0099±0.001
ΔPmio112ΔPgid113							
0.2% Casaa	43±2.58	59	1.46±0.05	63	74	10.33±4.65	0.0025±0.003
1% Casaa	42±1.92	55	1.36±0.09	58	65	6.86±3.38	0.0140±0.004
rpoB (Rif^R)							
-Thy	58±2.3						0.0056±0.0005
+Cm	58±2.3	74	1.78±0.1	103			
-Thy10´+Thy+Cm	58±2.3				110	20.11±0.04	
-Thy+Cm10´+Thy+Cm	58±2.3				71	0.017±0.03	
-Thy+Rif10´+Thy+Cm	58±2.3				90	9.20±0.1	
Different [Rif]							
-Thy+Rif 10 μg ml^{-1}					97	15.69±2.51	0.0040±0.000
-Thy+Rif 25 μg ml^{-1}					87	10.46±3.13	0.0485±0.0044
-Thy+Rif 50 μg ml^{-1}					76	3.74±2.09	0.3137±0.120
-Thy+Rif 150 μg ml^{-1}					72	1.00±0.03	1.1051±0.157
datA							
-Thy+Rif 1000 μg ml^{-1}	38±2.46	77	1.84±0.03	70	124	26.55±1.6	0.0017±0.007

Chapter 5

Active transcription from external promoter inhibits ChIC under thymine starvation, suppressing TLD

Abstract:

To further study of link between transcription and TLD we have tested a system where the level of chromosome transcription could be affected by external level of transcription from plasmid. We show that transcriptional activity from an internal promoter located in the plasmid slightly affect general bacterial transcription levels; while inversely correlates with the survival under thymine starvation and ChIC. Similar results were obtained by using induction of transcriptional activity from pBAD promoter. These results were very interesting as well as unexpected being a new approach verifying the relationship between transcription at initiation under thymine starvation and TLD.

To approach the link between the level of transcription and TLD we tested a system completely different from the usually performed. It is known that the structure of the nucleoid changes with environmental conditions [4] and, under some conditions, RNA polymerase appears to be concentrated into transcription foci or factories. Supporting this, Sánchez-Romero *et al.* reported that transcription affects the location of a DNA target in E. coli (Sanchez-Romero *et al.* 2012). According to that we speculated with the idea that the level of chromosome transcription could be affected by high level of transcription from plasmid.

We used pRK2 derivative pRW901, pRWSR1, pRWSR2 and pRWSR3 plasmids. The pRW plasmids were derivatives from pRW50, a low copy number (5-8 copies) broad host range RK2 (Thomas *et al.* 1980, Butala *et al.* 2009). Briefly, the pRW plasmids contain the gene for the plasmid replication initiator protein (*trfA*), the origin for vegetative DNA replication (*oriV*), the gene encoding resistance to tetracycline (*tetA*), the promoter-less *lacZYA* operon, and an EcoRI/HindIII insert, cloned immediately downstream of five LacI operator sites, with flanking 18-bp target sites for the yeast meganuclease I-SceI (Fig. 38). The pRW901, pRWSR2, used carry different inserts between the EcoRI and HindIII sites. pRW901 plasmid carries a promoter-less fragment, whereas pRWSR2 carries the fragment illustrated in Table 7R that contains the constitutively active KAB-CGTG promoter (Mitchell *et al.* 2003). Moreover, in this study, we used the plasmids pRWSR1 and pRWSR3, derivatives of pRWSR2 witch displays different promoter activities (Burr *et al.* 2000, Mitchell *et al.* 2003) having pRWSR1 the lower level and pRWSR2 the higher.

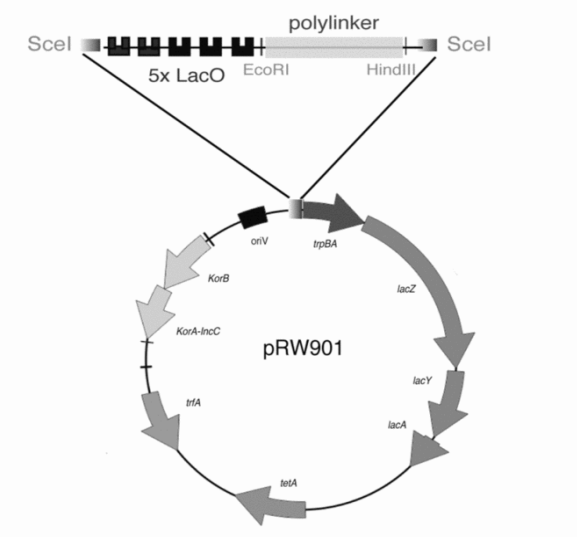

Figure. 38. Map of pRW901 plasmid.

Table 18. Promoter region sequences and activity relative to the pRWSR2 promoter. The table lists the sequence and activities of the three different KAB promoters for last study. Activities were deduced from β-galactosidase measurements and are expressed as a fraction of the measured activity of the pRWSR2 promoter (Miller units) (Michel *et al.* 2003, * this work).

Plasmids	Promoter sequences	β-gal	β-gal*
pRW901	GAATTCTAGACA--------**CGTGTTATGGT**----------AAGCTT		
	EcoR1 -35 -10 HindIII	nt	nt
pRWSR2	GAATTCTAGAAA--------**TGTGTTATCGT**----------AAGCTT	1.00	1.00
	EcoR1 -35 -10 HindIII		
pRWSR3	GAATTCTAGACA---------**AATGGAAAGA**----------AAGCTT	0.72	0.78
	EcoR1 -35 -10 HindIII		
pRWSR1	GAATTCTTGGGT---------**TGTGCTAAACT**----------AAGCTT	0.49	0.60
	EcoR1 -35 -10 HindIII		

5.1. Effect of pWR901 and derivatives on the bacterial general transcription and cell cycle parameters of MG1693

pRW901, pRWSR1, pRWSR2 and pRWSR3 plasmids were introduced in MG1693 competent cells obtained by $CaCl_2$ (see *Methods* section). Cultures of the strains containing plasmids were grown in M9 minimal medium at 37°C and RNA synthesis rate was determined at different times during exponential growth. To analyze whether the presence of the plasmids affects RNA rate normalization to the OD_{550} values were determined. Fig. 39 shows a reduction (10%) of RNA rate in the strain MG1693/ pRWSR2 compared to MG1693/ pRW901, indicating slight effect of active-transcription plasmid pRWSR2 over the general transcription of the cell comparing to the presence of non-transcription plasmid pRW901.

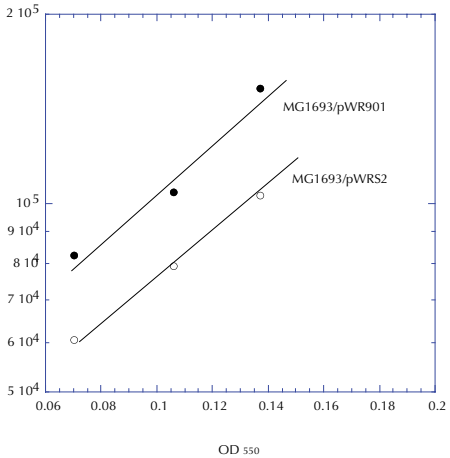

Figure. 39. RNA rate relative to OD_{550} in exponential growth of MG1693 containing pRW901 and pRWSR2 plasmids

In order to study a putative effect of the presence of these plasmids on the cell cycle parameters, the generation times from cultures exponentially growing was determined and the number of replication rounds, *n*, was calculated from ΔG values obtained from Rif treated cultures (see *Methods* section) and C period was estimated as $C=n.\tau$ (Table 8R).

These results show an increase of generation time and a reduction of either *n* and C values. Nevertheless these effects have to be ascribed to the presence of the plasmids but not related to their transcription activities, as pRW901 displayed these features.

5.2. TLD suppression by active transcription from plasmids

Results presented above (*Results* 1 and 3 chapter) showed that survival under thymine starvation inversely correlates to either the number of replication rounds, *n*, and the proportion of 'aborted' initiated origins under thymine starvation, *i*.

To test whether the presence of the different plasmids could affect TLD either by the lowering of *n* or *i* the already described experimental approach was developed (see *Methods* section). Cultures of MG1693 containing pRW901, pRWSR1, pRWSR2 and pRWSR3 plasmids were grown in M9 minimal medium at 37°C up to 0.2 OD_{550} then thymine starved and samples were taken at different times, diluted and plated in duplicate NAT plate that were incubated at 37°C. The numbers of colony on plate were accounted and CFU ml^{-1} was calculated for each time. Results are given as cell number relative to the beginning of the treatment (Fig. 40).

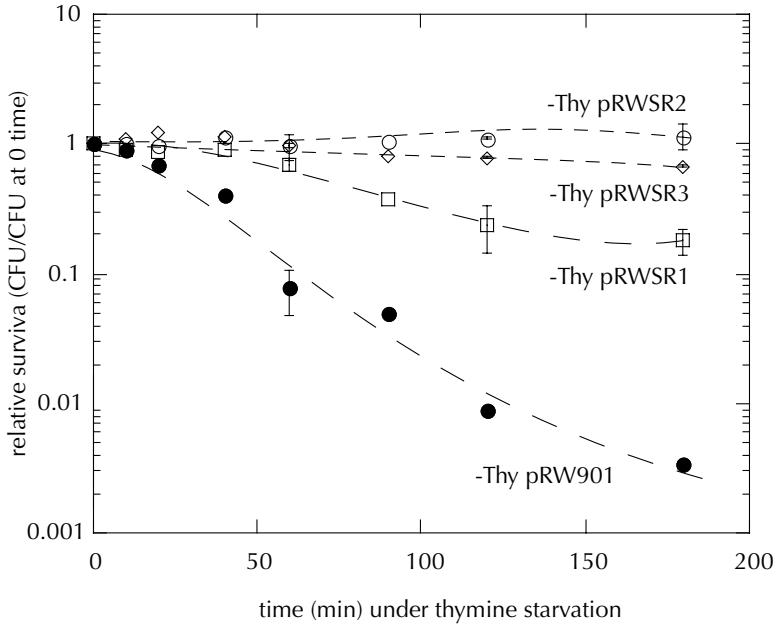

Figure 40. Relative cell survival under thymine starvation in MG1693 carrying plasmids with different transcriptional activity. pRW901 (◆), pRWSR2 (○), pRWSR1 (□), pRWSR3

Surprisingly, TLD was completely suppressed by the presence of pRWS2 being the thymine-starved survival inversely correlated to the level of transcription from the plasmids. The observed differences in the number of replication rounds (Table 8R) could not explain these dramatic results; hence the proportion of initiated origins under thymine starvation, *i* values, of these strains were determined to investigate whether this value correlated to TLD.

Cultures of MG1693 containing pRW901, pRWSR1, pRWSR2 and pRWSR3 plasmids were grown in M9 minimal medium in the presence of ^{3}H-Thy 1μCi ml^{-1} at 37°C up to 0.2 OD_{550}. Two aliquots were taken one was treated by Rif addition to obtain ΔG and the second one was thymine starved for 10 min then thymine was restored in the presence of Rif for 3 h to obtain $\Delta G'$ (see *Methods* section). Figure 41 shows that the proportion of reinitiated origins for 10 min thymine-starved cells, *i*, correlates to the level of transcription from the plasmid. These results indicate that transcription from the plasmid impairs ChIC under thymine starvation, alleviating or even suppressing TLD.

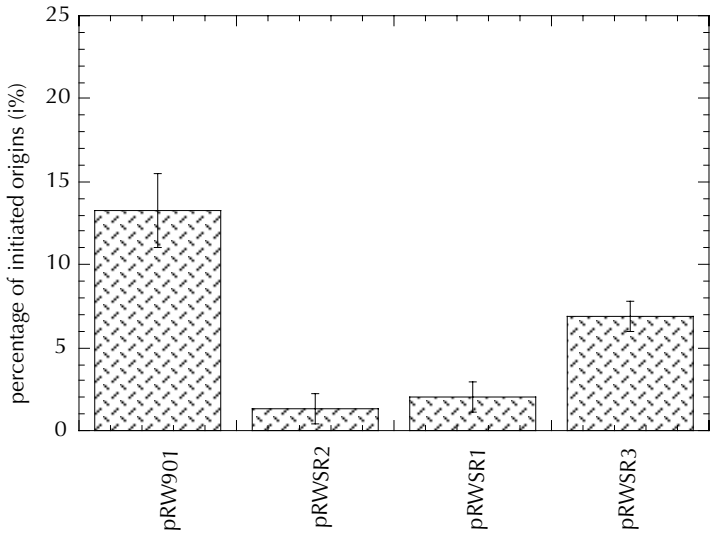

Figure 41. Quantification of ChIC for 10 min thymine-starved cells. MG1693 carrying plasmids with different transcriptional activity. pRW901, pRWSR2, pRWSR3, pRWSR1. Error bars represent the SD from 2 independent experiments.

5.3. TLD is suppressed under inducible transcriptional activity of pBAD promoter

We considered that the dramatic effect on TLD related to the transcription level from plasmids would require a different strategy to verify this relationship. We used inducible BAD regulation to the pKAB promoter inserted into pRW901 plasmid. In this plasmid pRW901pBAD, the arabinose promoter was inserted in the "polilinker" region where pWRS2 has constitutive promoter pKAB (Fig. 42).

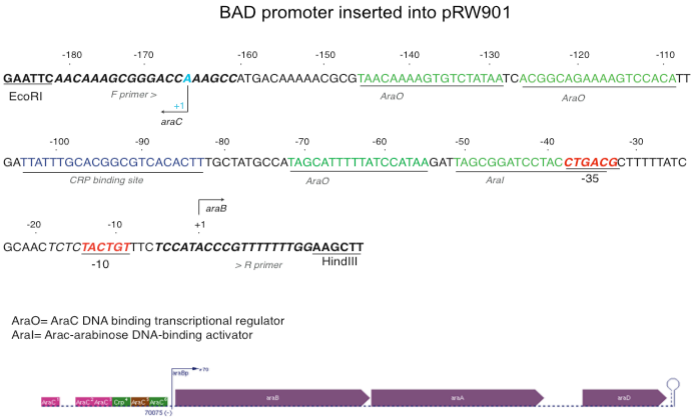

Figure 42. Detail of the insertion of arabinose inducible promoter into pRW901 to give pRW901BAD plasmid transcriptional activated by arabinose

MG1693/pBAD was grown in M9 minimal medium at 37°C up to 0.2 OD_{550} in presence or absence of arabinose 0.2% to induce transcriptional activity in promoter. Aliquots from both cultures were filtered and resuspended in fresh medium without thymine. The viability of the thymine-starved cells was estimated by determining CFU (Fig. 43)

These results show that induction of transcriptional activity in the plasmid suppress the lethality under thymine starvation.

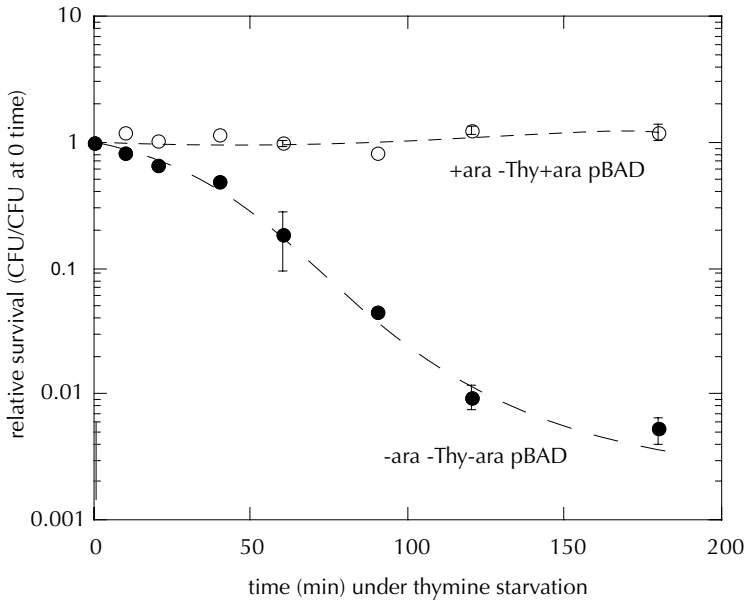

Figure 43. Relative cell survival under thymine starvation in presence of inducible activity transcriptional in pBAD promoter. Induction by arabinose 0.2% (○) or absence of induction (●).

To reinforce the correlation between TLD and the new 'aborted' initiation events during thymine starvation, ChIC was quantified as the proportion of initiated origins during 10 min thymine starvation, *i*, (see Methods section) in the presence or absence of arabinose 0.2% (Fig. 44, Table 19).

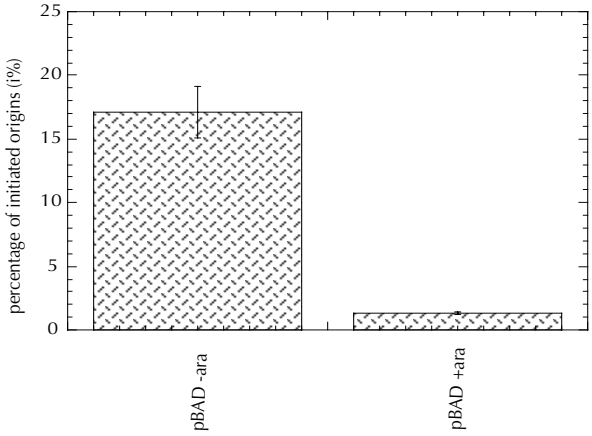

Figure 44. Quantification of ChIC for 10 min thymine-starved cells. MG1693 carrying plasmids pBAD in presence or absence of arabinose 0.2%. Error bars represent the SD from 2 independent experiments.

The results shown that, if the plasmid is transcriptionally active (+ arabinose) new initiation events under thymine starvation are inhibited and TLD is suppressed, even though the number of the replication rounds, *n*, are quite similar in the presence or absence of arabinose 0.2% (Table 8R). These results reveal that the proportion of origins initiated under thymine starvation is crucial for TLD, showing that it would not be only affected by *n*. Furthermore, these conditions show that ChIC not only depend on *n*, but also on the physiological stages of the cells, as slight difference was detected in *n* by growing in the presence or absence of arabinose.

Table 19. Cell cycle parameters and viability after 3 h of thymine starvation in MG1693 in presence or absence of plasmid with transcriptional activity promoter. Strains carrying plasmids growing in M9 minimal medium supplemented with Thy 20μg $ml^{-1}$and ^{3}H-Thy 1μCi ml^{-1}. $^{1}\tau$ generation time (min); $^{2}\Delta G$ DNA accumulated in the mid-log phase culture after Rif at 150 μg ml^{-1} for 3h (run-out); ^{3}n number of replication cycles per chromosome; ^{4}C period $^{5}\Delta G'$ is the DNA accumulated after the addition of Rif at 150 μg ml^{-1} for 3h to cultures starved 10 min for thymine; ^{6}The value of *i* was obtained from the empirical formula $\Delta G'/\Delta G = [2 (i + 1)]/2 = i+1$ (http//:genuex.unex.es/alf/c/ciclon.htm); ^{7}Cell survival after 3 h of thymine starvation relative to cero time of the treatment. SD is obtained from 2-4 independent experiments.

Strains	τ^{1}	ΔG^{2}	n^{3}	C^{4}	$\Delta G'^{5}$	i^{6}	^{7}Survival 3h –Thy
MG1693	39±1.2	72	1.75±0.11	68	108	19.46±4.5	0.0016±0.008
pRW901	46±1.9	43.3	1.11±0.23	52	62	13.28±2.2	0.004±0.000
pRWSR2	49±1.6	51.2	1.28±0.27	62	53	1.32±0.05	1.1234±0.009
pRWSR3	46±2.0	45	1.22±0.14	56	56	6.85±0.9	0.06748±0.007
pRWSR1	46±1.4	48	1.15±0.08	53	51	2.02±0.8	1.1844±0.0021
pBAD -ara	45±2.3	52.5	1.31±0.32	59	78	17.11±2.0	0.0052±0.0012
pBAD +ara	44±1.1	48.3	1.22±2.23	54	50	1.35±0.0	1.21±0.08

Chapter 6

Study of cell division under thymine starvation and restoring conditions

Abstract:

Cell dimensions and nucleoid morphology were determined during thymine starvation. Surprisingly, significant drops in cell length and area, associated with a smaller rise in diameter and doubling in the proportion of constricted cells were observed during the first 10 min. Longer starvation displayed the anticipated, well-known phenomena: division-inhibition associated with cell filamentation, nucleoid dispersion and appearance of anucleated cells. The short-term effects are consistent with induction of cell division and remodeling of cell dimensions during the first 10 min of thymine starvation being verified by viability experiments and counting cell numbers in Neubauer chamber. Similar qualitative results were obtained by blocking DNA replication with NAL or Hu. The presented results demonstrate that inhibiting DNA replication by various means enhances division of cells (most probably in D period) before the inhibition of cell division occurring under long term treatments. Furthermore, evolution of the cell morphology after restoration of thymine at different times was also analyzed.

6.1. Short-term thymine starvation enhances cell division

The last point developed in this work has been the study of nucleoid and cell morphology under thymine starvation by using phase contrast and fluorescence microscopy as experimental approach. Thymine starvation is known to inhibit cell division associated to filamentation. Nevertheless, we have shown in this work that important event, such aborted initiations; were displayed during the first minutes under thymine starvation. Here we have studied the morphological changes in the cells in a very precisely experiments to detect any change during the short-term thymine starvation treatment.

A culture of MG1693 was grew in M9 minimal medium at 37°C up to 0.2 OD_{550}. An aliquot was filtered and resuspended in medium without thymine. Samples were taken at different times (10, 20, 40, 60, 80, 120, 150, 180 and 210 min) of thymine starvation; then fixed in formaldehide 0.25% and kept at room temperature. The samples were stained with DAPI to final concentration 1µg ml^{-1} and immobilizated on smooth agarose 2% surface on an object slide (see *Methods* section). Images were acquired using an inverted Olymous microscope IX70 with a Micromax 512 camera.

Results from phase contrast and DAPI stained displayed the anticipated, well-known phenomena (Bi and Lutkenhaus 1991, Donachie 1968, Mulder and Woldringh 1989) such appearance of anucleated cells (Fig. 45), division-inhibition associated with cell filamentation and nucleoid dispersion (Fig. 46).

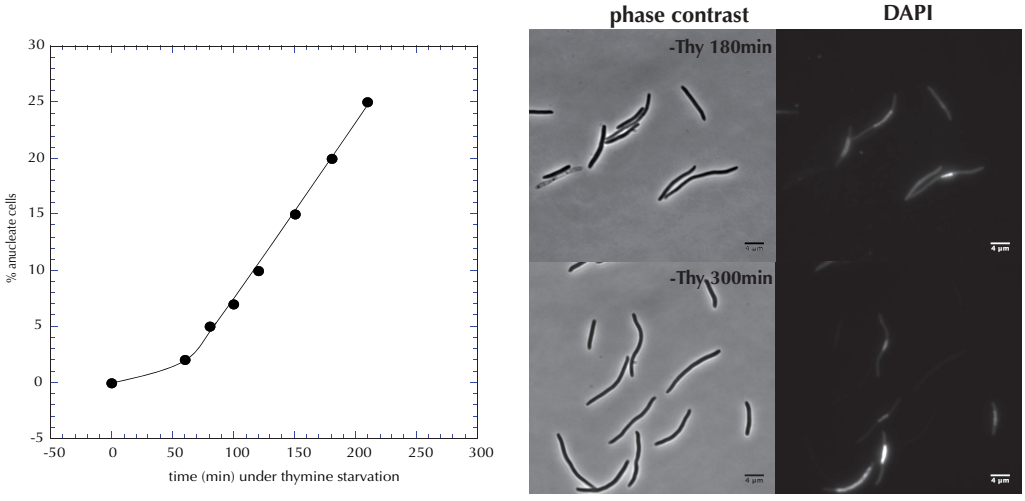

Figure 45. Anucleated cell under thymine starvation. (A) percentage (B) Micrographies of phase contrast and DAPI stained of 180 and 300 min starved-thymine cells.

Interestingly, 10 min thymine-starved cell were slightly smaller than untreated cells

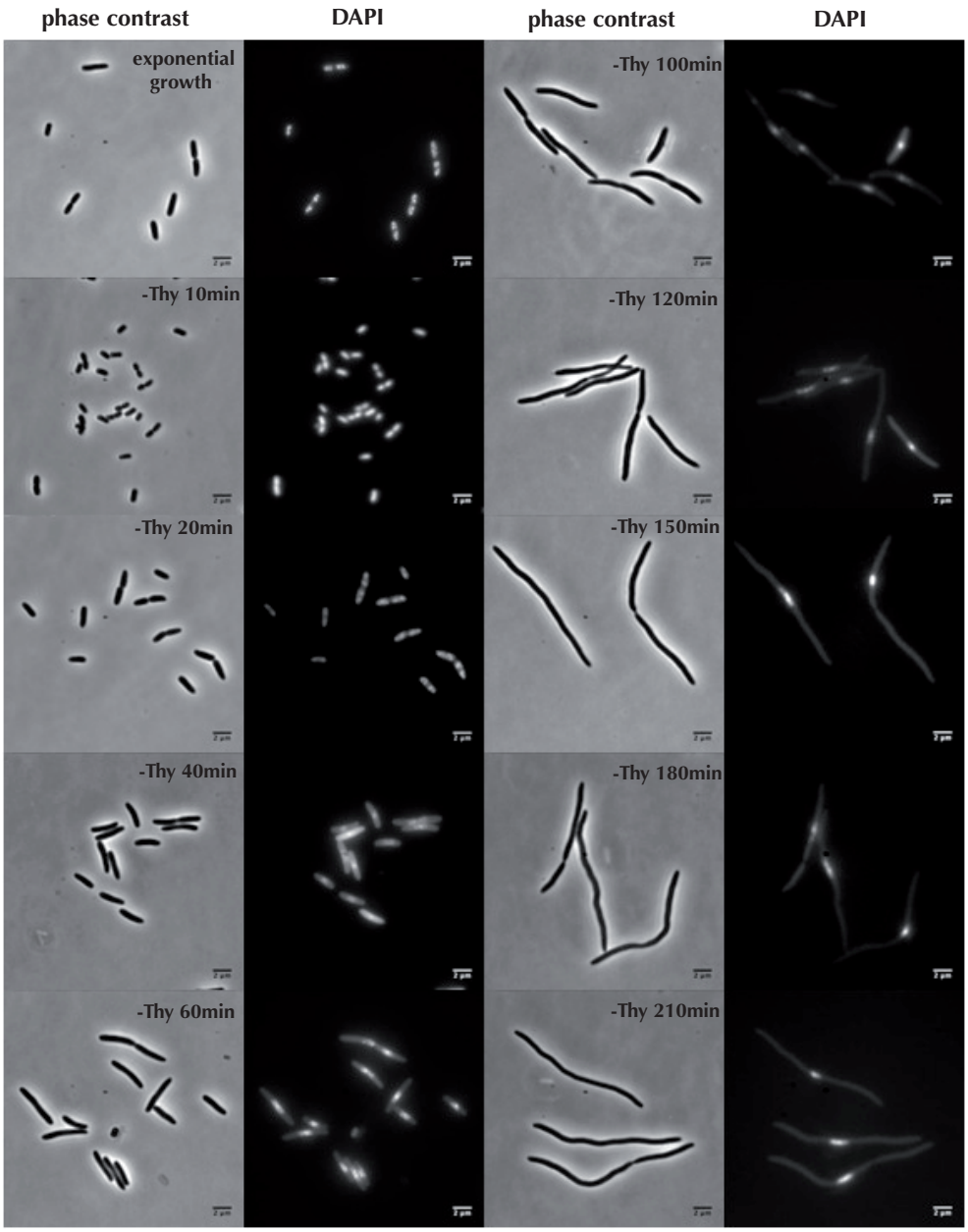

Figure 46. Effect of thymine starvation on the cell and nucleoid morphology by microscopy analysis. Phase contrast images of cells under thymine starvation and DAPI stained cells at different times of the treatment. Scale bars 2µm.

By using *Image J* and *Object J* programs a quantitative study of the cell parameters from 200-300 cells taken from different images was done. From each sample: cell length, diameter and area; in addition of nucleoid area were analyzed (Fig. 47). The results show that by long-term of thymine starvation cells elongate increasing its area by more than six times, cell diameter increases by a 15% and a compact nucleoid located at mid-cell was almost double from initial value (Fig. 47A).

Surprisingly but according to the observation of smaller cell size of 10 min thymine-starved cells, we observed significant drops (of about a 30-40%) in both cell length and area, nucleoid area, being associated with a smaller rise in diameter (Fig. 47B); suggesting and induction of cell division and remodeling of cell dimensions during the first 10 min of thymine starvation. Supporting this observation by a second experimental approach, we founded that viable cells increased by 10 min of thymine starvation, followed by the well described lost of viability (Fig. 47 C and D).

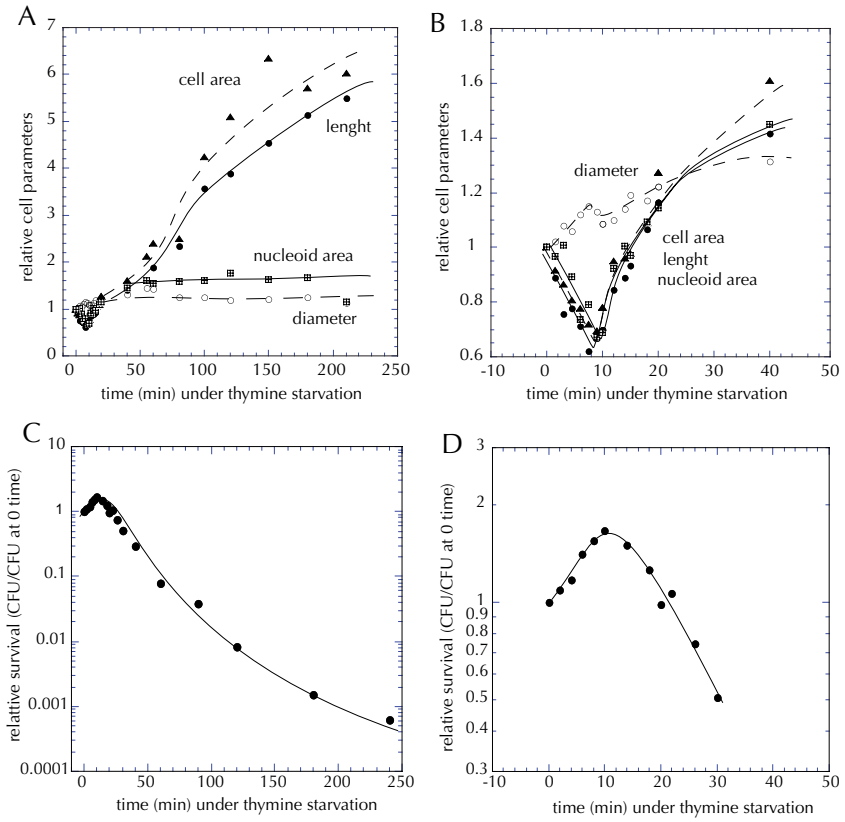

Figure 47. Effect of thymine starvation on cell and nucleoid parameters. cell length (●), diameter (○) and area (▲) in addition of nucleoid area (⊞) during 240 min (A). More precise, during the first 50 min (B). Relative survival under thymine starvation by counting of CFU (C), more precise during the first 50 min (D).

These effects were also observed in another K-12 derivative, strain CR34, while the response of *E. coli* 15 TAU-bar differed quantitatively (Fig. 48)

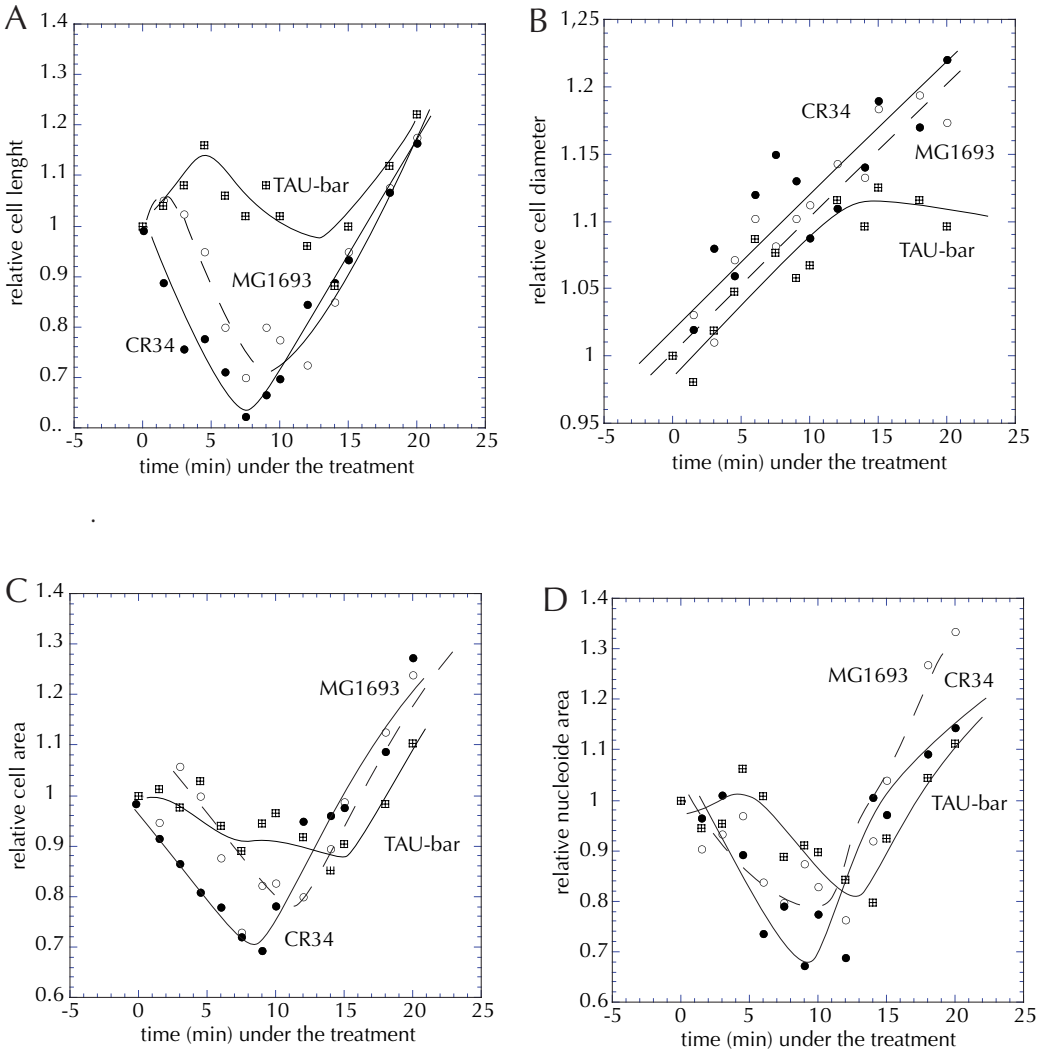

Figure 48. Effect of thymine starvation on cell and nucleoid parameters in different strains *thyA*. cell length (A), diameter (B) and area C) in addition of nucleoid area (D) during 20 min in the strain MG1693 (◆), CR34 (ς) and TAU-bar (⊞).

Induction of division in short-term thymine-starved cells was further verified by doubling in the proportion of constricted cells (from 20 to 45%) during the first 5 min of the treatment (Fig. 49).

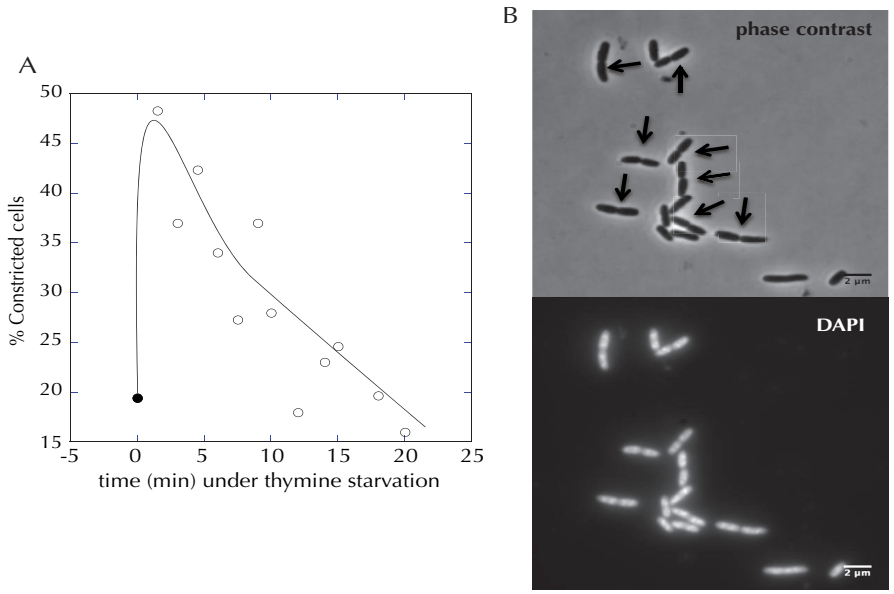

Figure 49. Constricted cells during thymine starvation. (A) Percentage (B) Micrographies of phase contrast and DAPI stained of 5 min starved-thymine cells. Arrows point to the constriction

Overall these results demonstrate that thymine starvation enhances division of cells, before the inhibition of cell division occurring under long-term treatments.

6.2. Instant cell division is related with arresting chromosome replication

In order to study whether the instant cell division observed during the first 10 min during thymine starvation was related with the arrest of chromosome replication: cell length, diameter and area in addition of nucleoid area were analyzed after inhibiting DNA replication by various means (i. e. Hu and NAL addition) by using microscopy approaching in the same experimental condition as described above for thymine starvation. Fig. 50 shows that, addition of Hu or NAL displayed similar pattern for the values of the different parameters.

Although quantitatively different, significant drops in both cell length and nucleoid area, associated with a smaller rise in diameter were observed; suggesting the enhancement of cell division and remodeling of cell dimensions is a general effect related to the first minutes of chromosomal replication blockage.

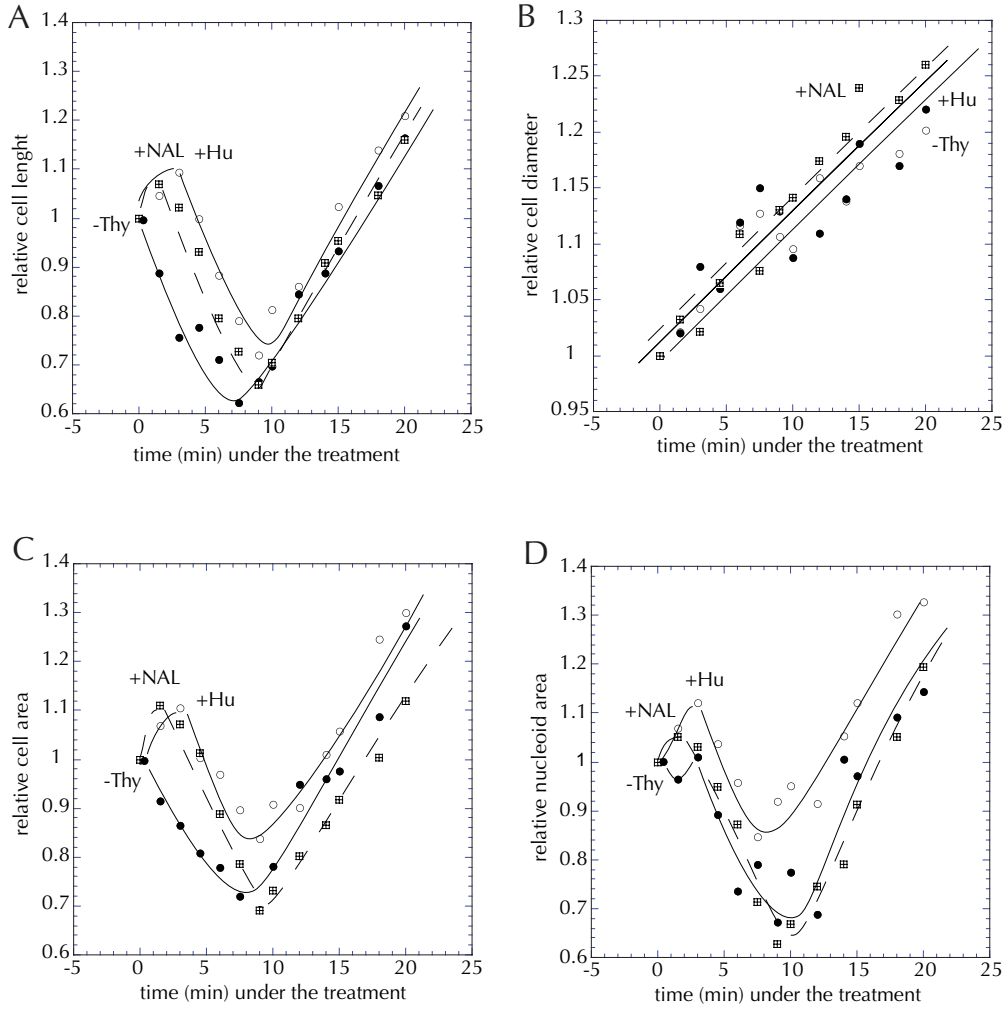

Figure 50. Effect of different treatments blocking DNA replication on cell and nucleoid parameters. Different treatment, thymine starvation (●), Hu addition (ς) and NAL addition (⊞)on cell and nucleoid parameters: cell length (A), diameter (B) and area (C) in addition of nucleoid area (D) during the first 20 min of the treatments.

A third different approach to verify this unexpectedly instant cell division under arrest of DNA replication was cell counting by using Neubauer chamber where cells are counted as particles independent of their viability (see *Methods* section). A culture of MG1693 was grew in M9 minimal medium at 37°C up to 0.2 OD_{550}. An aliquot was filtered and resuspended in medium without thymine in the presence or absence of Rif. Two more aliquots were treated either by addition of Hu or NAL. Samples were taken at different times (1.5, 3, 5, 8, 12, 15, 18, 20, 40 and 60 min) during the first hour of the treatments to verify in detail the effect on the number of cells. Cells were fixed in formaldehide 0.25% and kept at room temperature. The results show doubling cells number during the first 10 minutes of thymine starvation as well as Hu or NAL addition, but not for thymine starved cells in the presence of Rif (Fig. 51).

The presented results demonstrate that inhibiting DNA replication by various means (thymine starvation, Hu or NAL addition) enhances division of cells, before inhibition of cell division occurred under arrest of replication forks. No cell division was allowed if Rif was added during thymine starvation.

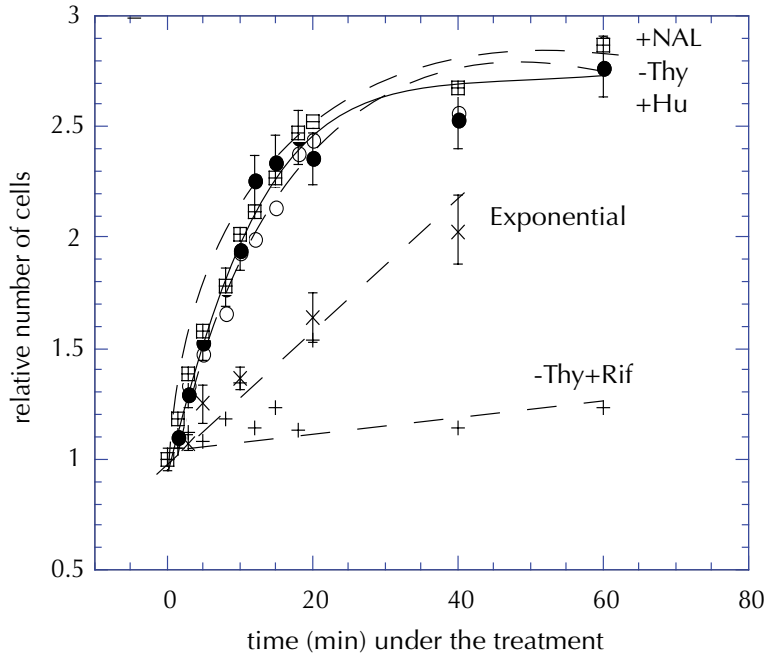

Figure 51. Cell counting in Neubauer chamber under different treatment. Cultures MG1693 in exponentially growing (✕), under thymine starvation in absence (●) or presence of Rif (+), Hu addition (○) and NAL addition (⊞).

6.3. Recovering from thymine starvation

Given that thymine-starved cells accumulate ChIC (*Results*, chapter 3) and display an instant cell division before the inhibition of this process occurred (above results this chapter), we wonder about the fate of cells after thymine restoration after different times of thymine starvation, when cells would be allowed to initiated those 'aborted' initiation events.

In order to get a general view of how cells recovered from TLD, viability experiments were performed by determining CFU. To account for that, MG1693 strain was grown in M9 minimal medium at 37°C up to 0.2 OD_{550}, an aliquot was filtered and resuspended in thymine free medium. Thymine was restored at different times (10 min, 1, 2, 4, 5, 6, 8, 12, 18, 24 and 36 h) of thymine-starvation (Fig. 52).

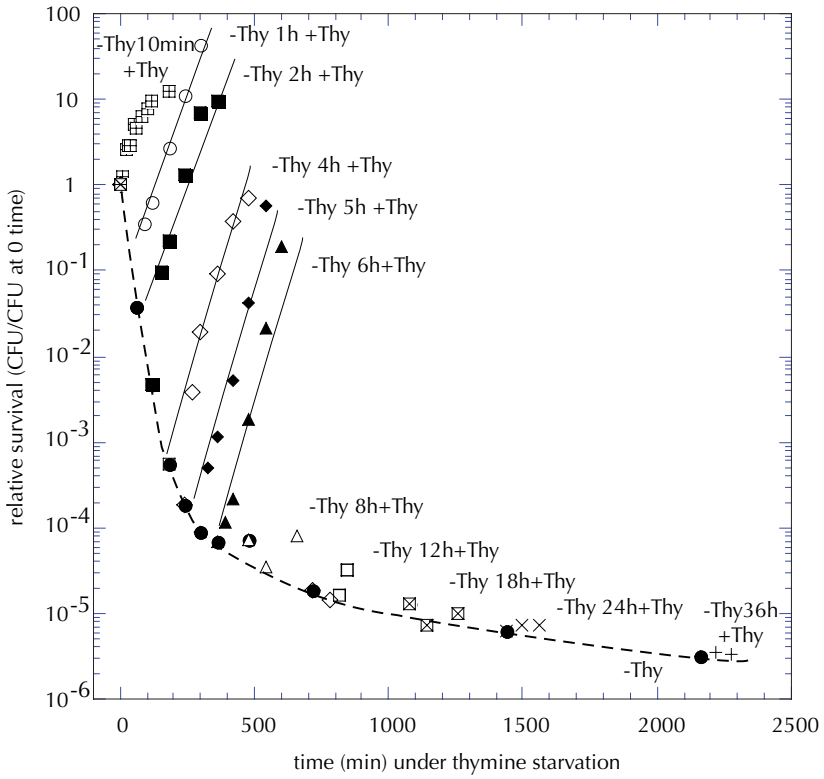

Figure 52. Relative survival after thymine starvation and restoration of thymine at different times. Thymine starved (●) and 10 min (⊞) 1 h (○) 2 h (■) 4h (◇) 5 h (◆) 6 h (▲) 8 h (△) 12 h (□) 18 h (⊠) 24 h (×) and 36 h (+) thymine addition to the starved culture.

Figure 52 shows that followed thymine addition to thymine starved cells up to 6 h, cells start dividing and the recovering of viable cells (CFU) displayed no apparent lag period. Nevertheless recovering from longer period of time of thymine starvation the recovering was not possible.

Developing a deeper analysis to study how the recovery proceeds, we determined cell morphology parameters by using microscopy approach similarly to above (6.1 section). A culture of MG1693 strain grew in M9 minimal medium up to 0.2 OD_{550} , then filtered and resuspended in thymine free medium. Thymine was restored at 10, 40, 55, 60, 70, 80, and 120 min of thymine-starvation and samples were withdrawn to determine length, area and diameter of the cell in addition to nucleoid area at different times of the recovering.

Quantification of cell length, diameter and area in addition to nucleoid area showed two patterns describing how morphological cell changes proceed; one for the recovering after 10 min under thymine starvation (short-term) and a second general one for the recovering from longer times of the treatment (40, 55, 60, 70, 80 and 120 min).

6.3.1. Recovering from short-term (10 min) of thymine starvation

When thymine was added to 10 min thymine-starved cells overall parameters were restored to those values from untreated cells values (Fig. 53A). Moreover, It is important to note that at 10 min of thymine starvation cells have already undertaken rapid changes in the morphology and cell division (Fig. 53B). At this point cells length and area or nucleoid area keep on increasing up to near initial values of untreated cells; cell diameter keep on increasing then decreases to reach the original value (Fig. 53).

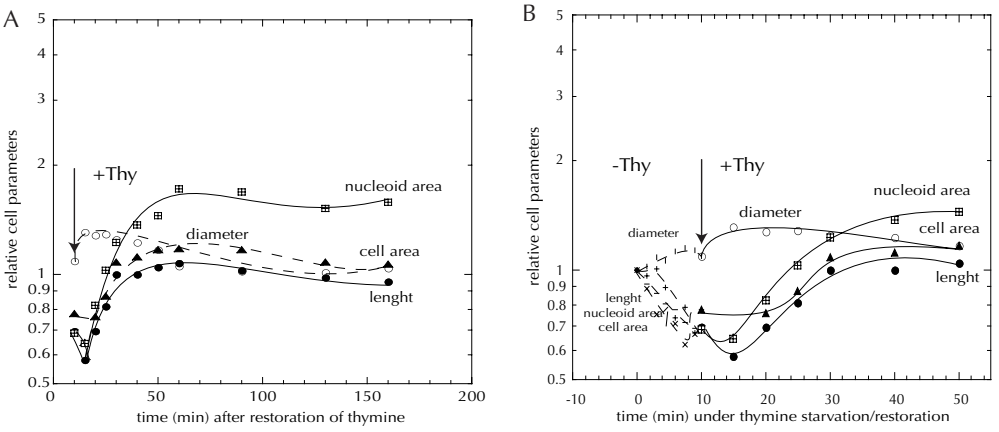

Figure 53. Effect of thymine restoration at 10 min (short time) of thymine starvation on different cell parameter. (○) diameter (⊞) nucleoid area (▲) cell area, (●) length.

6.3.2. Recovering from long-term of thymine starvation

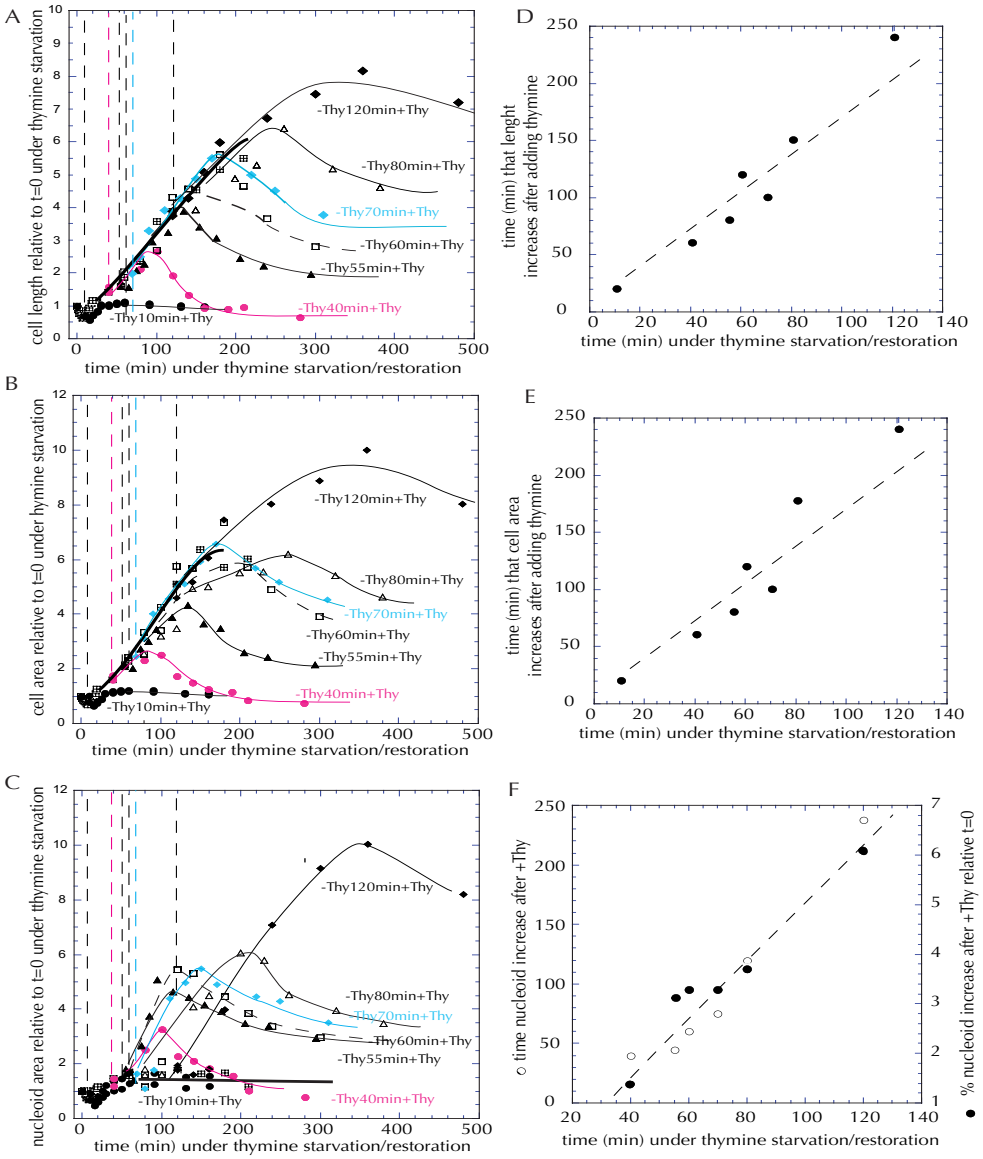

Figure 54. Effect of thymine restoration at different times of thymine starvation on cell and nucleoid parameters. Different times of thymine starvation (⬟) 0min (●) 40min (▲) 55min (□) 60 min (◆)70 min (▲) 80min (⬟) 120min. Left panels: cell length (A), cell area (B), nucleoid area (C) Solid thick line represent thymine starvation treatment. Right panels, relationship between the time under thymine starvation (D,E) and the time the different parameters keep on increasing (F).

These features of the recovering from long-term thymine starvation can be resumed as follow (i) the longer the time under thymine starvation, the longer the time cell length and area keep on increasing after thymine addition. Following that both parameters decrease and establish 3 to 8 times higher than untreated cells (Fig. 42AR, 42BR) (ii) cell diameter, increased 45% under thymine, decreased if thymine is restored, being established around 20% higher than untreated cells (Fig. 43R) (iii) the longer the time under thymine starvation, the longer the time nucleoid area keep on increasing after thymine addition (Fig. 42CR) (iv) As expected, the proportion of anucleated cells diminished after thymine was restored (Fig. 44R)

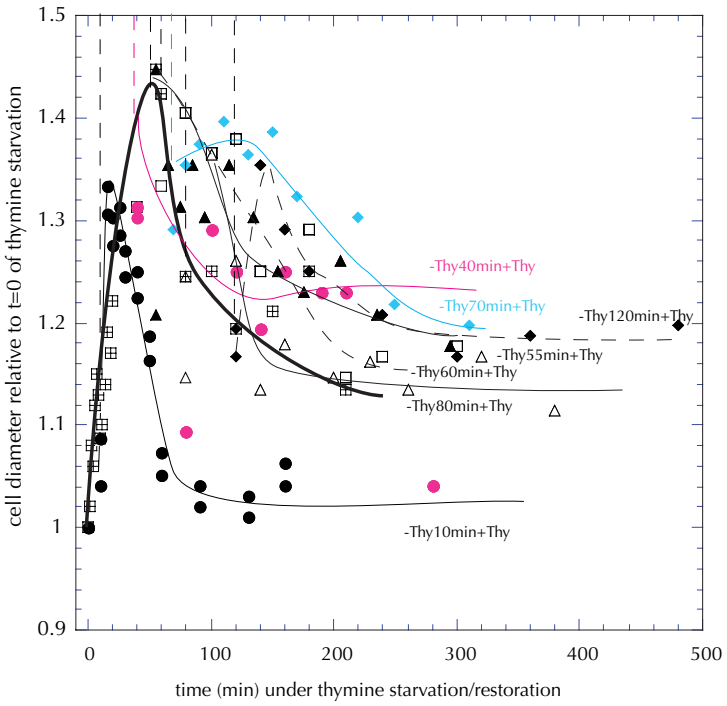

Figure 55. Effect of thymine restoration at different times of thymine starvation on cell diameter. Different times of thymine starvation (◆) 10 min (●) 40 min (▲) 55 min (□)) 60 min (◆) 70 min (△) 80 min (◆) 120 min. Solid thick line represent thymine starvation treatment (⊞).

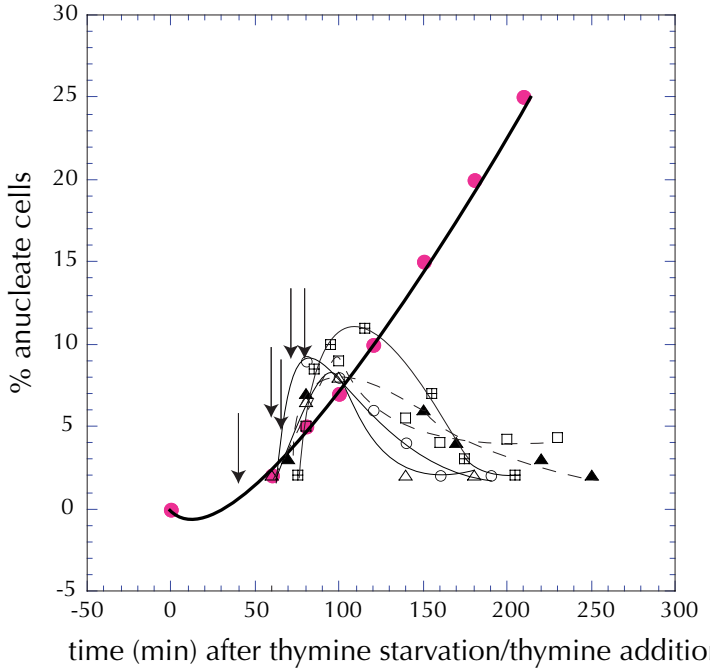

Figure 56. Anucleated cell under thymine starvation and after restoration**.** Percent of anucleated cells under thymine starvation (●) and after restoration under different times of the treatment (ᵔ) 40min, (⊞) 55min, (△) 60 min, (▲) 70 min, (□), 80 min.

The data presented in figures 41R, 42R and 43R were obtained from the micrographs of phase contrast and DAPI stained showed in figures 44R (recovering from 10, 20 and 40 min of thymine starvation) and figure 45R (recovering from 60, 80 and 120 min of thymine starvation).

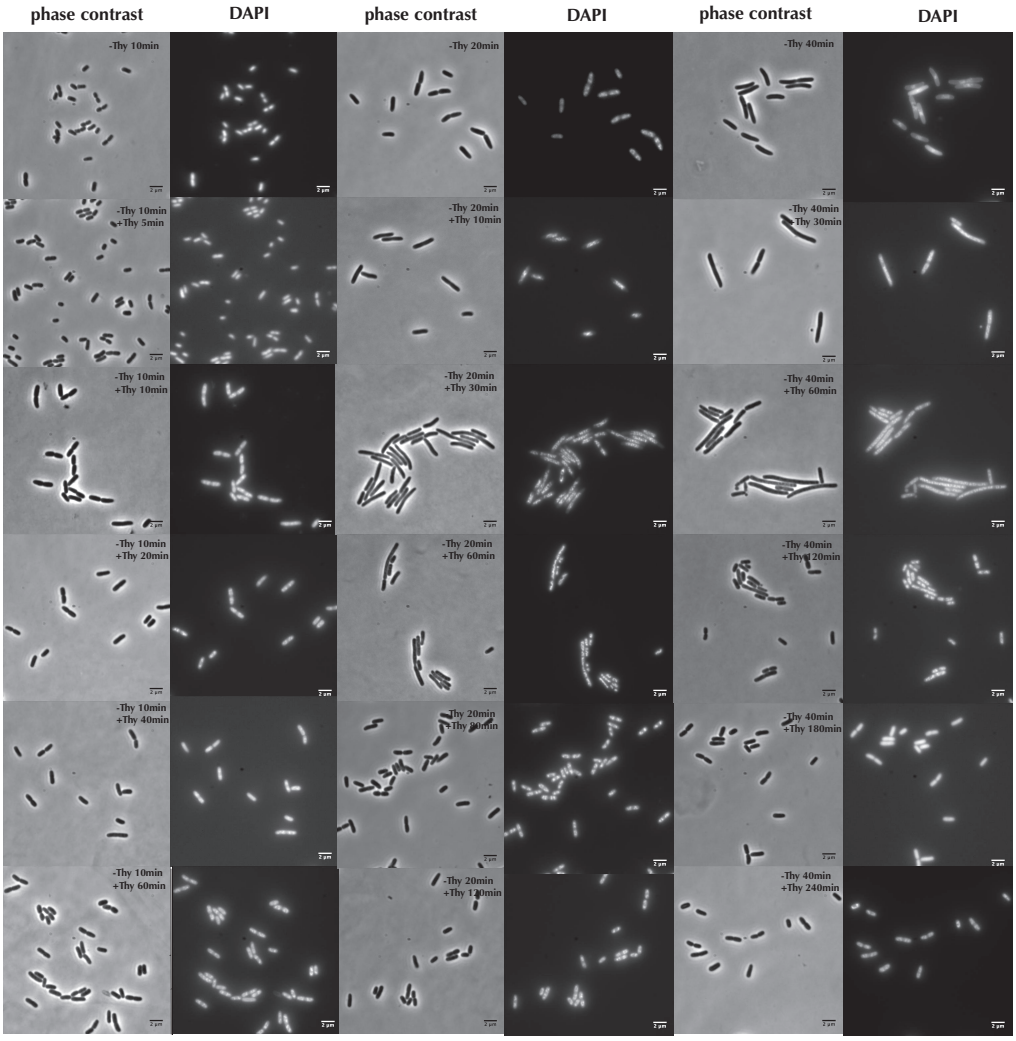

Figure 57. Effect of recovering from 10, 20 and 40 min thymine starvation on the cell and nucleoid morphology by microscopy analysis. Were analyzed by phase contrast and by fluorescence of DAPI stained cells at different times of the treatment. Scale bars 2μm

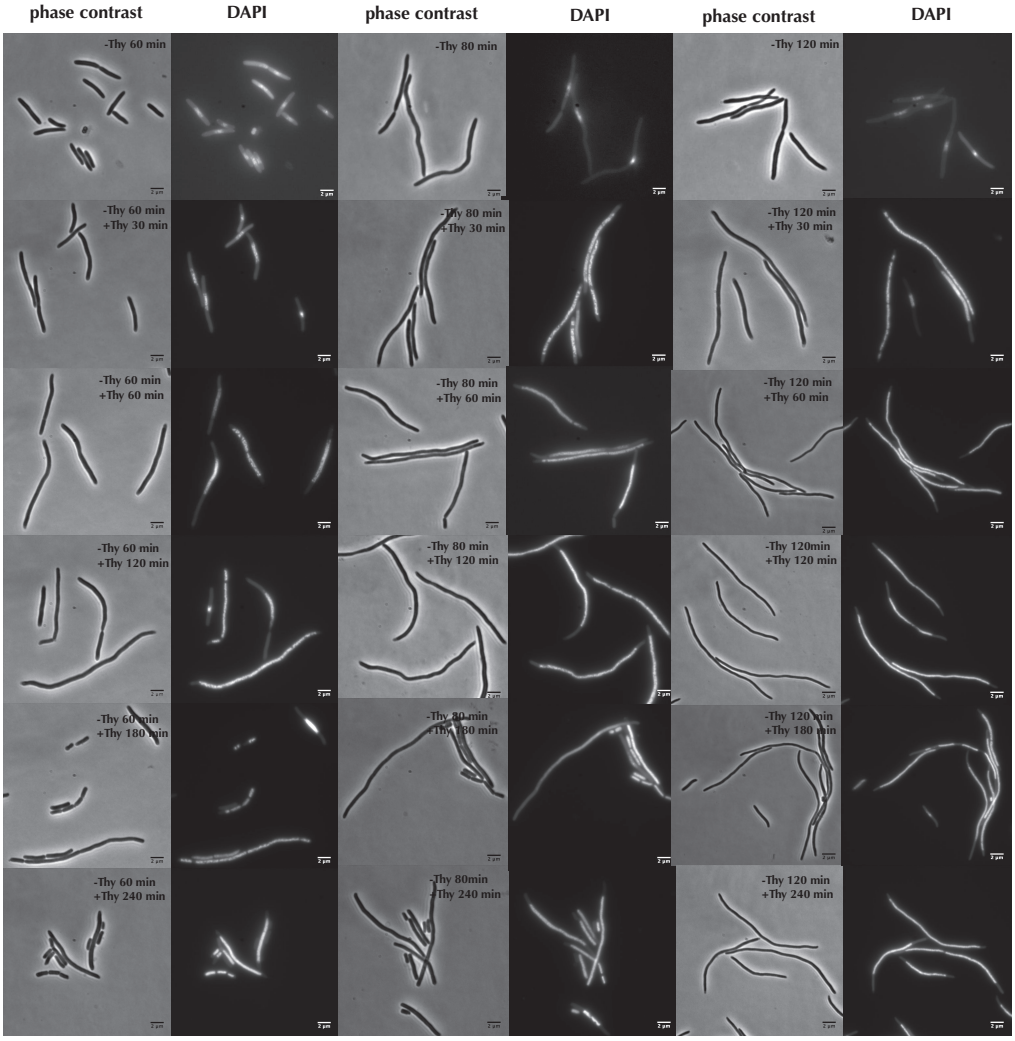

Figure 58. Effect of recovering from 60, 80 and 120 min thymine starvation on the cell and nucleoid morphology by microscopy analysis. Were analyzed by phase contrast and by fluorescence of DAPI stained cells at different times of the treatment. Scale bars 2μm

Discussion

The goal of this work has been to elucidate the molecular mechanism by which thymine starvation kills the cell; phenomenon best-known as *thymineless death* (TLD).

What is the target of TLD?

For many years TLD has been associated with replicating cells in prokaryotes and eukaryotes. Given that thymine is exclusively incorporated during the DNA replication this notion makes sense. Nevertheless two results made us to think it is not so simple; more elements should be in the scenario of TLD.

A) The first observation was that *TLD is not affected by Hu addition*. Given that Hu inhibits DNA replication, then cells in the presence of the drugs are not replicating an consequently TLD was expected to be suppressed.

We determined DNA synthesis by ^{3}H-Thy incorporation into de DNA after adding made fresh Hu 75 mM in MG1693 growing in M9 minimal medium supplemented with 0.2% casaminoacids at 37ºC. We found completely stop of ^{3}H-Thy incorporation. Given that small fraction of DNA synthesis in the presence of Hu could not be detected by this approach, in our experiment Hu was present 10 min before to the elimination of thymine from the growth medium; time that we considered enough to get full stopped replication forks under our experimental conditions.

Our results show that Hu addition 10 min before thymine starvation does not affect the kinetic of TLD either if thymine starvation of the pretreated cells with Hu was performed in the presence or absence of Hu (Fig. 15). These results would indicate that, according to Morganroth and Hanawalt, TLD does not require active replication forks.

Moreover the effect of Hu on DNA replication is not clear enough. Kuong and Kuzminov showed that Hu does not stop the replication at once (Kuong and Kuzminoz 2012); assuming this notion active replication forks would be present under thymine starvation in the presence of Hu and consequently it cannot be established whether active replication forks were required or not for TLD to occur. Nevertheless, it could be that, the critical point of the replication forks regarding TLD is not the 'state of replication fork' (ongoing or stalled) if not the presence of some residual pool of dNTPs maintaining aberrant DNA replication and recombination events at the replication fork. This pool could be generated either because Hu addition does not inhibit RNR at once *in vivo*, or because under thymine starvation there is a pool of other dNTPs being uracil incorporated instead of thymine (Kouzminova and Kuzminov 2008), or because residual pool of dNTPs was generated by small DNA degradation from the DSBs (Kuong and Kuzminov 2011). Actually, these

authors showed that under thymine starvation there is a residual 20% of new DNA synthesized after 30 min of thymine starvation.

Any of these possibilities could sustain the replication forks in an "indeterminacy state" i. e., ongoing and stalled at the same time under thymine starvation in the presence or absence of Hu; then explaining that the presence of Hu was irrelevant for TLD. This would suggest that TLD does not require the status of 'ongoing' for replication forks, although TLD requires replication forks as the number of replication rounds, and hence the replication forks correlates with the extent of TLD (Fig. 13). This results point to the replication fork (ongoing or stalled) as the principal target during thymine starvation; they will be necessary but not sufficient for TLD.

What effect could generate thymine starvation on the replication forks? Our results show that, neither the amount of DSBs nor nmDNA correlate with TLD although they are associated with this phenomenon. The consensus of several models to explain the molecular mechanism of TLD posits DNA damage and/or DNA recombination intermediates at the root of TLD. The results presented in this work show, in one hand, that the mere DNA damage is not the cause of TLD as DSBs were generated in thymine-starved cells in the presence of Rif that prevented TLD. This indicates that lethality would not be caused merely by DSBs at existing replication forks along the chromosome and hence, that the DSBs may be necessary but are not sufficient to cause TLD. On the other hand, in agreement with Nakayama *et al.* we found that complex DNA structures (nmDNA) are induced by thymine starvation (Nakayama *et al.* 1994). They proposed nmDNA to be associated with TLD, but we didn't observed nmDNA under thymine starvation when Hu was present. Giving that under thymine starvation in the presence of Hu there is lethality, this indicates nmDNA could be associated but is not necessary for TLD.

B) The second observation that made us to think about new elements, other than replication forks, to explain the mechanism undertaking TLD was that *TLD is suppressed in the presence of either Rif or Cm*. Under these treatments initiation is inhibited but replication forks are still active and progressing as well as in untreated cells. Regarding this, Rif and Cm would be eliminating a 'new element' related with the lethal effect of thymine starvation over the replication forks. Rif is known to inhibit RNA synthesis by binding to RNA polymerase and Cm inhibits protein synthesis. Both processes are required for chromosomal initiations to occur and we found a correlation between the occurrence of new replication rounds under thymine starvation and TLD. We propose the initiation of chromosomal replication to be the 'new element' in the scenario of TLD. We have established a causative link between DNA initiation occurred under thymine starvation and TLD. Several lines of evidence are presented.

(i) The runout of replications after addition of thymine in cultures starved 10 min for thymine in the presence or absence Hu indicates that initiations occurred under thymine starvation, while these events were suppressed in the presence of Rif or Cm; correlating with lethality (Fig. 20). The flow cytometry analysis of the runouts of the replication which initiation occurred under thymine starvation yielded completed chromosome, as discrete peaks were observed after the replication runout of cultures thymine starved for ten minutes after thymine restoring and 3 h of Rif+Cfx addition. This indicates that even though the *thymineless origins* were damaged, they were repaired being ready to give complete chromosomal replication after thymine addition. The analysis of the proportion of the number of cells containing four and eight chromosomes after the thymine starvation treatments suggests that the new initiated replications have occurred in a number of the cells containing four origins; according to the lowering in the proportion of cells with four chromosomes and the concomitant increase in the number of cells containing eight chromosomes.

(ii) ChIC accumulates under thymine starvation. Time-lapse experiments of thymine starvation show that the amount of initiated origins increases by the time pass by thymine-starved cells. Correlating with the lost of viability of the cell on plate (CFU). Flow cytometry profiles indicate that *thymineless initiations* occurred or are maintained during thymine starvation for more than 30 min but they were not able to complete chromosomal replication if thymine was added, most likely due to the irreversible DNA damage during the starvation.

At this point we wonder whether this initiation events were induced by thymine starvation treatment or they were the ones scheduled by the cell cycle parameters of MG1693 (τ 39 min, C period 68 min and D period 23.6 min). To approach this question the cell cycle of MG1693 was schematic illustrated in figure 59. From here it can be calculated the proportion of cells in different intervals by applying the formula $2^{(1-t1)} - 2^{(1-t2)}$ being t_1 and t_2 different times relative to cell cycle going from 0 (birth) to 1 (division) (Sueoka and Yosikawa 1965, *Ciclon* http://genuex.unex.es/alf/c/ciclon.html)

According to this the number of *ori/cell* is 5.028 in exponential growing cells. Assuming that the percent of cells that initiate are the cells stayed 10 min before the time of initiation relative to its cell cycle, *ti* is 24.8%, then the *ori/cell* after this time will be 6.02. From here it can be estimated that in 10 min the proportion of origins would increase 19.9% (that is the ratio 6.02/5.02). Giving that our experimental value for proportion of origins initiated during 10 min of thymine starvation is 19.4 it is consistent with the notion that initiations occurred under thymine starvation are not induced by the treatment but the scheduled following the cell cycle.

By using this approach it can be also predicted the percent of cells of the two peaks of the flow cytometry profile after 3h addition of Rif+Cfx. Knowing that the peak containing cells with 4chromosomes correspond to the cells between the interval (0-ti) and the second peak of the flow profile, cell with 8 chromosomes, correspond to the percent between the interval (ti-1), it can be estimated that in exponential growth flow cytometry profile would be 74.3% cells with 4 chromosomes and 25.7% with 8 chromosomes that would change to 49.5% and 54.1%, respectively. Our experimental data agree qualitatively as the percent of cells with 8 chromosomes increases and the percent of cells with 4 chromosomes decreases. Nevertheless they partially fix with the quantitative predictions, as the percent of cells with 8 chromosomes increases from 20% to 38.7% and the percent of cells with 4 chromosomes decrease from 80% to 61.2%

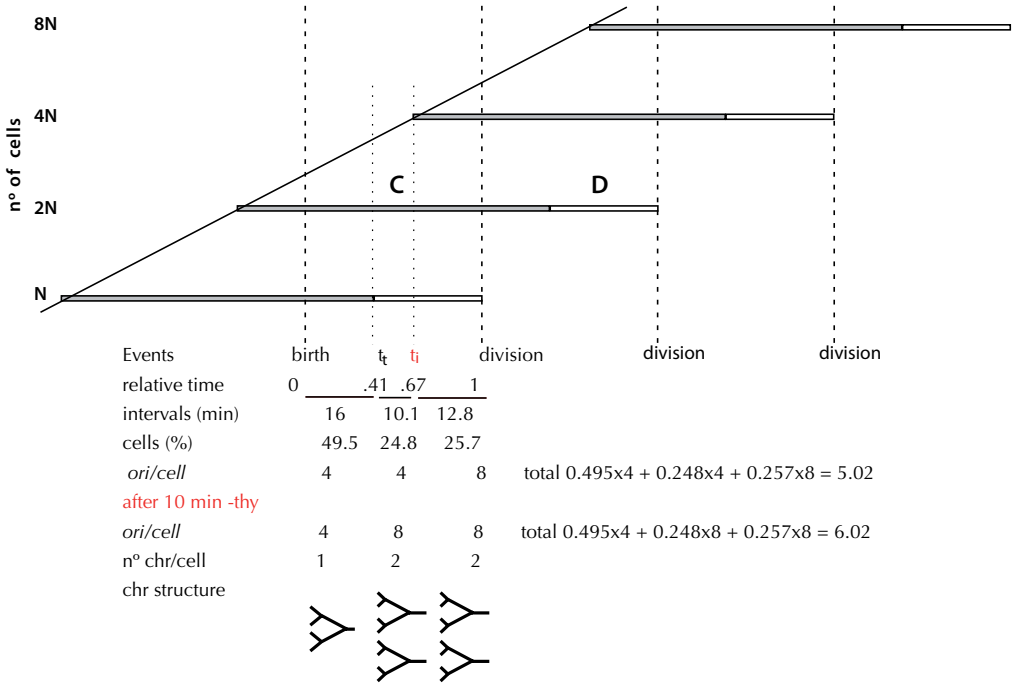

Figure 59. Schematic representation of cell cycle of MG1693. C and D period are showed by solid and empty bars. The time of initiation and termination of replication round are designed as (ti) and (tt) respectively. Percent of cells and ori/cell for the intervals (0-tt), (tt-ti) and (ti-1) in exponential growth and after 10 min of thymine starvation assuming the scheduled initiations are calculated following Sueoka and Yosikawa 1965. Structure of the chromosome is represented by showing half of the chromosome.

(iii) oriC replication intermediates accumulate under thymine starvation. Results showed by using 2D gels showed different DNA structures that figure out what is happening at *oriC* under thymine starvation. A bubble arc was detected after 40 min of thymine starvation, indicating initiations at *oriC,* and double-Y structures were also detected, most probably produced by two forks that encounter each other at *oriC*. These structures indicate that replications under thymine starvation are initiated in *oriC*, although initiations on different location could not be discarded. Actually, a simple-Y arc, corresponding to the accumulation of Y-shaped replication intermediates, was clearly detected after 10 min of thymine starvation, indicating the arrest of replication forks within the *oriC* region. This strongly suggests that there are initiations outside but near the origin entering into *oriC*.

(iv) Genetic approaches confirm the importance of the allowance of initiation under thymine starvation on TLD as the lethality was suppressed if new initiation were inhibited by inactivation of DnaA protein, which is absolutely required for initiation process. Furthermore TLD can be alleviated if the initiation process is not highly efficient, as in the case of *oriC* defective mutants. According to the suppression or alleviation of TLD under initiation inhibition conditions, 2D gels showed none of the DNA intermediaries observed in wild type strain where TLD occurred.

These results show that the targets of TLD are the new replication forks generated at *oriC* if initiation is allowed. This situation would generate lethal DNA intermediates containing DSBs. If initiation is inhibited or impaired, TLD is suppressed or alleviated, respectively. Supporting this notion recent results published by others have shown loss of *ori*-containing foci during early TLD (Fonville *et al*. 2010) and RecA-dependent degradation of the *oriC* region under thymine starvation (Sangurdekar *et al*. 2010, Kuong and Kuzminov 2012).

TLD correlates to the proportion of initiated origins under thymine starvation

By using all the data presented in this work (Table 20) we have deeply study the relationship between TLD and either *n* or *i*. This analysis could support a difference between the relevance of the location of the replication forks either, along the chromosome or at origin regarding TLD.

Taking in account altogether, the data from this work establish a weak correlation (R= 0.284) between the number of replication rounds per chromosome, *n*, and survival under thymine starvation (Fig. 60A). Nevertheless, we have show in chapter 1 there is a good correlation between these parameters (Fig. 13). If this relationship is analyzed by using all the data but eliminating the strains having serious problems to initiate the chromosome; such a defective

oriC mutants, *dnaA46* and *mioCp9* growing in 1% casaminoacids, figure 60B shows a substantial increase in the correlation coefficient (R= 0.734).

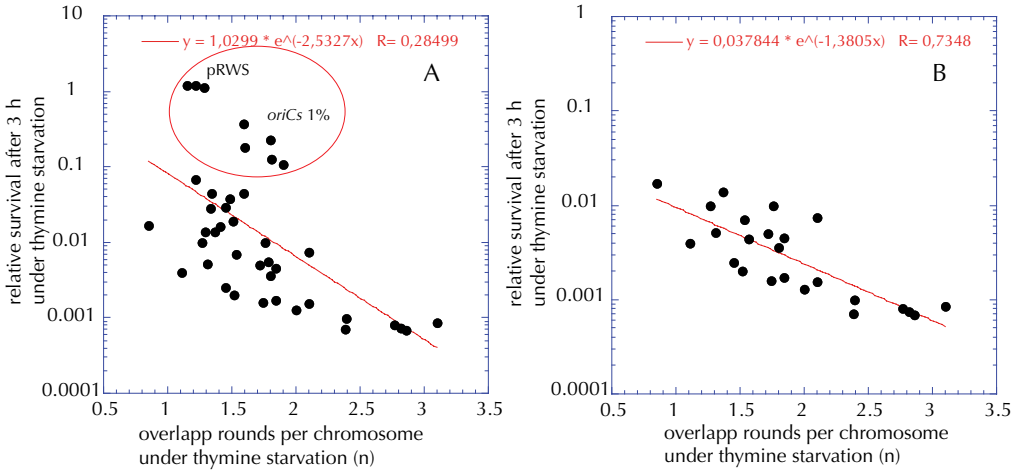

Figure 60. Relationship between survival after 3 h of thymine starvation and the number of replication rounds per chromosome, *n* (A) by using all the data from this work (Table 20) or (B) eliminating the data from defective *oriC* mutants, *dnaA46* and *mioCp9* growing in 1% casaminoacids

By contrast the percentage of initiated origins under thymine starvation, *i*, correlates much better to TLD (R= 0.878) either by taking for the analysis all the data (Fig. 61A) or by eliminating the strains having serious problem to initiate the chromosome (R= 0.749) (Fig. 61B).

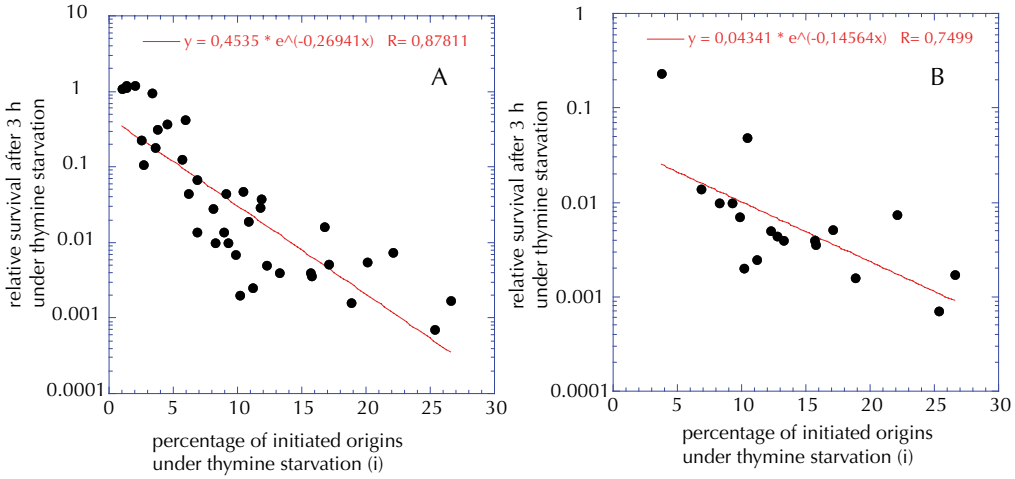

Figure 61. Relationship between survival after 3 h of thymine starvation and the proportion of initiated origins, *i* (A) by using all the data from this work (Table 20) or (B) eliminating the data from defective *oriC* mutants, *dnaA46* and *mioCp9* growing in 1% casaminoacids

These results suggest that TLD is not affected only by the number of replication rounds, *n*, (Fig. 60) but rather by the proportion of initiated origins under thymine starvation, *i* (Fig. 61). The higher the proportion of replication origins initiated during thymine starvation, the lower the survival under the treatment. The point is that for a given value of *n*, it can be initiated different proportion of origins, *i*. It would depend on the genetic backgrounds or growth conditions of the strains. Under conditions where the initiation of chromosome is greatly affected, the proportion of initiated origins under thymine starvation, *i* value, would be less than expected from that *n* value compared with the corresponding *n* value if initiation of chromosome was not limited. This would explain that in the strains having serious problem to initiate the chromosome, such a defective *oriC* mutants, *dnaA46* and *mioCp9* growing in 1% casaminoacids, the magnitude of TLD does not correlate with the value of the overlap of the replication rounds.

Consequently with these results and regarding the three elements occurred under thymine starvation; i. e. DSBs, nmDNA and initiations, we propose that nmDNA and/or DSBs at the origin region, originated by new *thymineless initiation* event would provide the lethal effect observed under thymine starvation. The nmDNA and/or DSBs generated by thymine starvation should be located not only at the origin region, but also at the replication forks and recombination-repair points throughout the chromosome. According to our proposal, those located outside the origin region would not be lethal, explaining that the level of DSBs does not correlate with lethality. To account for the features observed under thymine starvation in the presence or absence of Rif and Hu we propose a general model (Figure 62).

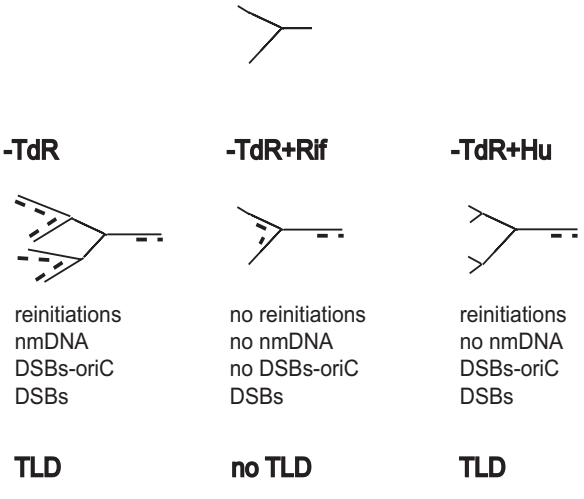

Figure 62. model presenting the various molecular events related to thymine starvation in the presence or absence of Rif or Hu. The molecular events presented here relate the newly induced initiation events and the presence or absence of nmDNA structures or DSBs in the origin region and outside the origin region. The dashed line represents DNA synthesis under thymine starvation at the new replication fork at the origin region, at the forks along the chromosome or as a consequence of recombination-repair processes.

Why *oriC* sequence could be highly sensitive to thymine starvation?

Prokaryotic and eukaryotic chromosomal origin regions share, at least, two features that could be related with their capacity to promote lethality when thymine is absent. First, they possess a region with a higher than average number of adenine and thymine residues (the AT-rich region) where initial destabilization and opening of the double helix takes place at the initiation of replication [Warner and Rockstroh 1980, Lari *et al*. 2006).. The initiation of the replications occurred during thymine starvation would generate a high incorporation of uracil into this sequence [Ahmad *et al*. 1998, Aherne and Brown *et al*.1999, Sangurdekar *et al*. 2010).] that will be removed from DNA by the uracil DNA glycosylase of the BER machinery. This elimination of uracils concentrated at the AT-rich origin regions would eventually generate a large number of irreparable DSBs. A second feature is the binding of several proteins that promote the opening of this sequence to initiate replication [Leonard and Grimwade 2005), that would make reasonable to think this region to be highly accessible to nucleases. We suggest that the occurrence of new initiation events under thymine starvation would promote uracil incorporation that could allow the formation of lethal *oriC-multiforked* DNA structures containing DSBs. A related model has been recently proposed by Kuong and Kuzminov; where *thymineless cells* convert persistent single-strand gaps behind replication forks into double strands using the released thymine for new initiations, whereas subsequent disintegration of small replication bubbles causes replication origin destruction (Kuong and Kuzminov 2012). According to any of these models DSBs created at origin would be concomitant with nmDNA and eventually would result in cellular death under thymine starvation (TLD). By contrast, Hu addition under thymine starvation would allow open access to the origin region but not aberrant progression of the replication forks as all dNTPs (not only thymine) would exhaust at the same rate. This would result in lethal DSBs at the open origin region but not nmDNA, explaining the TLD that occurs in the absence of nmDNA. According to this, the inhibition of new initiation events under thymine starvation in the presence of Rif would prevent DNA initiation and hence the formation of lethal multiforked DNA structures (nmDNA) and DSBs (Figure 62).

Is the transcription-dependent step of initiation the target for Rif suppression of TLD?

TLD suppression by Rif was observed early in the study of TLD (Hanawalt 1963) but its mechanism of action has not yet been elucidated. Given that Rif inhibits RNA polymerase activity throughout the bacterial genome, the suppression of TLD exerted by Rif could be explained either by its effect on the inhibition of RNA synthesis required for chromosomal initiation at *oriC* or by the inhibition of other as-yet-unidentified processes that could be required for TLD in some other way.

This problem has been analyzed by studying the effect of Rif addition under different concentrations and genetic backgrounds; concluding that partial inhibition of the activity of the RNA polymerase in thymine-starved cells modulates ChIC_and TLD. This suggests that transcription process required at initiation would be the mechanism by which Rif suppressed TLD. Although it cannot be excluded that other effects of Rif addition could contribute to TLD suppression, our results support that the effect of Rif addition on RNA synthesis affects the efficiency of initiation process and that inversely correlates with the magnitude of TLD.

By using *rpoB* (Rif^R) we tried first to eliminate any other effect of Rif from that of inhibition of RNA synthesis. If the abortive initiations under thymine starvation were the ultimate cause of the lethality under thymine starvation, then similar TLD would be expected in Rif^R strains in the absence or in the presence of the drug. Surprisingly we found a partial suppression of TLD in the presence of Rif, corresponding to a lower proportion of origins initiated under thymine starvation, *i* value, in the presence of Rif than in the absence. RNA rate was affected by Rif addition, indicating that RNA polymerase was partially sensitive to Rif. The partial sensitivity of Rif did not alter the growth rate, while initiation events were specifically impaired. We did not get our objective, that is separate any other effect of Rif different from inhibition of RNA polymerase, nevertheless we got a condition that specifically related TLD to the DNA initiation without greatly affect the rate of the bacterial growth. The partial activity of the RNA polymerase was then mimicked in wild type strain by adding different Rif concentrations, obtaining a correlation between the concentration of Rif, RNA rate, TLD and proportion of initiated origins under thymine starvation.

For a second approach we look for conditions where TLD was affected by affecting specifically initiation process. We used *ΔdatA* defective strains, which is described to initiate in the presence of 150 μg ml^{-1} of Rif, getting complete inhibition of chromosome initiation by adding Rif 1,000 μg ml^{-1} Our results show the correlation of TLD and the proportion of initiated origins under thymine starvation in the presence of the different Rif concentrations, being the

level of transcription rate similar after addition of either 150 µg ml^{-1} or 1,000 µg ml^{-1} of Rif. It has been suggested in the *ΔdatA* defective more DnaA protein would be available to open *oriC* sequence during the initiation process and consequently less RNA transcription around *oriC* will be required for initiation to occur. Supporting this our results showed higher ChIC under 150 µg ml^{-1} than 1,000 µg ml^{-1} of Rif. According to this, our results indicate that the effect of Rif was on the chromosomal initiation and TLD but not with its effect on the general transcription.

The third genetic approach was to study the effect of thymine starvation on the *mioC* and *gid* genes either defective or overregulated transcription surrounded *oriC* sequence. We showed that under growth conditions where the altered *mioC* and *gid* transcription could limit the initiation process, TLD was alleviated. The relevance of these results relies on the fact that TLD alleviation in these mutants must be related to the alteration of the normal transcription levels around *oriC* (Fig. 36). This effect on TLD, together with the broad reduction of chromosome replication initiation intermediates by these mutants, strongly implicates the transcriptional-dependent step of replication initiation as being critical for TLD, even though there is not a strong quantitative correlation between both parameters. This could be due to the different effect on the transcription activity that has been reported for these mutants. Thus, *mioCp9* is a mutation that constitutively promotes transcription from *mioC* promoter entering to *oriC*; while P*mioC* and P*gid* are defective alleles in the promoter region, i.e. no transcription is supported from these regions around *oriC*. This would imply that even though the proportion of initiated origins under thymine starvation were quite similar in all mutants, constitutive transcription entering into *oriC* that occurs in the *mioCp9* could protect somehow *oriC* region and eventually improve the viability of the thymine-starved cells with respect to P*mioC112* and P*gid113* mutant strains.

The last genetic approach was an example of serendipity. Recently, It was proposed that transcription from plasmids could affect some way transcription from the chromosome and we check this strategy to see the effect on TLD. The results were very clear, the level of transcription from the plasmid has no significant effect on the general transcription from the chromosome by measuring RNA rate; nevertheless the correlation with the survival under thymine starvation was dramatic. The results showed that the presence of actively-transcribed plasmid, suppressed TLD (Fig. 40). We verified this effect was specifically due to the transcription from the plasmid by using pBAD construction carrying the inducible arabinose promoter. The result showed that in the presence of arabinose, when the promoter was induced; TLD was suppressed (Fig. 43). Proved the relationship between transcription from the

plasmid and TLD, we showed that chromosomal initiation under thymine starvation was fully inhibited. It is Important to note that transcription from the plasmid did not impair the initiation process in exponentially growing cells by (Table 19). Being reasonable to expect that transcription from plasmids should alter transcription (and no other different process) from the chromosome, our results suggest that it is the transcription required for chromosomal initiation the process affected. This effect could be explained by recruitment of bacterial RNA polymerase to the plasmid, in such a way that the general transcription level was no altered but the more sensitive transcription process required for initiation to occur under thymine starvation was impaired, consequently suppressing TLD. Other speculating explanations cannot be excluded, one of them could be the transertion, the coupling of transcription, translation and insertion of nascent proteins into the membrane; has been proposed as a global regulator in bacteria and particularly for cell cycle (Fishov and Norris 2012). Nevertheless, much work has to be done to explain how transcription from one replicon could alter the transcription from a different replicon.

About the singularity of thymine starvation

A critical and ancient question about TLD is whether the lethality imposed by the starvation of thymine is thymine-specific. Two approaches have been developed in order to answer this question; nevertheless complexity of participating and contributing events related to them revealed not clear answer to the initial question.

The first approach has been to compare the effect of the starvation for all dNTPs with the starvation of thymine. In theory it is reasonable to think that should have similar effects, as both conditions would stop DNA synthesis. Two ways to achieve starvation of all dNTPs are described. One is the addition Hu; that chemically inactivates RNR, which eventually regulates the biosynthesis of all dNTPs and the second one is the thermal inactivation of RNR by using *nrdA101* mutant, which has been extensively studied in our lab (Guzmán *et al*., 2002, Guzmán *et al*., 2003, Guarino *et al*., 2007a, Guarino *et al*., 2007b, Riola *et al*., 2007, Sánchez Romero *et al*. 2010, 2011)

Effect of Hu is complicated by the secondary effect on RNA synthesis depending on the way and the time it was prepared and the additional observation that new DNA seems to be synthesized for at least 30 min after addition. Inactivation of RNR by using *nrdA101* mutant strains revealed the RNR as a component of the replication hyperstructure, in such a way that thermal inactivation of RNR was related with the processivity of the replisome.

The second approach has been recently tested by Itstko and Schaaper.

These authors has analyzed the effect of the lowering of the cellular dGTP pool claiming the similarity of this condition with thymine starvation; nevertheless neither the lethality nor the effect on the accumulation of DNA nor the effect on the initiation of chromosomal replication under lowering dGTP pool are similar to thymine starvation, although DNA damage and SOS response is observed (Itstko and Schaaper 2014).

At this point we could ask about, 'what' define TLD phenomenon? From the results presented in this work and other recently published we could established there are two main conditions crucial for TLD. One is the allowance of initiation events during thymine starvation and the other is the transitory residual unbalanced-dNTPs pool. Both conditions generate DSBs and DNA - branched structures at origins that become lethal to the cells. These two events define the singularity of thymine starvation as neither starvation for all dNTPs nor starvation of dGTP fulfill these requirements and consequently none of them kill the cells following the molecular mechanism described for TLD.

Overtaking of cell division by stop DNA replication

Thymine starvation is known to inhibit cell division associated to filamentation, as other inhibitors of elongation of DNA replication do. In this work we showed that long thymine starvation period displayed the anticipated, well-known phenomena: division-inhibition associated with cell filamentation, nucleoid dispersion and appearance of anucleated cells.

Additionally, in a very precisely study cell dimensions and nucleoid morphology were determined during the first 20 min under thymine starvation. Surprisingly, significant drops in cell length and area, associated with a smaller rise in diameter and doubling in the proportion of constricted cells were observed during the first 10 min (Fig. 47B and Fig. 49). The short-term effects are consistent with the overtaking of cell division and remodeling of cell dimensions during the first 10 min of thymine starvation being verified by viability experiments and counting cell numbers in Neubauer chamber (Fig. 47D and Fig. 51). Similar qualitative results were obtained by blocking DNA replication with NAL or Hu. The presented results demonstrate that inhibiting DNA replication by various means enhances cell division in a given number of cells before inhibiting cell division after long treatments.

Understanding what would be the features or physiological state of the cells that are induced to divide under thymine starvation we speculated to be cells that had finished the round of replication in the cell cycle, that are the cells in D period (Fig. 59). The proportion of cells being at this interval relative to cell

cycle can be calculated as the proportion of cells between *tt* (time by the chromosome finished replication in the cell cycle) and *1* (end of cell cycle when the cells divide); according to the MG1693 data 50.5% of the cells are in D period (Fig. 59). By using this value, a prediction of the estimated mean cell area of the culture could be done as follow: if 50.5% of the cells are dividing (named as *x*), they consequently would present half of the cell area/length (0.5 relative number) and the rest 49.6% of the cells (named as *1-x*) would display the area/length of untreated culture (1, relative number). According to that, the expected mean area for the cells culture (N) could be calculated as, *mean area/length*=[N(1-x)1+2Nx.0.5]/[N(1-x)+(2Nx)]= 1/(1+x) According to Fig. 47 *mean are/length* =1/(1+x)= 0.65 and from here, x= 0.538 or 53.8% of cells.

Giving that, using the experimental value for the minimum mean area/length (Fig. 47) the proportion of dividing cells corresponds to the proportion of cells in D period, this would suggest that cell staying in D period could fit the physiological state required to go for instant cell division, although some cells would divide even without finished chromosome replication. These calculations support that thymine starvation would enhance division of those cells had terminated replication or near to this moment.

According to the calculations made from cell cycle parameters, the observed instant cell division after replication blockage most probably occurred in cell staying in D period, that is to say, cells that have already finished the round of replication corresponding to that cell cycle, hence they would not require thymine any more. According to all models, initiation of FtsZ-ring formation occurred slightly before replication was completed and concluded at termination, or near termination of replication and by this time FtsZ-ring should be positioned at mid-cell and ready to contract to yield two daughters cells (den Blaauwen *et al*. 1999, Wang *et al* 2005, Inoue *et al.* 2009, Moriya *et al*. 2010, Rodrigues and Harry 2012). According to all of them, FtsZ-ring is established at the termination stage of replication.

How DNA replication blockage could trigger cell division? To explain the occurrence of this instant cell division our proposal relays in FtsK translocase, that has been recently proposed as new bacterial checkpoint for cell division (Grainge 2010). FtsK is widely distributed in bacterial species and is a membrane-bound DNA translocase capable of rapid directional movement on the bacterial chromosome (Bigot *et al*. 2007). In addition to acting as a DNA pump, it has a number of other functions: it is involved in the proper assembly of the divisome; it promotes chromosome unlinking following replication; and it stimulates chromosome dimer resolution. These features work together to ensure timely chromosome segregation and cell division (Dubarry et al., 2010). Further, FtsK has been proposed to regulate the late stages of septation acting as a checkpoint to ensure DNA is fully cleared from the septum before it is allowed

to close, as well as being the driving force to unlink the chromosomes and segregate the DNA away from the septum. Looking at these features, activation of FtsK could accelerate the movement of replicated chromosomes away from the mid-cell and the FtsZ-ring to constrict instantaneously. According to our results, activation of FtsK should be immediate after replication inhibition.

Several mechanisms, SOS dependent or independent, have been proposed to explain the inhibition of cell division after DNA damage or when replication is interrupted. Nevertheless here we have shown that in a given fraction of the cells (those with a finished replication cycle) division is activated. Our results indicate the existence of at least two kinds of mechanisms connecting DNA replication to cell division, depending on the location of the replication forks on the chromosome. One would inhibit cell division if replication forks were all along the chromosome, here all the proposal (SOS dependent or independent) are included explaining the blockage of cell division after inhibition of DNA replication. A second kind of mechanism would accelerate cell division if the replication round corresponding to that cell cycle has already finished; hence this mechanism would be working during the D period. This mechanism would act very quickly and only in a fraction of cells, hence it will be detected only by precise time-lapsed measurements as performed in this work.

Analysis of the evolution of the cell morphology after re-addition of thymine after 10 min of starvation showed a rapid restoration of cell length, area and diameter to the values of the untreated cells. This rapid recovering is in agreement to the results from DNA synthesis where it shown that the initiations occurred under thymine starvation are able to complete that round of replication without problem in the progression of the replication forks (Fig. 25).

Recovering from longer period of thymine starvation showed that cells continue increasing in size for a period of time directly proportional to the time of starvation, suggesting that the mechanism implied in the inhibition of cell division has an accumulative effect most likely related to the accumulation of the inhibitor of division SulA protein.

In this work we have studied the mechanism by which thymine starvation kills the cells, to propose a model introducing a new key element, the initiation of replication into the already complex_scenario of TLD. This model is relevant not only in prokaryotes but also in eukaryotes where chemotherapeutic treatments of cancerous cells have been developed involving the inhibition of thymidylate synthase (TS) Heidelberger *et al*. 1957, Leonard and Grimwade 2005,) However, despite the fact that inhibition of TS has served for many years

as a chemotherapeutic strategy, the mechanism by which thymine depletion leads to death has yet to be fully elucidated. The lethal effects of the anti-metabolite 5-fluorouracil (5FU) and the antifolates methotrexate and raltitrexed have been related to the induction of DSBs in chromosomal DNA Ayusawa *et al*. 1983, Longley *et al*. 1998]. However, even though chromosome breaks are a relevant candidate to explain ~~the~~ lethality, it is unclear whether they play a causal role in TLD. The possibility that TS inhibition increases homologous recombination has been receiving special attention because the resulting instability may contribute to cell death Ayusawa *et al*. 1983, Hori *et al*. 1984, Nakayama *et al*. 1994) but its relationship with TLD in eukaryotes remains controversial. According to our model, DSBs and nmDNA contribute to TLD, but only when they were positioned at the chromosomal origins of replication. In eukaryotes, where there are multiple origins along multiple chromosomes, the firing of these origins (or newly induced origins) under TS inhibition would generate higher number of DSBs and DNA recombination structures that will cause its lethal effect. We believe that understanding the mechanism of action of chemotherapeutic drugs that interfere with thymine metabolism and the process of *thymineless death* could be essential for the development of effective tumor and anticancer treatments.

Conclusions

1. TLD does not require ongoing DNA replication. Replication forks are required but no sufficient for TLD.

2. Survival from thymine starvation inversely correlates with the number of replication rounds and forks per chromosome.

3. Thymine starvation generates DSBs; nevertheless they are not sufficient to cause TLD.

4. Complex DNA intermediates are generated under thymine starvation; moreover they are not required to TLD.

5. Initiation events occurred in thymine-starved cells, but they only progress to replicate the chromosome if thymine is restored.

6. Addition of Rif or Cm or thermal inactivation of DnaA protein inhibited new initiation events under thymine starvation, suppressing TLD.

7. Partial deletions of *oriC* sequence alleviate TLD.

8. Initiations generated during more than 30 min under thymine starvation are not able to complete chromosome replication.

9. We defined ChIC as the *Chromosomal Initiation Capacity* accumulated during the time of treatment. ChIC increases during thymine starvation and is inhibited in the presence of Rif or inactivated DnaA protein.

10. By using 2D gels *oriC* it showed that replication intermediates corresponding to Y-shaped DNA structures accumulate during thymine starvation. This DNA intermediates are not observed under thymine starvation in the presence of Rif or inactivated DnaA protein.

11. The targets of TLD are the new replication forks at *oriC*. They generate DNA intermediates only if initiation of chromosomal replication is allowed.

12. TLD can be modulated by RNA rate.

13. Altering transcriptional activity around *oriC* decreases ChIC alleviating TLD.

14. Active transcription from a external plasmid does not greatly affect bacterial RNA rate, but suppressed TLD.

15. From 12 to 14 it could be concluded that RNA polymerase activity on initiation of chromosome replication does not display the same features than in general transcription process.

16. Thymine starvation induces an instant cell division during the first 10- 20 min of the treatment before TLD occurred.

17. Induction of cell division under thymine starvation is not strain-dependent.

18. Induction of cell division is related to inhibition of elongation of chromosomal replication.

19. Viability of thymine-starved cells up to 6 h is recovered if thymine is reposted. Recovering is not possible for cells treated longer period under thymine starvation.

20. There is a correlation between the time under thymine starvation and the time cells keep on increasing their size after the thymine addition.

21. Overall results it can be concluded that aborted initiations of chromosome replication occurred under thymine starvation are crucial for TLD. These initiations would generate DSBs and replication intermediates at *oriC* region that are lethal for the cell. If initiation events are inhibited or impaired, TLD is suppressed or alleviated, respectively.

Apendix

Assessment and interpretation of bacterial viability under thymine starvation by using the LIVE/DEAD BacLight Kit in combination with flow cytometry and microscopy.

For microbes, the distinction between life and death is problematic. The answer to this question is far from simple and the question remains unanswered after 20 years of intense research and permanent controversy (Barer and Harwood 1999). Life is generally characterized by: (1) the presence of structure; (2) changeable genetic information; (3) metabolism or functional activity, and (4) the ability to reproduce and grow (Nebe-von Caron and Badley 1995). However, living bacteria are generally only characterized by their ability to divide forming colonies as determined by plating.

In 1976, John Postgate stated: "At present one must accept that the death of a microbe can only be discovered retrospectively: a population is exposed to a recovery medium, incubate, and those individuals which do not divide to form progeny (colony) are taken to be dead. There exist at present no short cuts which would permit assessment of the moment of death" (Postgate 1969,1976).

Notwithstanding this, however, many attempts have been made to develop rapid methods different from plating. The development of alternative methods has been also concomitant with advances in fluorescent dye technology offering probes for a variety of cellular functions. Fluorescence-based methods have remained very useful for a wide diversity of applications ranging from industrial to environmental microbiology. These tools are used for viability/activity assessment and the increased use of fluorescent probes is also due to improvements in the quantitative and qualitative sensitivity of instruments.

The commercially available LIVE/DEAD BacLight kit (Invitrogen) is enjoying increased popularity among researchers in various fields (Boulos 1999). The kit consists of two stains, propidium iodide (PI) and SYTO9, which both stain nucleic acids. Green fluorescing SYTO9 is able to enter all cells and is used for assessing total cell counts, whereas red fluorescing PI enters only cells with damaged cytoplasmic membranes.

Although this kit enables differentiation only between bacteria with intact and damaged cytoplasmic membranes, it is often used to differentiate between live and dead cells (Gasol *et al.* 1999, Sachidanandham and Poh 2005). This correspondence is assumed because membrane-compromised bacterial cells can be considered dead (Nebe-von-Caron *et al.* 2000; Berney *et al.* 2006 a,b) while the correspondence between intact and live cells is not necessarily true (Joux and Lebaron 2000). This would indicate that cell membrane is not damaged.

Figure I shows standard resolution for the LIVE/DEAD stain of viable cells of MG1693 wild type growing exponentially in M9 minimal medium (Fig. IA) and for ethanol precipitated aliquot (Fig. IB). It can be seen strong green fluorescence (R1 quadrant) and weak red fluorescence (R2 quadrant), while population of MG1693 cells precipitated in ethanol 100% showed weak green fluorescence and strong red fluorescence as this treatment completely permeabilizes the cell membrane (Berney *et al.* 2006 a, b).

To test whether TLD could be quantified by LIVE/DEAD kit, a culture of MG1693 was grown up to 0.2 OD_{550}, was filtered and the cells resuspended in medium without thymine. Samples were withdrawn at different times, cells stained with LIVE/DEAD kits and analyzed by flow cytometry (see *Methods* section). Figure I shows the flow cytometric analysis for different times under thymine starvation (1, 2, 3, 4, 6 and 24 h).

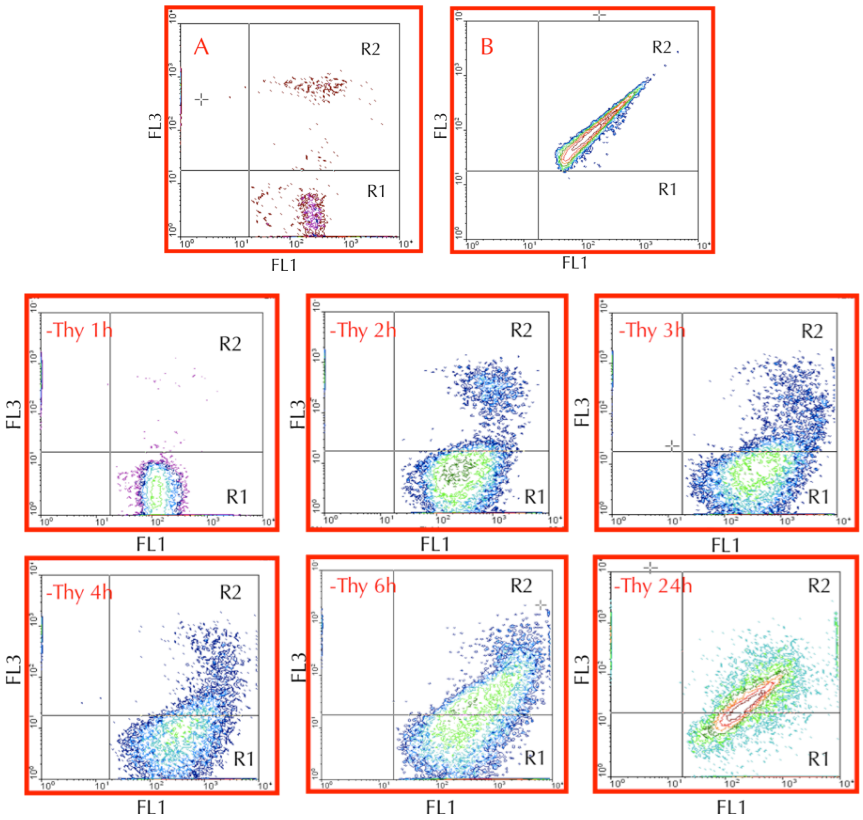

Figure I. Flow cytometric analysis of MG1693 by using LIVE/DEAD kit. Upper panels: (A) Untreated cells (B) ethanol-precipitated cells. Lower panels: Bacterial culture under different times of thymine starvation (1, 2, 3, 4, 6 and 24 h). Bacterial cells samples were stained with a mixture of SYTO9 plus PI and analyzed by flow

The pattern indicates the movement of the bacterial cluster from R1 to R2 quadrant, strongly suggesting that intermediate states of live/dead are occurring. These states are characterized by different intracellular concentrations of SYTO9 and PI. Membrane integrity was lost after thymine starvation, however, in the process of cells becoming permeabilized, the bacterial cluster on the two-dimensional dot plot moved in a distinctive curve-shaped manner from strong green and weak red fluorescence intensity (region 1 [R1]) to weaker green fluorescence intensity (2 (R2)).

Assuming that R1 quadrant corresponds to live cells and R2 quadrant includes dead cell, the relative percentage of live and dead cell in exponentially growing culture and thymine-starved cells was calculated. Figure II

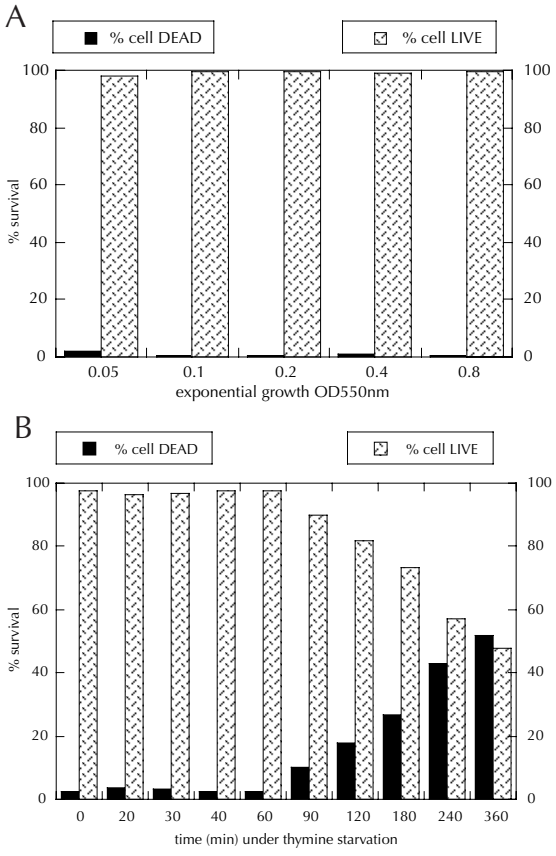

Figure II. Relative percentage relative of live and dead cell by flow cytometry analysis of stained cells with LIVE/DEAD kit. (A) in exponentially growing culture (B) after analysis of thymine starvation.

By comparing the above results from LIVE/DEAD kit analysis of TLD and viability results of TLD as determined by CFU very different quantitative results can be observed (Fig. III).

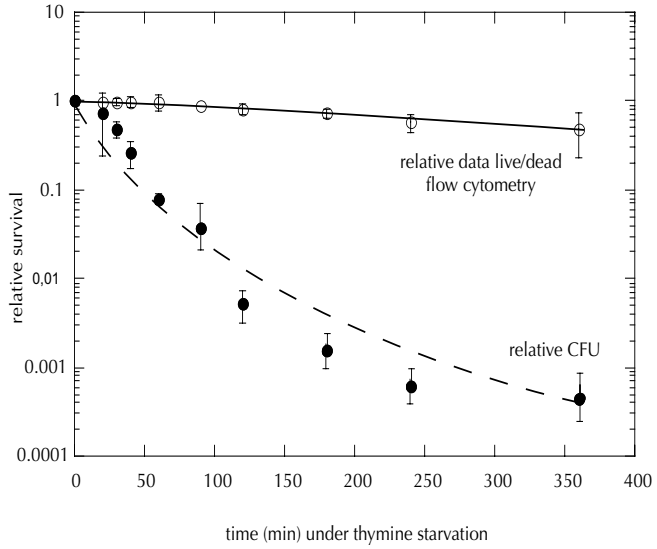

Figure III. Viability analysis under thymine starvation by determining relative CFU (●) or relative viable cells by flow cytometry stainined cells by LIVE/DEAD kit (○).

According to these results, stained cells with LIVE/DEAD kit would not be suitable to quantify the effect of thymine starvation in bacterial cultures as only 0.5 of relative survival can be detected by LIVE/DEAD kit, while by using CFU the relative survival was estimated to be 0.005. These differences could be due to the concept of viability for each approach. Determining CFU measures the availability of the cell to divide eventually forming a colony; while LIVE/DEAD staining depends on the permeability of the membrane. These results would indicate that, although metabolism and physiology of the cells under thymine starvation is completely imbalanced, the integrity of the membrane is not damaged at the same level and hence IP stain is not able to enter into the cells to give red color. The discrepancy between reproductive viability, as measured by colony formation on agar, and direct cytometric counts has been reported by others authors (Dawes 1985, Pickup 1991, Kaprelyants and Kell 1993, Kaprelyants *et al*. 1993, Morita 1997).

LIVE/DEAD stained bacterial cells can be also assessed by fluorescence microscopy that usually simplified to either green-labeled cells (live) or red-labeled cells (dead). To verify the intermediate states of live/dead detected by flow cytometry analysis in MG1693 thymine-starved cells (Fig. I), the same aliquots stained with LIVE/DEAD kit was analyzed by microscopy. The cells were mounted onto a bed of 1% agarose in H_2O for viewing under the microscope. Images were acquired using a Nikon, model Eclipse E600 that has coupled digital camera Hammamatsu, C4742-95-10NR model and the acquisition software NIS-Elements F2.30 by image capture and *Image J* program was used for the analysis of photographs (Fig. IV). Microscopy analysis shows green (live) cells for viable cells of MG1693 wild type growing exponentially in M9 minimal medium and red (dead) cells for ethanol precipitated aliquot (Fig. VI upper panels). By increasing the time under thymine starvation cells experience an evolution of color from green to red. According to flow cytometry analysis, cells go through intermediate states, showing a graduation of yellow stained at 1, 2, 3, 4 and 6 h. These intermediate states have been also observed (Virta *et al*. 1998; Joux and Lebaron 2000; Barbesti *et al*. 2000; Christiansen *et al*. 2003; Hoefel *et al*. 2003; Berney *et al*. 2006 a,b,c). According to the kit manufacturer's manual, the region of intermediate states is referred to as *unknown*. This can lead to difficulties in the interpretation of results and can be critical.

Overall these results we concluded that LIVE/DEAD stained could be used only for qualitative analysis of TLD. Nevertheless it could be interesting to deeply analyze and studying the intermediate state defined by LIVE/DEAD staining. Sorting experiment using single cell (or small population) at this stage of TLD would allow a direct link between the reproductive viability and the staining pattern of the bacteria. Knowing what the fate of these cells on plating, the physiological processes and molecular reactions that were affected in these cells would give us new data to better understand TLD phenomenon.

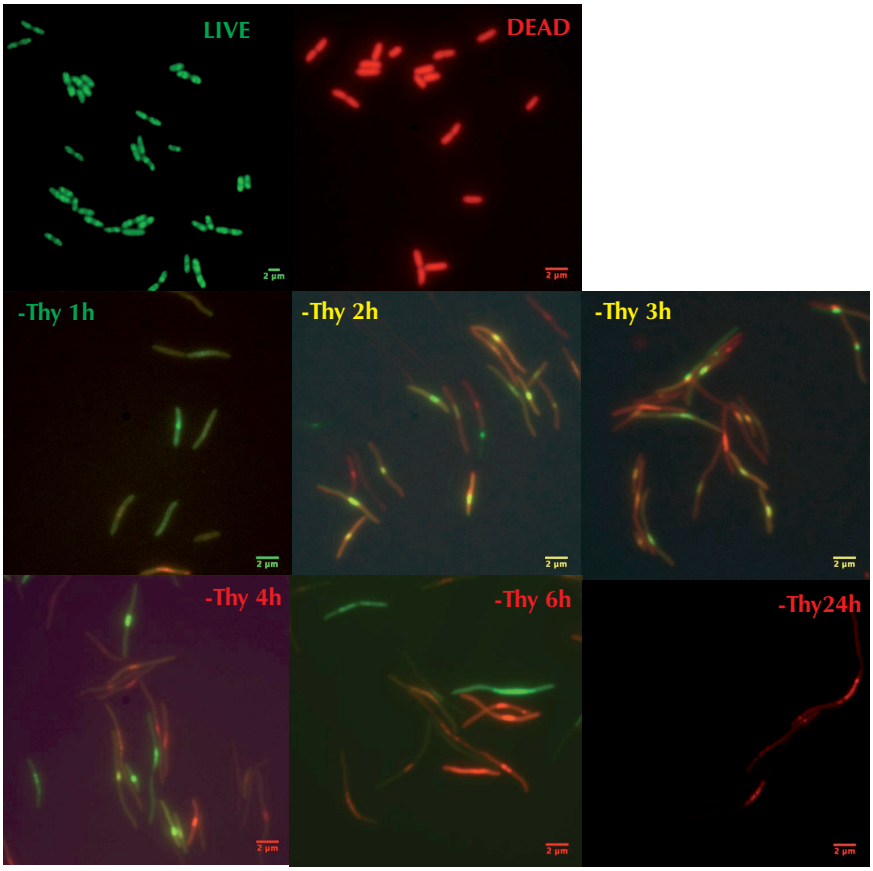

Figure IV. Fluorescence microscopy analysis of MG1693 by using LIVE/DEAD kit. Upper panels: (A) Untreated cells (B) ethanol-precipitated cells. Lower panels: Bacterial culture under different times of thymine starvation (1, 2, 3, 4, 6 and 24 h). Bacterial cells samples were stained with a mixture of SYTO9 plus PI and analyzed by fluorescence microscopy. FL1,520±10nm; FL3,≥630nm.

For microbes, the distinction between life and death is problematic, on both a practical and a philosophical level. While we are safe, at least for now, in the assertion that "The only certainty in life is death," the definitions of the two states remain somewhat nebulous; the route from life to death, and the potential for reversing part of the route, remains uncertain.

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DNA replication initiation as a key element in thymineless death

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ABSTRACT

Thymine deprivation results in the loss of viability in cells from bacteria to eukaryotes. Numerous studies have identified a variety of molecular processes and cellular responses associated with *thymineless death* (TLD). It has been observed that TLD occurs in actively growing cells, and DNA damage and DNA recombination structures have been associated with cells undergoing TLD. We measured the loss of viability in thymine-starved cells differing in the number of overlapping replication cycles (*n*), and we found that the magnitude of TLD correlates with the number of replication forks. By using pulsed field gel electrophoresis (PFGE), we determined the proportion of linear DNA (DSBs) and the amount of DNA remaining in the well after treatment with *XbaI* (nmDNA) under thymine starvation in the absence or presence of both rifampicin (suppressing TLD) and hydroxyurea (maintaining TLD). Our results indicate that DSBs and nmDNA are induced by thymine starvation, but they do not correlate with the lethality observed in the presence of the drugs.

We asked whether TLD was related to chromosomal DNA initiation. DNA labeling experiments and flow cytometric analyses showed that new initiation events were induced under thymine starvation. These new DNA replication initiation events were inhibited in the presence of rifampicin but not in the presence of hydroxyurea, indicating that TLD correlates with the induction of new initiation events in *Escherichia coli*. In support of this finding, cells carrying a deletion of the *datA* site, in which DNA initiation is allowed in the presence of rifampicin, underwent TLD in the presence of rifampicin. We propose that thymineless-induced DNA initiation generates a fraction of DNA damage and/or nmDNA at origins that is critical for TLD. Our model provides new elements to be considered when testing mammalian chemotherapies that are based on the inhibition of thymidylate synthetase.

1. Introduction

"Thymineless death (TLD) is the loss of viability that takes place when growing cells of thymine requiring *Escherichia coli* are transferred to a medium lacking thymine but otherwise sufficient for continued growth." [1] This phenomenon was discovered by Barner and Cohen in the 1950s [2], and the essential mechanism is still being investigated. In the past few years, a number of researchers have become interested in determining the mechanism underlying TLD, and significant results concerning a loss of the *oriC* region in cells undergoing TLD have recently been reported [3,4]. One of the original observations regarding TLD was that RNA and protein syntheses continue under thymine starvation, and both are required for TLD [5–7]. A myriad of molecular and physiological events have been observed under thymine starvation, including the inhibition of cell division, a severe dNTP pool imbalance, the induction of the SOS system, the induction of prophages, increased mutagenesis and recombination frequencies, and DNA damage (reviewed in [8]). The role of the suicide module *mazEF* has been controversial [9–11]. It has been observed that TLD is related to actively replicating DNA [5,6], but the role of DNA replication is not well understood because ongoing chromosomal replication does not appear to be required [10]. These pleiotropic effects have posed major problems for revealing the ultimate cause of this lethality. Several models that have been proposed to explain TLD have considered two primary sources for the lethality that are not mutually exclusive: (i) DNA breaks and (ii) complex DNA structures [1]. (i) Multiple models point to DNA breakage and/or fragmentation as the initial event causing TLD, including: a nuclease induced under thymine starvation [12], futile cycles of DNA damage repair induced by RNA synthesis [13], AP-sites created when uracil incorporation under thymine starvation is excised by glycosylase [14], and the processing of induced DSBs [15]. (ii) Complex DNA structures are generated by attempts to repair gaps, including recombination processes [1,16–18]. By the time this paper was under review, two groups had observed a loss of the *oriC* region in cells undergoing TLD. Rosenberg and colleagues proposed three TLD pathways: death by SOS turning on the SulA inhibitor of cell division, death by recombination, and death involving RecQ helicase and RecJ 5′ exonuclease [3]. Khodursky and colleagues have proposed that DNA damage around

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the *oriC* region is a consequence of the transcription-dependent initiation of replication in thymine-starved cells and may be the underlying cause of TLD [4].

In the present work, we analyzed the role of chromosomal replication in TLD. We found that TLD correlates with the average number of replication cycles (*n*). We found that the mere induction of the two primary sources of lethality (DSBs and/or complex DNA structures) is not sufficient to explain TLD. We confirmed that thymine starvation induces new replication cycles. This induction was inhibited under conditions in which TLD was prevented, verifying that TLD correlates with new initiation events in *E. coli*. These results support a model in which induced initiation events are the main requirement for TLD, in agreement with the proposal by Khodursky and colleagues [4]. We propose that induced chromosomal replication initiation events generate the scaffold wherein DSBs and/or nmDNA become critical for TLD, and that such lesions located at the new *oriC* regions that are generated by thymine starvation are the cornerstones of the lethality.

2. Materials and methods

2.1. Bacterial strains and growth conditions

E. coli K12 MG1693 (F–, λ^-, *thyA715*, *rph-1*, *deo?*) is a spontaneous Thy$^-$ derivative of the MG1655 strain (selected with trimethoprim) obtained from the Genetics Stock Center. We founded this strain to be sensitive to high concentrations (100 μg/ml) of thymidine (TdR). Our guess is that strain MG1693 could be defective in the *deo* operon [19].

The deficient strains, MG1693 *recB258::Tn10* and MG1693 *ΔdatA::kam*, were constructed by the standard P1 transduction method and selection for the appropriate resistance. The donor strains were AB1157 *recB258::Tn10*/pDWS2 (pBR322 *recBCD+ thyA+ argA+*), obtained from B. Michel and W3110 *ΔdatA::kam*, obtained from K. Skarstad.

Bacteria were grown by shaking at 37 °C in M9 minimal medium (MM9) containing M9 salts, 2 μg/ml thiamine, 0.4% glucose, 0.2% casamino acids, 5 μg/ml TdR and 1.5 mM UR. Growth was monitored by assessing the absorbance at 550 nm (OD_{550}). Thymine starvation was achieved by collecting the cells on a Millipore filter (pore size 0.45 μm) and washing and resuspending them in the same MM9 medium without TdR or UR.

2.2. DNA synthesis and determination of the number of replication cycles (n) and the chromosomal replication period (C)

DNA synthesis was determined by growing the cells in MM9 medium containing 1 μCi/ml of [*methyl*-^{3}H]-TdR (20 Ci/mmol) (ICN) and assaying the radioactive acid-insoluble material. The number of replication cycles, *n*, was determined by runout replication experiments after adding 150 μg/ml rifampicin to a mid-log growing culture. From the amount of runout DNA synthesis (ΔG), the value of *n* was obtained from the empirical formula $\Delta G = [2^n n \ln 2/(2^n - 1)] - 1$ [20–22] by using computer software implemented in our lab [23]. The *C* period value in steady state cultures was determined to be $C = n\tau$ [24]. τ is the generation time, defined as the mass doubling time in minutes as measured by OD_{550}.

2.3. Flow cytometry

The DNA content per cell was measured by flow cytometry using a Bryte HS (Bio-Rad) flow cytometer essentially as previously described [25,26]. When the OD550 of each culture growing at 37 °C in M9 minimal medium reached 0.2, a portion of the culture was transferred into another flask, and rifampicin (150 μg/ml) and cephalexin (50 μg/ml) were added to inhibit new rounds of chromosomal replication and cell division, respectively. These treated cultures were grown for an additional 3 h with continuous shaking, after which 400 μl of each culture was added to 7 ml of 74% ethanol. Approximately 1.5 ml of each fixed sample was centrifuged, and the pellets were washed in 1 ml of ice cold staining buffer (10 mM Tris, 10 mM $MgCl_2$, pH 7.4, in sterile dH_2O), and resuspended in 65 μl of staining buffer and 65 μl of staining solution (40 μg/ml ethidium bromide and 200 μg/ml Mithramycin A). The cells were incubated on ice in the dark for at least 30 min and run in the Flow Cytometer Bryte-HS (Bio-Rad) at 390–440 nm.

2.4. Viability studies

Bacteria were grown by shaking at 37 °C in M9 minimal medium (MM9) containing M9 salts, 2 μg/ml thiamine, 0.4% glucose, 0.2% casamino acids, 5 μg/ml TdR and 1.5 mM UR. At various time intervals, 50 μl aliquots were serially diluted and plated in duplicate on Luria–Bertani plates supplemented with 10 μg/ml thymine. Plates were incubated overnight at 37 °C and colony forming units (cfus) were counted the next day.

2.5. Measurement of DSBs by PFGE

DSB measurements were performed as described in the literature [27–29]. Briefly, for chromosome labeling, cells were grown in minimal medium with 0.2% casamino acids in the presence of 5 μCi/ml [*methyl*-^{3}H]-TdR (20 Ci/mmol) (ICN) until the culture reached 0.2 OD_{550}. The cells were collected, washed, and embedded in agarose plugs. Gentle lysis was performed in the plugs before using them for PFGE. The proportion of migrating DNA was determined by cutting each lane into slices and counting the tritium present in the wells and in the gel slices. The linear DNA values were expressed as the percentage (%) of the cpm gel slices in the total gel slices plus the well.

2.6. Measurement of non-migrating DNA (nmDNA) by PFGE

Measurements of nmDNA were performed as described [13] and modified according to the literature [30,31]. Briefly, when the culture reached OD_{550} 0.2, the cells were collected, washed, and embedded in agarose plugs. Gentle lysis and *XbaI* treatment (50 U/100 μl) for 2 h was performed in the plugs before using them for PFGE. The visualization of DNA bands was achieved by ethidium bromide staining. The amount of nmDNA in MG1693 was quantified by densitometry by using the *Imagen J* program. The nmDNA values were expressed as the percentage (%) of arbitrary densitometric units of the gel well in the total the gel line plus the well.

3. Results

3.1. TLD correlates with the number of replication cycles

TLD has been related to DNA replication [5,6], but surprisingly, ongoing replication does not appear to be required for TLD [10]. These two assessments led us to investigate why TLD requires actively growing cells. We first asked whether the number of replication forks could be related to TLD. To establish a relationship between the magnitude of the lethality under thymine starvation and the number of replication forks, both parameters were determined in the strain MG1693 grown under different conditions to achieve different numbers of overlapping replication cycles. In contrast with eukaryotic organisms, in *E. coli* the time required to replicate the single chromosome (the *C* period) can be longer than the generation time. This results in overlapping replication cycles in the same chromosome. The larger the ratio between the length of

Table 1
Cell cycle parameters and viability after 3 h of thymine starvation for the strain MG1693 growing in MM9 medium supplemented with different concentrations of TdR in the presence or absence of 1.5 mM UR.

Growth condition	τ[a]	C[b]	n[c]	3-h TLD[d]
TdR 5 μg/ml	40	123 ± 13	3.10 ± 0.26	$8.5 \times 10^{-4} \pm 2.0 \times 10^{-4}$
TdR 10 μg/ml	40	84 ± 10	2.10 ± 0.31	$1.5 \times 10^{-3} \pm 0.7 \times 10^{-3}$
TdR 2 μg/ml + UR	44	124 ± 11	2.81 ± 0.16	$7.4 \times 10^{-4} \pm 0.1 \times 10^{-4}$
TdR 5 μg/ml + UR	38	73 ± 4	1.93 ± 0.12	$6.4 \times 10^{-3} \pm 1.1 \times 10^{-3}$
TdR 10 μg/ml + UR	39	111 ± 16[e]	2.85 ± 0.36	$6.8 \times 10^{-4} \pm 1.0 \times 10^{-4}$
TdR 5 μg/ml + UR M9 glycerol	98	83 ± 8	0.85 ± 0.08	$1.6 \times 10^{-2} \pm 0.2 \times 10^{-2}$
TdR 5 μg/ml + UR 30 °C	52	80 ± 7	1.51 ± 0.18	$5.6 \times 10^{-2} \pm 1.3 \times 10^{-2}$

The values of C, n and cfu/ml are expressed as the mean ± standard deviation.
[a] Generation time (min).
[b] Period (min).
[c] Number of replication cycles per chromosome.
[d] Relative number of viable cells/ml (cfu/ml) after 3 h of thymine starvation.
[e] Our guess is that the strain MG1693 could be defective in the *deo* operon [19]. This would explain why TdR10 (at 10 μg/ml in the presence of UR) is high enough to inhibit its own synthesis and therefore cause a longer C.

the C period and the generation time (τ), the greater the overlap of consecutive replication cycles [32]. The degree of overlap is quantified by n, which is the ratio between the C period and the generation time, $n = C/\tau$ [24]. To determine the number of overlapping replication cycles, we used the experimental approach that was followed by Pritchard and Zaritsky [20,21]. Briefly, n has been described as a function of ΔG, which is defined as the amount of DNA that is synthesized following the inhibition of new initiations events while ongoing forks are allowed to finish [20]. This condition can be achieved by the addition of rifampicin (150 μg/ml), as this drug inhibits RNA polymerase and transcription, which are required for the initiation of replication. From the experimental value of ΔG, n can be obtained from: $\Delta G = [2^n n \ln 2/(2^n - 1)] - 1$ [20,21]. Thus, the amount of DNA that is synthesized after initiation is inhibited solely by the number of replication forks per chromosome. After rifampicin addition, the greater the number of replication forks running along the chromosome, the greater the accumulation of DNA. Cultures of the strain MG1693 were grown in different media to enforce different numbers of replication cycles. Different n values can be obtained by modifying either C or τ. It is well documented that in *thy*- strains, the replication time (C) can be increased without affecting τ by reducing the thymine or TdR concentration [21], and it has also been observed that C can be reduced by adding UR in the presence of TdR [19,33]. Strain MG1693 was grown in MM9 medium supplemented with 2, 5 or 10 μg/ml TdR in the presence or absence of 1.5 mM UR and 1 μCi/ml [methyl-^{3}H]-TdR to label DNA. To get n to vary in accordance with τ, the cultures were grown in glycerol as the only carbon source, or they were incubated at 30 °C instead of 37 °C. At mid-logarithmic phase (up to OD_{550} 0.2), an aliquot of each culture was treated with rifampicin and the values of ΔG and n were calculated (Table 1). The presence of fully replicated chromosomes was verified by flow cytometric analysis of a second aliquot of the cultures after 3 h of rifampicin and cephalexin treatment (see Section 2) (Fig. S1 supplementary material).

A third aliquot of the cultures was starved for thymine for 3 h (see Section 2), and viable cells were estimated by plating on rich medium. The values are expressed relative to values obtained before treatment (Table 1). We found a correlation between the number of replication cycles and the magnitude of TLD, suggesting that the number of replication forks or multiforked DNA is correlated with TLD (Fig. 1).

3.2. DSBs induced by thymine starvation do not correlate with TLD

DNA damage is associated with TLD [13–16], although the primary source of this damage is not well established. Stalled replication forks generated by the absence of TTP could lead to DNA damage, as stalled forks are known to promote DSBs [15,34]. To determine whether DNA damage correlates with TLD, we quantified the amount of linear DNA and the viability of the thymine-starved cells under different conditions. It has been reported that TLD is prevented by the addition of cloramphenicol at 200 μg/ml or rifampicin at 150 μg/ml; TLD is not affected by the addition of hydroxyurea at 75 mM [7,10]. We verified these previous observations in our genetic background MG1693 (Fig. 2A).

The levels of DSBs were quantified by the amount of linear DNA as determined by using PFGE combined with cell lysis in agarose plugs [27]. To determine the amount of broken DNA produced, it is necessary to prevent the degradation of linear DNA and the repair of DSBs. This was achieved by using a *recB*-deficient MG1693 strain in which DSBs generated by either treatment are not repaired, allowing them to be evaluated [15,27–29]. Cell viability and the level of DSBs in MG1693 *recB* were quantified from exponential cultures and after 2 h of thymine starvation in the presence or absence of rifampicin or hydroxyurea (Fig. 2B and Table 2).

The results showed that the number of DSBs increased under thymine starvation in the *recB* mutant, but the level was independent of the lethality caused by either treatment, even though they were not repaired. These results indicate that mere DNA damage is

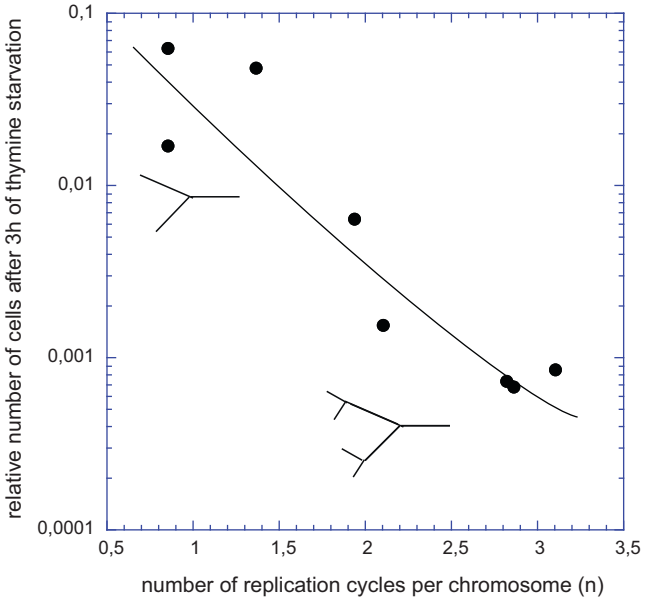

Fig. 1. Relationship between the number of replication cycles per chromosome (n) and the magnitude of TLD after 3 h of thymine starvation. A schematic of the replication forks is presented.

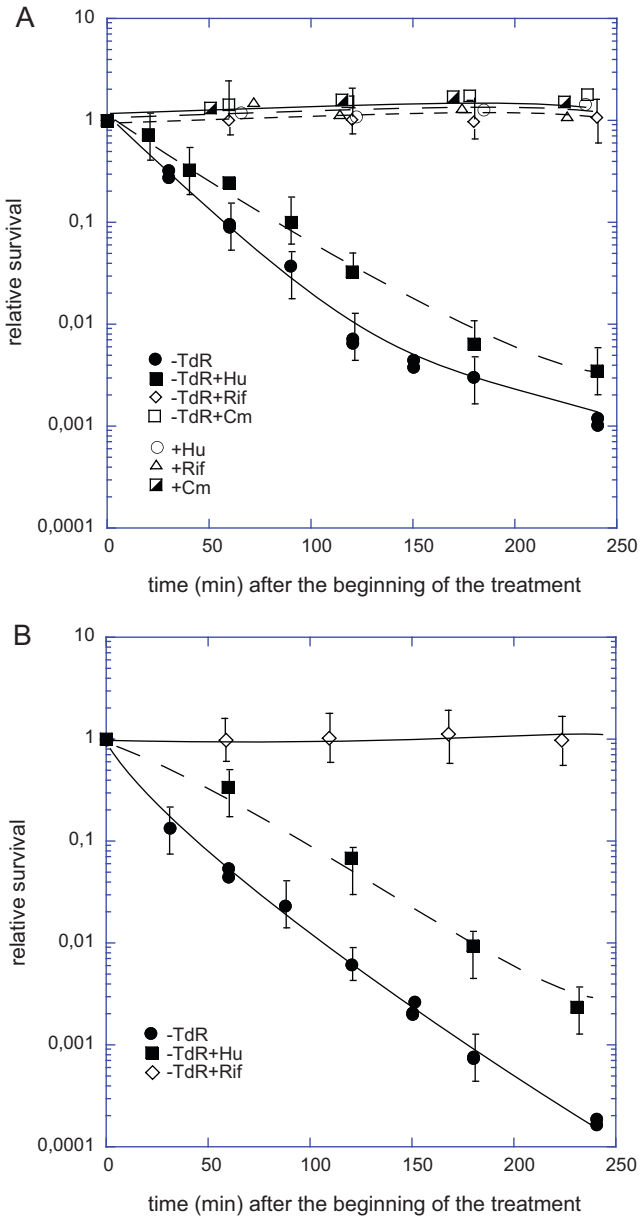

Fig. 2. (A) Viability of exponentially growing MG1693 cultures after addition of hydroxyurea (○), rifampicin (△) or cloramphenicol (◪). Viability of thymine-starved MG1693 cultures with no treatment (●), thymine-starved in the presence of rifampicin (◊), in the presence of cloramphenicol (□) or in the presence of hydroxyurea (■). (B) Viability of exponentially growing MG1693 *recB258::Tn10* cultures incubated under thymine starvation (●), incubated under thymine starvation in the presence of rifampicin (◊) or in the presence of hydroxyurea (■). The values are expressed as the mean ± standard deviation of at least three independent experiments.

Table 2
Percentage of linear DNA in MG1693 *recB* cells after 2 h of thymine starvation in the presence or absence of rifampicin or hydroxyurea.

Treatment	% linear DNA[a]	Treatment effect[b]	Lethality
Exponential	36.1 ± 9	1	No
−TdR	60.9 ± 11	1.68	Yes
−TdR + 75 mM hydroxyurea	57.7 ± 8	1.59	Yes
−TdR + 150 μg/ml rifampicin	66.2 ± 10	1.83	No

[a] The values are expressed as means ± standard deviation.
[b] The percentage of linear DNA relative to the exponential culture.

Table 3
Percentage of nmDNA in MG1693 cells after 2 h of thymine starvation in the presence or absence of hydroxyurea, rifampicin or chloramphenicol.

Treatment	% nmDNA[a]	Treatment effect[b]	Lethality
Exponential	8.6 ± 3.6	1	No
−TdR	21.4 ± 4.7	2.48	Yes
−TdR + 75 mM hydroxyurea	11.2 ± 3.8	1.30	Yes
−TdR + 150 μg/ml rifampicin	9.0 ± 3.9	1.04	No
−TdR + 200 μg/ml chloramphenicol	9.5 ± 4.4	1.10	No

[a] The percentage of nmDNA is expressed as the mean ± standard deviation.
[b] The percentage of nmDNA relative to the exponential culture.

not sufficient to cause TLD, and they suggest that some features of DNA damage could be different when generated under the different treatments.

3.3. The nmDNA induced by thymine starvation is not necessary for TLD

Some proposals postulated the formation of branched DNA as the source of the toxic effect of thymine starvation [1]. Nakayama et al. demonstrated the presence of complex DNA structures by digesting DNA from cultures under thymine starvation with the restriction enzyme *XbaI* and separating it by PFGE [16]. They called these structures "non-migrating DNA" (nmDNA), defined as the DNA that is unable to enter the gel and gets stuck in the well. The nmDNA was characterized as having single-stranded tails or gaps and branching with single-stranded arms. It is important to notice that when using enzymes that yield smaller fragments, non-starved and thymine-starved cells have the same pattern characteristic of the enzyme, with little DNA remaining in the well [16].

To study whether the formation of nmDNA correlates with TLD, we assayed nmDNA after thymine starvation in the presence or absence of rifampicin, cloramphenicol, or hydroxyurea. Mid-exponentially growing cultures of MG1693 were starved for thymine in the presence or absence of these drugs. Two hours after the treatment, cells were collected, washed, embedded in agarose plugs, gently lysed and treated with *XbaI* before being used for PFGE. The amount of nmDNA was quantified by densitometry of the PFGE (Table 3 and Fig. S2). By using this experimental approach, we show in Table 3 that nmDNA was generated under thymine starvation, and it was absent in the presence of rifampicin or chloramphenicol, as previously reported [16]. This might suggest that TLD correlates with the generation of nmDNA. However, we found no nmDNA under thymine starvation in the presence of hydroxyurea, indicating that the generation of nmDNA is not a requirement for TLD.

3.4. New initiation events correlate with TLD

Because TLD was related to the number of the replication forks, but cannot be explained solely by the induction of DSBs and/or nmDNA, we speculated that the putative re-initiations induced by thymine starvation [35] could generate the lesion-causing TLD. This relationship is supported by the recent results showing a loss of DNA from the *ori* region after 2–3 h under thymine starvation [3,4]. This conjecture required the verification of two questions. The first one was whether thymine starvation induces new replication cycles (i.e., initiation events). The second one was whether the induced replication initiations occur in the presence of rifampicin and/or hydroxyurea. To approach these points, we determined the value of ΔG after the addition of rifampicin in the presence of thymine and in cells that are thymine starved for 10 min in the presence or absence of rifampicin or hydroxyurea.

Exponentially growing cultures of MG1693 were starved for thymine in the presence or absence of rifampicin or hydroxyurea

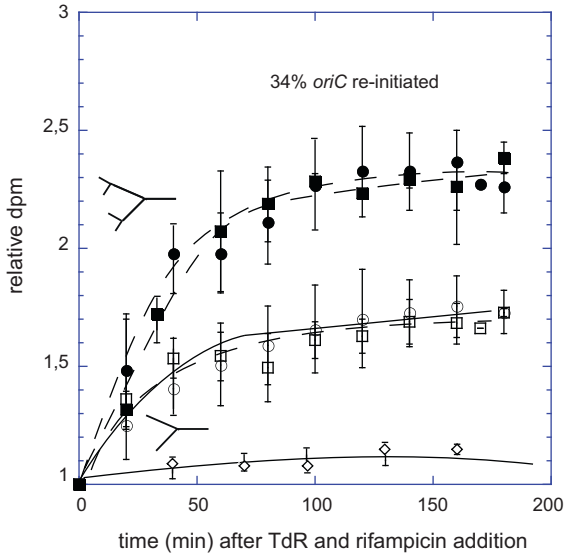

Fig. 3. DNA synthesis in exponentially growing MG1693 cultures after addition of hydroxyurea (◊), run-out DNA synthesis after addition of rifampicin (□), and run-out DNA synthesis after addition of TdR and rifampicin (zero timepoint) in cultures incubated under thymine starvation for 10 min (●), incubated under thymine starvation in the presence of rifampicin for 10 min (○), and incubated under thymine starvation in the presence of hydroxyurea for 10 min (■). A schematic of the replication initiation events is presented.

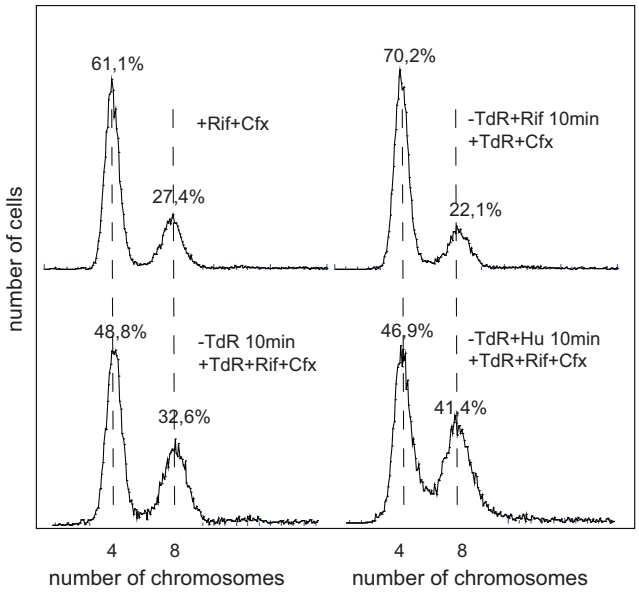

Fig. 4. DNA content per cell measured by flow cytometry after 3 h of incubation in the presence of rifampicin and cephalexin in the MG1693 strain incubated under thymine starvation for 10 min in the presence or absence of rifampicin or hydroxyurea.

for 10 min. After the treatments, aliquots were washed by filtering, and the cells were incubated in the presence of TdR and rifampicin to allow for the completion of DNA replication rounds that were already initiated, while inhibiting the initiation of new events. The results showed that, after 10 min of thymine starvation, ΔG was higher without any treatment, verifying that there is induction of new replication cycles (i.e., initiation events) by thymine starvation (Fig. 3). The fraction of the origins existing before the thymine starvation that were re-initiated was estimated to be 34% [23].

Regarding the effect of the antibiotics on the induction of re-initiation under thymine starvation, we found that the number of induced origins after 10 min of thymine starvation in the presence of hydroxyurea was the same as that observed under thymine starvation. By contrast, no new initiations were induced after 10 min of thymine starvation in the presence of rifampicin (Fig. 3). These results indicate that the presence of rifampicin inhibits the induction of new replication cycles, but hydroxyurea allowed new initiations events to occur in the absence of thymine.

In order to determine whether the induced DNA initiations were able to complete the replication of the whole chromosome, we performed flow cytometric analysis of the cells after the treatments. Fig. 4 shows the DNA histogram showing discrete peaks corresponding to four and eight chromosomes after the runout that followed the 10 min of thymine starvation in the absence or presence of rifampicin or hydroxyurea. These results indicate that chromosomal DNA initiations induced by thymine starvation in the presence or absence of hydroxyurea are able to complete the replication of the whole chromosome. It is interesting to note the relative increase in the number of cells with eight chromosomes, suggesting the re-initiation and full replication of a number of chromosomes containing four origins. These results explain the ancient observation that TLD is suppressed in the presence of rifampicin or chloramphenicol [7], as the induction of new replication cycles under thymine starvation is inhibited by the addition of these drugs. Furthermore, these results account for the observation that the presence of hydroxyurea does not alter the magnitude of TLD [10], as hydroxyurea allows the new initiation events induced by thymine starvation.

3.5. Strains carrying deletion of the datA region undergo TLD in the presence of rifampicin

It has been reported that mutant strains carrying a deletion of the *datA* region allow a proportion of DNA chromosomal initiation in the presence of the standard concentration of rifampicin (150 μg/ml). Nevertheless, in the presence of higher concentration of rifampicin (1200 μg/ml), new initiation events can be inhibited [36]. This phenotype gave us a means of verifying a direct relationship between the suppression of TLD and the inhibition of chromosomal DNA initiation. We first analyzed the viability of *ΔdatA* mutant cells under thymine starvation in the presence of rifampicin at 150 μg/ml, 300 μg/ml and 1200 μg/ml (Fig. 5A). The results showed that the *ΔdatA* mutant loses viability under thymine starvation in the presence of 150 μg/ml rifampicin and that the lethality is gradually prevented in the presence of higher rifampicin concentrations. This was an interesting result, as it showed the first genetic condition in which TLD is observed in the presence of the standard concentration of rifampicin. Given that with increasing rifampicin concentrations DNA initiation would be inhibited, this result suggests a correlation between the suppression of TLD and the inhibition of DNA initiation. Moreover, we verified the relationship between TLD and the induction of DNA initiation via labeling experiments. This approach was performed as above but using rifampicin at 1200 μg/ml after the pretreatments in order to inhibit chromosome initiation [36]. Cultures of exponentially growing MG1693 *datA* were starved for thymine in the presence or absence of rifampicin at 150 μg/ml or 1200 μg/ml. Ten minutes after these treatments, TdR was added and cells were incubated in the presence rifampicin at 1200 μg/ml. Fig. 5B shows that in *ΔdatA* strains, new replication cycles (i.e., initiation events) were induced not only by thymine starvation but also under thymine starvation in the presence of rifampicin at 150 μg/ml. Addition of rifampicin at the higher concentration inhibited the induction of the new replication cycles under thymine starvation, as was observed in the wild-type strain with the standard concentration of rifampicin (Fig. 3). These results show that the inhibition of new rounds of replication correlates with the prevention of the loss of

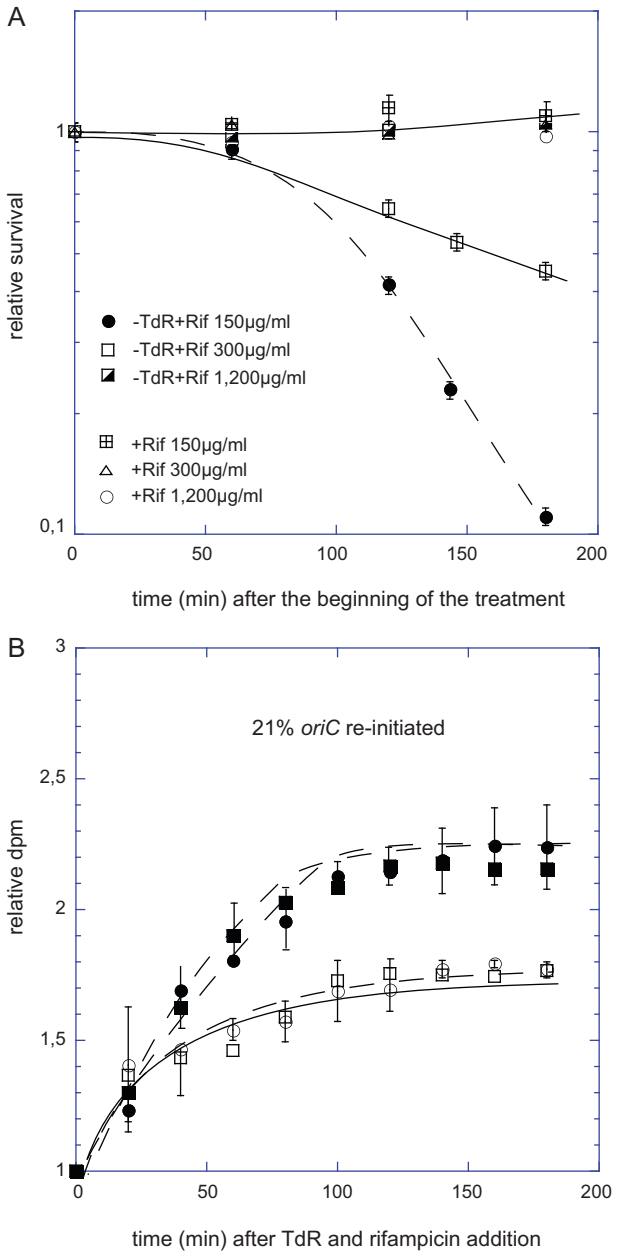

Fig. 5. (A) Viability of exponentially growing MG1693 *ΔdatA* cultures after addition of rifampicin at 150 μg/ml, 300 μg/ml or 1200 μg/ml (⊞, △, □) and incubated under thymine starvation in the presence of rifampicin at 150 μg/ml, 300 μg/ml or 1200 μg/ml (●, □, ◪). (B) DNA synthesis in exponentially growing MG1693 *ΔdatA* cultures after runout DNA synthesis after addition of rifampicin at 1200 μg/ml (□), and runout DNA synthesis after the addition of TdR and rifampicin (zero timepoint) in cultures incubated under thymine starvation for 10 min (●), incubated under thymine starvation in the presence of 1200 μg/ml rifampicin for 10 min (○), and incubated under thymine starvation in the presence of rifampicin 150 μg/ml for 10 min (■). The values are expressed as the mean ± standard deviation of at least three independent experiments.

viability under thymine starvation, indicating that the initiation of chromosomal replication is an essential condition for TLD.

4. Discussion

For many years, TLD has been associated with dividing cells in prokaryotes and eukaryotes. Morganroth and Hanawalt [10] showed that TLD was not associated with the chromosomal replication process *per se*. In the present work, we show that, even though ongoing replication is not required for TLD, the number of overlapped replication cycles (and hence the number replication forks) correlates with the extent of TLD (Fig. 1). Our results show that, even though DSBs and nmDNA could be associated with TLD, the levels of DSBs and nmDNA do not correlate with the lethality observed under thymine starvation in the presence of rifampicin, cloramphenicol or hydroxyurea. We found a precise correlation between the induction of new replication cycles under thymine starvation and TLD.

The consensus of several models of the molecular mechanism of TLD posits that DNA damage and/or DNA recombination intermediates are at the root of TLD. The results presented in this work show that DNA damage *per se* is not the cause of TLD because DSBs were generated in thymine-starved cells in the presence of rifampicin, which prevented TLD. This indicates that lethality is not caused merely by DSBs at existing replication forks along the chromosome, and hence that DSBs may be necessary but are not sufficient to cause TLD. In agreement with Nakayama et al. [16], we found that complex DNA structures (nmDNA) are induced by thymine starvation. They proposed that nmDNA is associated with TLD, but we did not observe nmDNA under thymine starvation when hydroxyurea was present. This indicates that nmDNA could be associated with TLD but is not necessary for TLD.

We have established a causative link between DNA initiation induced under thymine starvation and TLD. Three lines of evidence are presented: (i) the amount of runout chromosomal replication after the addition of thymine in cultures starved for thymine for 10 min in the presence or absence of rifampicin or hydroxyurea correlates with lethality. (ii) Flow cytometric analysis of the runout DNA replication induced by thymine starvation yielded completed chromosomes, as discrete peaks were observed after the replication runout of cultures thymine starved for 10 min. This would suggest that the induced replication rounds most likely are initiated at *oriC*, as neither aberrant chromosome numbers nor broad peak shapes were observed. The proportion of cells containing four and eight chromosomes after the thymine starvation treatments suggests that the re-initiated rounds of replication occurred in a number of cells containing four origins. This would explain the reduction in the proportion of cells with four chromosomes and the concomitant increase in the number of cells containing eight chromosomes. (iii) In *ΔdatA* strains, rifampicin is less effective at inhibiting DNA initiation than in wild type, and it requires higher concentrations of rifampicin [36]. We found that in that mutant strain, TLD is suppressed only by the highest rifampicin concentration.

Consequently, based on the results regarding the three elements induced by thymine starvation; i.e., DSBs, nmDNA and re-initiations, we propose that nmDNA and/or DSBs positioned at the origin region provide the lethal effect observed under thymine starvation. Supporting this notion, Fonville et al. have observed loss of *ori*-containing foci during early TLD [3] and Sangurdekar et al. [4] have precisely shown a loss of the genetic material around replication origins under thymine starvation [4], linking TLD to the initiation of replication. The nmDNA and/or DSBs generated by thymine starvation should be located not only at the origin region, but also at the replication forks and recombination-repair points throughout the chromosome. According to our proposal, those located outside of the origin region would not be lethal, explaining why the level of DSBs does not correlate with lethality. To account for the features observed under thymine starvation in the presence and absence of rifampicin and hydroxyurea, we propose a general model (Fig. 6).

Prokaryotic and eukaryotic chromosomal origin regions have at least two features that are consistent with their capacity to promote lethality when thymine is absent. The first is that they possess a region with a higher than average number of adenine and thymine residues (the AT-rich region) where initial destabiliza-

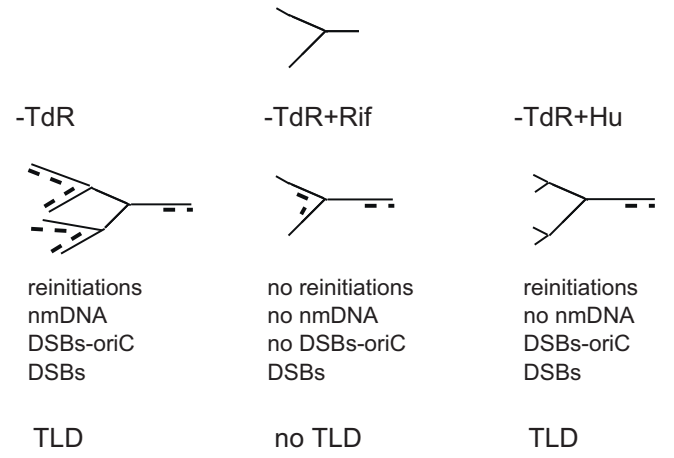

Fig. 6. A model presenting the various molecular events related to thymine starvation in the presence or absence of rifampicin or hydroxyurea. The molecular events presented here relate the newly induced initiation events and the presence or absence of nmDNA structures or DSBs in the origin region and outside the origin region. The dashed line represents DNA synthesis under thymine starvation at the new replication fork at the origin region, at the forks along the chromosome or as a consequence of recombination-repair processes.

tion and opening of the double helix takes place at the initiation of replication [37]. Chromosomal replication initiation that is induced by thymine starvation would result in the incorporation of uracil into this sequence [38,39]. Genomic uracil is removed from DNA by the uracil DNA glycosylase of the BER machinery, and the reintroduction of uracil at the strand break intermediates created by this system will eventually generate a large number of irreparable DSBs and multiforked and gap-containing DNA structures (nmDNA). The second feature of the origin region is the binding of several proteins that promote the opening of this sequence to initiate chromosomal replication [40], and it would be reasonable to think that this region is highly accessible to nucleases. We suggest that the induction of new initiation events under thymine starvation promotes uracil incorporation, which can lead to the formation of lethal multiforked DNA structures. Furthermore, under thymine starvation, DSBs created at origins would be concomitant with nmDNA and eventually would result in cellular death. By contrast, hydroxyurea addition under thymine starvation allows for open access to the origin region but blocks the progression of the replication forks. This would result in lethal DSBs at the origin region but not nmDNA, explaining the TLD that occurs in the absence of nmDNA. Accordingly, the inhibition of the induction of new initiation events under thymine starvation in the presence of rifampicin prevents re-initiation and hence the formation of lethal multiforked DNA structures (nmDNA) and/or DSBs (Fig. 6).

This model is relevant not only in prokaryotes, but also in eukaryotes, in which chemotherapeutic treatments involving the inhibition of thymidylate synthase (TS) in cancerous cells have been developed [41,42]. However, despite the fact that the inhibition of TS has served for many years as a chemotherapeutic strategy, the mechanism by which thymine depletion leads to death has yet to be fully elucidated. The lethal effects of the antimetabolite 5-fluorouracil (5FU) and the antifolates methotrexate and raltitrexed have been related to the induction of DSBs in chromosomal DNA [43,44]. However, even though chromosome breaks are relevant candidates to explain the lethality, it is unclear whether they play a causal role in TLD. The possibility that TS inhibition increases homologous recombination has been receiving special attention because the resulting instability may contribute to cell death [14,44,45]. However, its relationship to TLD in eukaryotes remains controversial [46]. In contrast, Fonville et al. have shown that RecA and the SOS response are required for a substantial fraction of TLD in *E. coli* [3]. According to our model, DSBs and nmDNA contribute to TLD only when they are positioned at a chromosomal origin of replication. In eukaryotes, in which there are multiple origins along multiple chromosomes, the firing of these origins (or newly induced origins under TS inhibition) generates DSBs and DNA recombination structures that cause the lethal effect of the TS inhibitors. We believe that understanding the mechanism of action of chemotherapeutic drugs that interfere with thymine metabolism and the process of thymineless death can be a key to the development of effective tumor treatments.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.dnarep.2010.10.005.

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Rifampicin suppresses *thymineless death* by blocking the transcription-dependent step of chromosome initiation

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ABSTRACT

Thymineless death (TLD), a phenomenon in which thymine auxotrophy becomes lethal when cells are starved of thymine, can be prevented by the presence of rifampicin, an RNA polymerase inhibitor. Several lines of evidence link TLD to chromosome initiation events. This suggests that rifampicin-mediated TLD suppression could be due to the inhibition of RNA synthesis required for DNA chromosomal initiation at *oriC*, although other mechanisms cannot be discarded. In this work, we show that the addition of different rifampicin concentrations to thymine-starved cells modulates TLD and chromosomal initiation capacity (ChIC). Time-lapse experiments find increasing levels of ChIC during thymine starvation correlated with the accumulation of simple-Y, double-Y and bubble arc replication intermediates at the *oriC* region as visualized by two-dimensional DNA agarose gel electrophoresis. None of these structures were observed following rifampicin addition or under genetic-physiological conditions that suppress TLD, indicating that abortive chromosome replication initiations under thymine starvation are crucial for this lethality. Significantly, the introduction of *mioC* and *gid* mutations which alter transcription levels around *oriC*, reduces ChIC and alleviates TLD. These results show that the impairment of transcription-dependent initiation caused by rifampicin addition, is responsible for TLD suppression. Our findings here may provide new avenues for the development of improved antibacterial treatments and chemotherapies based on thymine starvation-induced cell death.

1. Introduction

Thymineless death (TLD) is a phenomenon whereby cells rapidly lose viability when starved of thymine. It was first observed when thymine auxotroph *E. coli*, i.e., *thyA* mutants, were transferred to a medium lacking thymine but otherwise sufficient for growth [1]. It was subsequently shown to be a widespread phenomenon, occurring in bacteria, yeast and human cells [2]. TLD underlies the mode of action of several common chemotherapeutic drugs including anticancer agents (5-fluorouracil, raltitrexed, methotrexate) and the antibiotic trimethoprim, although the mechanism of action of these drugs is still unclear as they are bacteriostatic [3–5]. A myriad of molecular and physiological events have been observed under thymine starvation, including the inhibition of cell division, severe imbalance of dNTP pools, induction of the SOS system, prophage induction, increased mutagenesis and recombination frequency, and DNA damage, among other effects (reviewed in [2]). Different mechanisms linking DNA breakage and fragmentation to TLD have been proposed including: DNA breaks and complex DNA structures, either alone or in combination [6]. Different causes of DNA breakage and fragmentation as an initial trigger of TLD have been proposed including induction of a nuclease [7], the futile DNA damage repair cycles induced by RNA synthesis [8], the AP-sites created when uracil incorporated under thymine starvation is excised by glycosylases [9], or the processing of double-strand breaks (DSBs) induced by the lack of thymine [10]. On the other hand, complex DNA structures generated by attempts to repair gaps formed during thymine starvation, including recombination processes, have been described [6,11]. Efforts to uncover the molecular mechanisms underlying TLD have recently yielded evidence linking the initiation of chromosome replication and the loss of the *oriC* region of cells undergoing TLD [12–15]. This origin-specific degradation requires RecF, RecJ, RecO [13], RecA and RecBCD [15] suggesting a role for recombination in TLD. Additionally, different mechanisms depending on SOS and recombination functions at replication forks have been proposed to explain the TLD process [12,16,17].

TLD suppression by rifampicin was observed early in the study of TLD [18], but its mechanism of action has not yet been

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elucidated. Initially, it was proposed that the DNA repair system might be unable to repair the large number of transcription-associated strand breaks generated by thymine starvation [8]. However, it has been shown that rifampicin addition does not suppress DSB formation associated with TLD [14]. Hence, the mechanism underlying the suppression of TLD by the presence of rifampicin has remained elusive. The replication initiation events associated with TLD require transcription. Given that rifampicin inhibits RNA polymerase activity throughout the bacterial genome the suppression of TLD exerted by rifampicin could be explained either by its effect on the transcription process in general, inhibition of RNA synthesis required for DNA chromosomal initiation at *oriC* or inhibition of other as-yet-unidentified gene(s) that are required for TLD in some other way.

Here, we analyze the effect of rifampicin on TLD in detail with the aim of identifying the basis of its action. We present evidence indicating that the target of this suppression is the rifampicin-sensitive step of chromosome initiation. Time-lapse experiments following the effects of thymine-starvation found increasing levels of ChIC correlated with an accumulation of simple-Y and bubble arc replication intermediates around *oriC* under thymine starvation, but only if initiation events were allowed. None of these structures appeared under conditions that inhibit replication initiation such as the presence of rifampicin or in DnaA-inactivated conditional mutants. Moreover, we demonstrate that TLD can be alleviated under conditions in which new initiation events are impaired by *cis*-acting elements that abolish or increase the transcription of genes surrounding *oriC* (described in [19,20]).

2. Materials and methods

2.1. Bacterial strains and growth conditions

E. coli K12 MG1693 (F-, λ⁻, *thyA715*, *rph-1*) is a spontaneous Thy⁻ derivative of the MG1655 strain (selected with trimethoprim), which was obtained from the Genetic Stock Center (Yale University). The MG1693 strains carrying defective alleles were constructed using the standard P1 transduction method and selection for appropriate antibiotic resistance.

The donor strains were as follows: AQ10293 (P*mioC112asnA101*::*cat*), AQ9652 (P*gid103asnA101*::*cat*) and AQ10614 (P*gid103*P*mioC112*), obtained from E. Boye (Bates *et al.*, 1997); EMO20-1 (*mioCp9::Tn5*), obtained from T. Katayama (Su'etsugu et al. [20]) and JRW27 (*dnaA46 tna::Tn10*), obtained from J. Walker.

Bacteria were grown by shaking at 37 °C in M9 minimal medium (MM9) containing M9 salts, 2 μg ml^{-1} thiamine, 0.4% glucose, 0.2% or 1% casamino acids (CAA) and 20 μg ml^{-1} thymine. Growth was monitored by measuring the absorbance at 550 nm (OD_{550}). Thymine starvation was achieved by collecting the cells on a Millipore filter (pore size 0.45 μm), then washing and resuspending them in the same MM9 medium without thymine in the presence or absence of rifampicin at various concentrations.

2.2. Viability studies

Bacteria were grown with shaking at 37 °C in MM9 medium. After thymine starvation for 3 h or at various time intervals, 50 μl aliquots were serially diluted and plated in duplicate on Luria-Bertani plates supplemented with 10 μg ml^{-1} thymine. Plates were incubated overnight at 37 °C, and colony-forming units (cfus) were counted the next day.

2.3. Determination of the proportion of origins initiated under thymine starvation, i, by measuring DNA synthesis

DNA synthesis was determined by growing bacterial cells in MM9 medium containing 1 μCi ml^{-1} of [*methyl*-^{3}H]-thymine (20 Ci/mmol) (ITISA-HartBiomedica) and assaying the amount of radioactive TCA-insoluble material.

The proportion of origins initiated under thymine starvation, *i* was calculated from two experimental values: ΔG and $\Delta G'$. In a mid-log phase culture ΔG is proportional to the number of overlapping replication rounds per chromosome, *n*, where 2^n represents the final DNA content [21]. Thus, ΔG was determined by measuring the relative amount of DNA accumulated after adding rifampicin at 150 μg ml^{-1} to exponentially growing cells (i.e., runout replication). Knowing ΔG, the value of *n* was derived from the empirical formula $\Delta G = [2^n n \ln 2/(2^n - 1)] - 1$ [22,23] using the computer software developed in our lab [24].

If after a period of time under thymine starvation an additional initiation occurs in a fraction of *i* origins and further new initiations are prevented by adding rifampicin, the final content of DNA will be $2^n(i+1)$. The synthesized DNA relative to its initial amount will be $\Delta G' = [2^n(i+1)n\ln 2/(2^n - 1)] - 1$ where *n* is obtained from ΔG in the exponential culture and *i* being the only variable in this formula [24]. Thus, ChIC was quantified as the fraction of origins, *i*, that were able to initiate and yield fully replicated chromosomes.

In order to do a comparative analysis of all the strains, *i* value was calculated after 10 min of thymine starvation. This time is not arbitrary as longer periods of thymine starvation undergo incomplete chromosome replication upon thymine re-addition in the presence of 150 μg ml^{-1} rifampicin (see Fig. 2C). If this were the case, *i* values would not be properly resolved.

2.4. Flow cytometry

DNA content per cell was measured by flow cytometry using a Beckman Coulter Cytomics FC 500 as previously described [25]. When cultures reached an OD_{550} of 0.2, a portion was starved of thymine in the presence or absence of different rifampicin concentrations. After starvation, thymine was provided together with rifampicin (150 μg ml^{-1}) and cephalexin (50 μg ml^{-1}) to inhibit new rounds of chromosome replication and cell division, respectively. These treated cultures were grown for an additional 3 h with continuous shaking, after which 400 μl of each culture was added to 7 ml of 74% ethanol. Approximately 1.5 ml of each fixed sample was centrifuged, and pellets were washed in 1 ml of ice-cold staining buffer (10 mM Tris, 10 mM MgCl2, pH 7.4, in sterile dH_2O) and resuspended in 1 ml of the same buffer. Cells were incubated on ice in the dark for at least 30 min in the presence of 1 μl of SYBR-Green (Life Technology) diluted 1:1000 and then analyzed on the Beckman flow cytometer at 480 nm.

2.5. RNA transcription rates

RNA transcription rates were determined by growing the cells in MM9 medium. After thymine starvation in the presence or absence of rifampicin, 1 ml samples were removed at various time intervals, incubated with 1 μCi ml^{-1} of [*methyl*-^{3}H]-uridine (20 Ci/mmol) (ITISA-HartBiomedica) and subsequently assayed for radioactive acid-insoluble material.

2.6. Two-dimensional agarose gel electrophoresis (2D gel)

Cells were lysed in plugs as described elsewhere [26]. Chromosomes embedded in plugs were treated with *Pvu*II restriction

enzyme overnight at 37 °C under gentle agitation. 2D gel migration was performed in 1 X TBE as follows: 1st dimension 0.4% agarose, 0.9 V/cm, 22 h at room temperature (RT); 2nd dimension 1% agarose, ethidium bromide (EtBr) 0.3 μg ml^{-1}, 5 V/cm, 7.5 h at 4 °C. DNA was transferred from 2D gels to a nylon membrane and hybridized using the Southern technique. The 419 bp *Xho*I-*Sma*I *oriC*-fragment generated by PCR was used as a probe.

Primer *ori1:* 5′-TTTGTCGGCTTGAGAAAGACC

Primer *ori-2:* 5′ TGCAAAACAGACAGGCGAAAC

Images were visualized using a Molecular Imager FX scanner (Bio-Rad) and analyzed with Quantity One software (Bio-Rad).

3. Results and discussion

3.1. Survival of thymine-starved cells inversely correlates with the rate of RNA synthesis and initiation efficiency

TLD is fully suppressed by the addition of 150 μg ml^{-1} rifampicin [18], a concentration which also inhibits the initiation of replication. We investigated whether survival under thymine starvation was affected by rifampicin concentration. An *E. coli* MG1693 culture was grown to an OD_{550} of 0.2. Several aliquots were removed and starved of thymine in the presence of 0, 5, 10, 25, 50, 100 or 150 μg ml^{-1} rifampicin. The viability of thymine-starved cells treated with different concentrations of rifampicin was estimated by determining the number of colony forming units (*cfu*) (Fig. 1A). RNA synthesis rates after rifampicin addition were analyzed by measuring ^{3}H-uridine incorporation during a 2 min incubation (Fig. 1B). We observed that cell survival under thymine starvation inversely correlated with the rate of RNA synthesis and showed a dose-dependent response to rifampicin (Fig. 1A, B).

We have previously observed that chromosomal initiation capacity (ChIC) is reduced upon rifampicin-treatment in thymine-starvation conditions [14]. Given the evidence linking TLD to initiation events, we hypothesized that there may be a causal relationship between reduced ChIC and rifampicin-mediated suppression of TLD. We quantify ChIC as the proportion of origins that are initiated after the addition of thymine to thymine-starved cells, denoted as *i* value.

To verify the correlation between ChIC and TLD, we determined *i* values for thymine starvation in the presence of different rifampicin concentrations. The experimental approach is detailed in Section 2. Briefly, *i* is calculated from two experimental values: ΔG and $\Delta G'$. ΔG is the relative increase in the amount of DNA after the inhibition of new rounds of chromosome replication, condition that can be achieved by the addition of 150 μg ml^{-1} rifampicin to the exponentially growing culture. $\Delta G'$ represents the relative increase in the amount of DNA of the same culture treated during 10 min for thymine starvation, and then return the thymine in the presence of 150 μg ml^{-1} rifampicin. The *i* value for a given treated culture can then be calculated from the formula $\Delta G' = [2^n(i+1)n\ln 2/(2^n-1)] - 1$ [24]. To resolve *i* value from this formula we used the experimental value of $\Delta G'$ and the value of *n* calculated from ΔG using the formula $\Delta G = [2^n n\ln 2/(2^n-1)] - 1$ [22,23].

A ^{3}*H-thymine*-labeled MG1639 culture was grown to an OD_{550} of 0.2, aliquots removed and thymine-starved for 10 min in the presence of 0, 10, 25, 50, 100 and 150 μg ml^{-1} rifampicin, followed by the addition of thymine and 150 μg ml^{-1} rifampicin for 3 h where the initiated origins can achieve complete chromosomal replication, but new initiation events are not allowed (Fig. 1C). Samples were then collected, and the relative increase in DNA content determined from the radioactivity of TCA-precipitated material ($\Delta G'$, Table 1). The fraction of origins initiated under thymine starvation, *i*, was calculated from $\Delta G'$ and ΔG values (Table 1) as explained in Section 2.

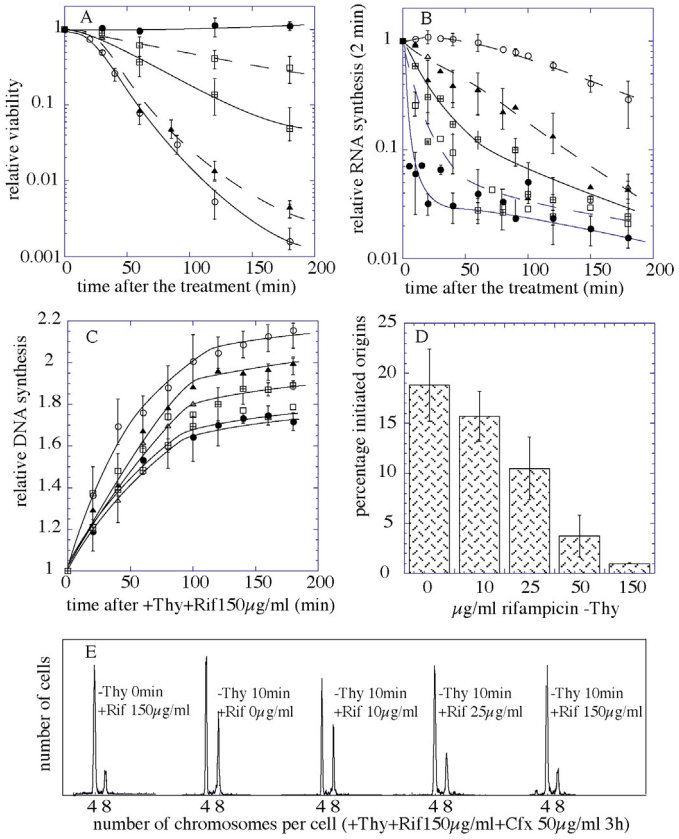

Fig. 1. Addition of rifampicin at different concentrations alleviates TLD diminishing ChIC during thymine starvation. (A) Relative cell survival after thymine starvation (TLD) in the presence of different rifampicin concentrations. (B) Relative RNA synthesis as determined by ^{3}*H-uridine* incorporation. (C) Runout of DNA synthesis after addition of thymine and 150 μg ml^{-1} rifampicin to a culture that was thymine-starved for 10 min in the presence of different rifampicin concentrations. Symbols: (○) 0, (▲) 10, (⊞) 25, (□) 50 and (●) 150 μg ml^{-1} rifampicin. (D) Quantification of ChIC as a percentage of initiated origins after adding thymine to 10 min thymine starved cells in the presence of different rifampicin concentrations (see Section 2). Error bars represent the SD from 2 to 4 independent experiments. (E) Flow cytometric profiles after adding thymine and 150 μg ml^{-1} rifampicin to a culture that was thymine-starved for 10 min in the presence of different rifampicin concentrations.

As shown in Fig. 1C, DNA synthesis decreased after thymine and rifampicin addition with increasing rifampicin concentration in thymine-starved cells ($\Delta G'$ in Table 1). ChIC was calculated as the fraction of origins initiated during the 10 min thymine starvation (Fig. 1D, *i* in Table 1). Significantly, sub-inhibitory rifampicin concentrations associated with decreased ChIC (Fig. 1D, *i* in Table 1) were also associated with the alleviation of TLD. This suggests that a partial inhibition of RNA polymerase might alleviate TLD by impairing a transcription-dependent step of chromosome initiation under thymine starvation.

To determine whether the origins that initiated during thymine starvation in the presence of rifampicin were able to complete chromosomal replication, we performed flow cytometry analysis on cells treated as described above. Fig. 1E shows discrete peaks corresponding to four and eight chromosomes after runout following 10 min of thymine starvation in the presence or absence of rifampicin. These results indicate that the rounds of chromosomal DNA replication initiated during the first 10 min of thymine starvation were able to finish following the addition of thymine in the presence of inhibitory levels of rifampicin, giving fully replicated chromosomes.

Table 1
Cell cycle parameters and TLD of MG1693 derivatives.

Strains	τ[a]	ΔG[b]	n[c]	$\Delta G'$[d]	i[e]	Survival 3 h – Thy[f]
MG1693-wt						
0.2% Casaa	39 ± 1.25	72	1.75 ± 0.11	108	19.46 ± 4.55	0.0016 ± 0.008
-Thy + Rif10 μg				97	15.69 ± 2.51	0.0040 ± 0.000
-Thy + Rif25 μg				87	10.46 ± 3.13	0.0485 ± 0.044
-Thy + Rif50 μg				76	3.74 ± 2.09	0.3137 ± 0.120
-Thy + Rif150 μg				72	1.00 ± 0.03	1.1051 ± 0.157
1% Casaa	37 ± 1.79	100	2.38 ± 0.09	157	25.30 ± 0.91	0.0007 ± 0.000
mioCp9						
0.2% Casaa	42 ± 1.79	52	1.27 ± 0.09	65	8.22 ± 0.92	0.0100 ± 0.003
1% Casaa	43 ± 6.42	53	1.33 ± 0.09	68	9.56 ± 4.41	0.0450 ± 0.030
PmioC						
0.2% Casaa	38 ± 1.63	62	1.52 ± 0.16	78	10.18 ± 5.69	0.0020 ± 0.000
1% Casaa	40 ± 1.92	71	1.71 ± 0.01	87	9.67 ± 2.29	0.0058 ± 0.002
Pgid						
0.2% Casaa	40 ± 2.00	62	1.53 ± 0.00	78	9.88 ± 4.32	0.0070 ± 0.004
1% Casaa	43 ± 2.31	73	1.75 ± 0.01	81	4.82 ± 4.64	0.0099 ± 0.001
PmioCPgid						
0.2% Casaa	43 ± 2.58	59	1.46 ± 0.05	74	10.33 ± 4.65	0.0025 ± 0.003
1% Casaa	42 ± 1.92	55	1.36 ± 0.09	65	6.86 ± 3.38	0.0140 ± 0.004
dnaA46ts						
30 °C	72 ± 5.66	65	1.60 ± 0.01	77	7.25 ± 2.60	0.0137 ± 0.009
42 °C				71	3.33 ± 1.31	0.9431 ± 0.182

[a] τ, Doubling time.
[b] ΔG, is the DNA content accumulated by mid-log phase culture after 150 μg ml^{-1} rifampicin treatment for 3 h (i.e., runout).
[c] n, was obtained from the empirical formula $\Delta G = [2^n n \ln 2/(2^n - 1)] - 1$ [22–23] using the computer software developed in our lab (see [24] for more details).
[d] $\Delta G'$, is the DNA content accumulated by cultures after 10 min thymine starvation followed by re-addition of thymine in the presence of 150 μg ml^{-1} rifampicin for 3 h.
[e] The value of i was obtained from the empirical formula $\Delta G' = [2^n(i+1)n \ln 2/(2^n - 1)] - 1$ using the computer software developed in our lab.
[f] Cell survival after 3 h of thymine starvation relative to time zero of the treatment. SD was obtained from 2–4 independent experiments.

3.2. Rifampicin inhibits the accumulation of abortive initiations generated under thymine starvation

The completion of chromosome replication described above suggests that the DNA damage and recombination structures generated during the first 10 min under thymine starvation are not critical to the replication process. Given that degradation of *oriC* was observed after extended thymine starvation, we studied ChIC and the potential protective effect of the presence of rifampicin by performing time-lapse experiments on thymine-starved cells. To this end, we thymine starved MG1693 *E. coli* cultures for 0, 30, 60 and 120 min and measured relative DNA synthesis after runout in the presence of thymine and rifampicin (150 μg ml^{-1}). Fig. 2A shows that the runout values ($\Delta G'$) increased with the length of thymine starvation. This indicates that more origins were initiated after thymine addition if the time of thymine starvation was extended, demonstrating that ChIC increases in thymine-starved cells. Flow cytometry analysis detected discrete peaks following the runout of the cells thymine-starved for 10–30 min but not for those starved for 60 or 120 min (Fig. 2C). This suggests that thymine starvation for more than 30–60 min impedes the completion of chromosome replication most probably due to the collapse of newly initiated rounds of replication from thymineless origins. To verify the association between rifampicin-mediated TLD suppression and the inhibition of initiation events accumulated during thymine starvation, the same approach was carried out in the presence of 150 μg ml^{-1} of rifampicin to inhibit new initiations in the absence of thymine. In agreement with our hypothesis, the addition of rifampicin during thymine starvation blocked the increase in DNA synthesis upon runout (Table 1; Fig. 2B). Moreover, flow cytometry revealed discrete peaks after replication runout under these conditions, indicating that chromosome replication was completed even following 2 h of thymine starvation in the presence of rifampicin (Fig. 2D). This suggests that rifampicin protects against TLD in two ways: by preventing the generation of thymineless origins, as new initiation events are inhibited by rifampicin, and consequently avoiding the collapse of the ongoing replication forks when thymine is added.

3.3. The presence of rifampicin inhibits the accumulation of oriC replication intermediates during thymine starvation

The results presented above establish a relationship between TLD suppression and the inhibition of chromosomal initiation by rifampicin addition. Hence, it is reasonable to think that abortive initiation at the *oriC* sequence could be an effect of thymine starvation. If this is true, then replication intermediates should accumulate at *oriC* during thymine starvation, being prevented in the presence of rifampicin. We examined the progression of replication forks at *oriC* by two-dimensional agarose gel electrophoresis (2D gel) [27] under different treatments and genetic backgrounds (Figs. 3 and 4).

An exponential phase MG1693 culture was starved of thymine, and samples were removed after 10, 20, 30, 40, 50, 60 and 120 min and analyzed by 2D gel (Fig. 3B). Chromosomal DNA was digested with the *Pvu*II restriction enzyme and hybridized to a labeled 419 bp *Xho*I-*Sma*I *oriC*-fragment used as a probe (Fig. 3A, see Section 2). Because of the speed of replication forks in *E. coli*, replication intermediates are not detectable in the chromosomes of exponentially growing cells [28]. Fig. 3B shows several features generated during thymine starvation. A simple-Y arc, corresponding to the accumulation of Y-shaped replication intermediates, was detected after 10 min of thymine starvation, indicating the arrest of replication forks within the *oriC* region. This simple-Y arc became more intense as the period of thymine starvation increased. A bubble arc was detected after 40 min of thymine starvation, indicating initiations at *oriC*. In addition double-Y structures were also detected, probably the result of two forks encountering each other at *oriC*. Schematic representations of these DNA structures identified by 2D gel are presented in Fig. 3D. These results show that under

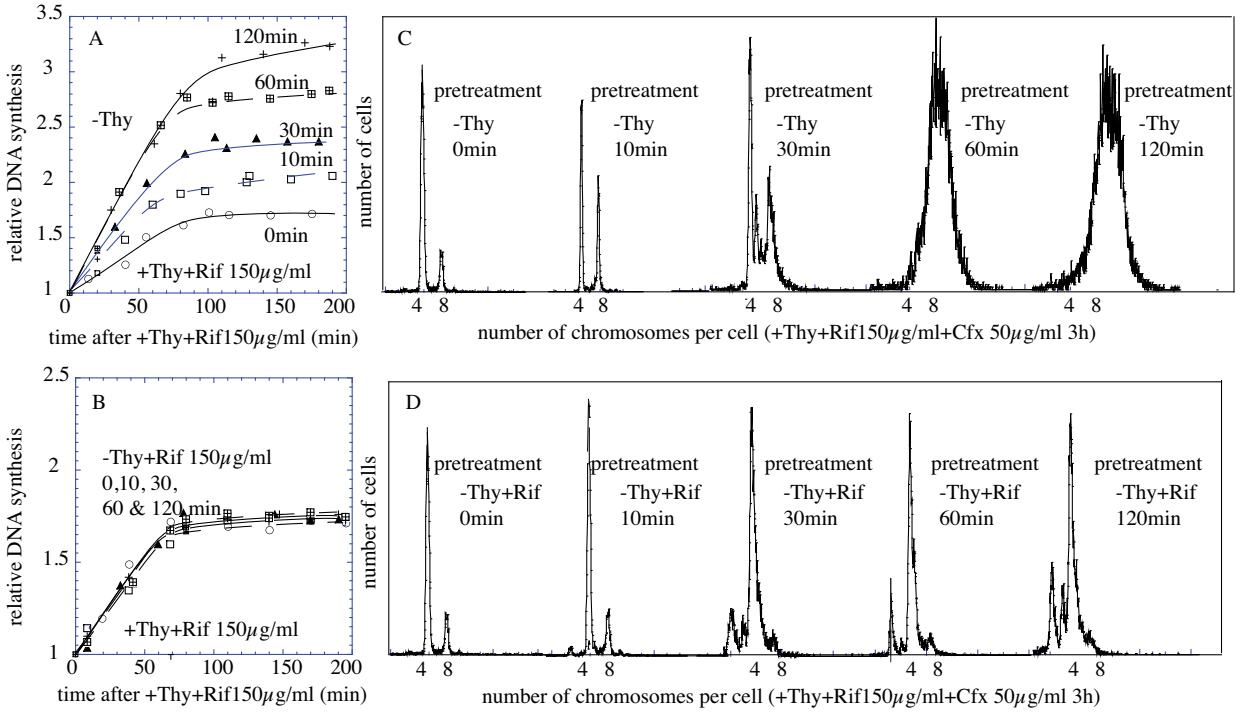

Fig. 2. ChIC increases during thymine starvation. (A) And (B) Runout of DNA synthesis after the addition of thymine and 150 μg ml^{-1} rifampicin to cultures that were thymine-starved for (○) 0, (□) 10, (▲) 30, (⊞) 60 and (+) 120 min in the absence (A) or the presence (B) of 150 μg ml^{-1} rifampicin. (C) and (D) Flow cytometry profiles after adding thymine, 150 μg ml^{-1} rifampicin and cephalexin to cultures starved of thymine for (○) 0, (□) 10, (▲) 30, (⊞) 60 and, (+) 120 min in the absence (B) or the presence (D) of 150 μg ml^{-1} rifampicin.

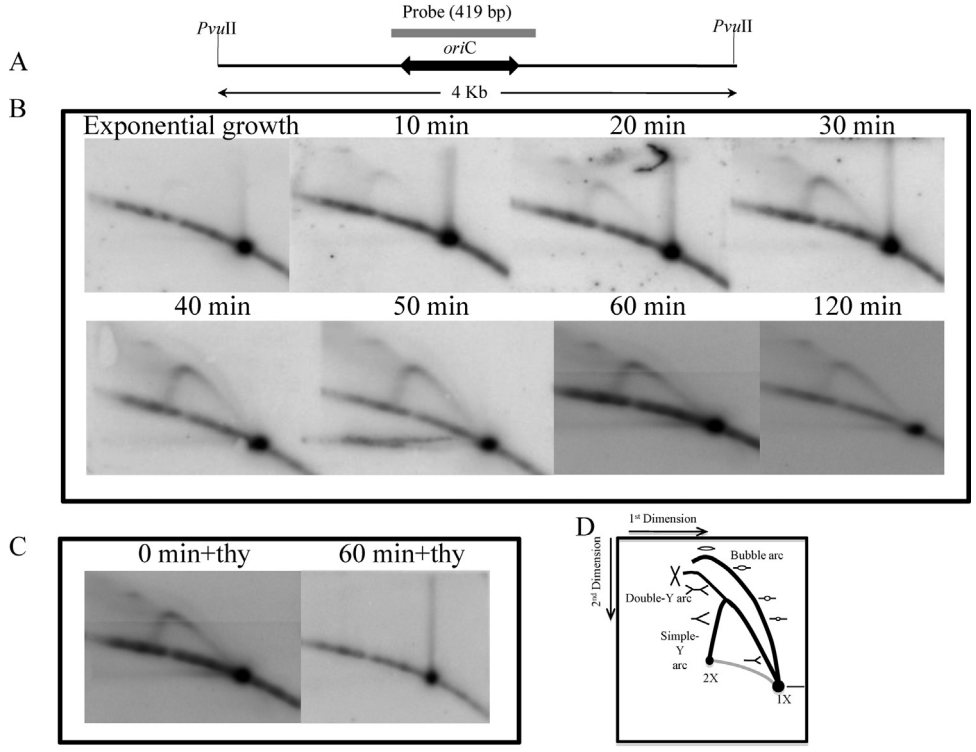

Fig. 3. Replication forks accumulate at *oriC* during thymine starvation. 2D gels were used to analyze DNA replication on a restriction fragment containing the *E. coli* replication origin *oriC*. DNA from a wild type strain was digested with *Pvu*II, analyzed by 2D gel electrophoresis and probed with a sequence containing the *oriC* region. (A) *Pvu*II fragment and *oriC* probe used for Southern blot analysis. (B) From left to right, 2D gel DNA analysis from exponentially growing cells following 0, 10, 20, 30, 40, 50, 60 and 120 min of thymine starvation. (C) 2D gel DNA analysis from cells starved for 60 min (left) and following by thymine addition (right). (D) Schematic representation of the DNA structures identified in 2D gels.

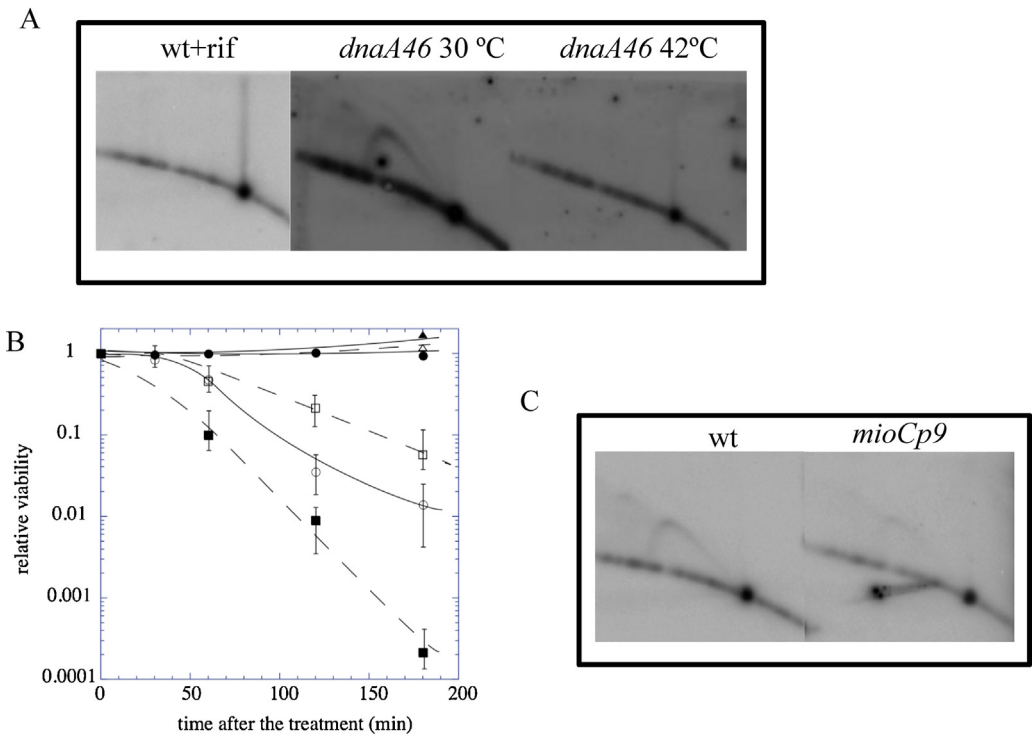

Fig. 4. Inhibition of chromosomal initiation suppresses TLD by preventing the accumulation of replication intermediates at *oriC*. (A) From left to right, 2D gel DNA analysis of the wild type MG1693 strain in the presence of rifampicin at 37 °C and the *dnaA46* mutant strain after 60 min of thymine starvation at 30 °C or 42 °C. (B) Relative cell survival of the *dnaA46ts* mutant strain after thymine starvation at 30 °C (○) in the presence of rifampicin (●) and at 42 °C (△) in the presence of rifampicin (▲) and wild type MG1693 after thymine starvation at 30 °C, (□) or 42ºC (■) (C) From left to right, 2D gel DNA analysis after 60 min of thymine starvation of wild type MG1693 and the *mioCp9* constitutive transcriptional mutant strain grown in MM9 medium supplemented with 1% CAA.

thymine starvation, replication initiation occurs both canonically at the *oriC* sequence and non-canonically outside of it. In the latter case, *oriC* would be passively replicated by a single fork or by two forks that encounter each other after being initiated outside of the *oriC Pvu*II-fragment (Fig. 3A). These forks are most likely generated as a consequence of futile *thymineless*-replication around the *oriC* sequence, supported by the verification of *thymineless*-DNA synthesis by Kuong et al. [15].

These results verify two important ideas: firstly, that the *oriC* sequence is replicated under thymine starvation, and secondly, that replication intermediates are accumulated progressively. Significantly, when we add thymine after 60 min of thymine deprivation, all the structures disappeared (Fig. 3C). This demonstrates the progression of replication forks leaving the *oriC* region, although from our earlier experiments we know that not all of them can fully complete the replication round (Fig. 2C). In contrast, none of these structures were found in thymine-starved wild type cells in the presence of rifampicin for 60 min (Fig. 4A), where TLD is suppressed (Fig. 1A). This observation further supports a relationship between TLD suppression and the inhibition of abortive replication of *oriC* by rifampicin addition during thymine starvation.

To confirm these results, we examined the *dnaA46* mutant strain by 2D gel. The *dnaA46* allele encodes a thermosensitive DnaA protein, whereby incubation at 42 °C blocks chromosome replication initiation but permits replication elongation [29]. Consistent with previous observations [30], we found that TLD occurred in thymine-starved *dnaA46* mutant cells grown at 30 °C but suppressed at 42 °C with rifampicin, while TLD observed in *dnaA*+ strain MG1693 under thymine starvation at 42 °C is even greater than at 30 °C (Fig. 4B). At permissive temperature the *dnaA46* mutant strain shows a bubble and a simple-Y arc under thymine starvation conditions (Fig. 4A), indicating that replication forks accumulated at the *oriC* region. However, when thymine-starved *dnaA46* mutant cells were cultured at 42 °C, thus blocking chromosome replication, none of these chromosome replication intermediates were observed. These observations directly link the formation of replication intermediates under thymine starvation conditions to chromosome replication initiation, supporting a central role for chromosomal initiation in TLD.

3.4. Altering transcriptional activity around oriC decreases ChIC and alleviates TLD

The rifampicin-sensitive chromosomal initiation step in *E. coli* is widely accepted to be transcription of the *mioC* and *gid* genes near *oriC* [31–34]. Neither, deletion of the *mioC* or *gidAB* promoters nor constitutive transcription from the *mioC* promoter have a large effect on the cell cycle, but some reduction in initiation efficiency has been observed in rich medium [19,20,35,36]. This effect indicates that transcription from these genes is not absolutely necessary, but can improve the initiation efficiency when cells are grown in rich medium, which is required under some initiation conditions such as those described above. Here, we take advantage of the detrimental effect on chromosomal initiation observed in these mutants to verify whether altered transcription around the *oriC* region modulates TLD.

We constructed isogenic MG1693 strains carrying P*mioC112*, P*gid113*, and the double mutant P*mioC112* P*gid113* defective alleles in the promoter region [19] and a *mioCp9* allele that confers constitutive transcription from *mioC* entering into the *oriC* region [20]. TLD was measured in wild type and mutant cultures grown in MM9 medium supplemented with 0.2% and 1% CAA without thymine for three hours. We found significant differences in bacterial viability after 3 h of thymine starvation in rich medium (Fig. 5A, Table 1). The strongest effect was observed in the *mioCp9* mutant strain, which had 60 times the viability of starved wild type cells.

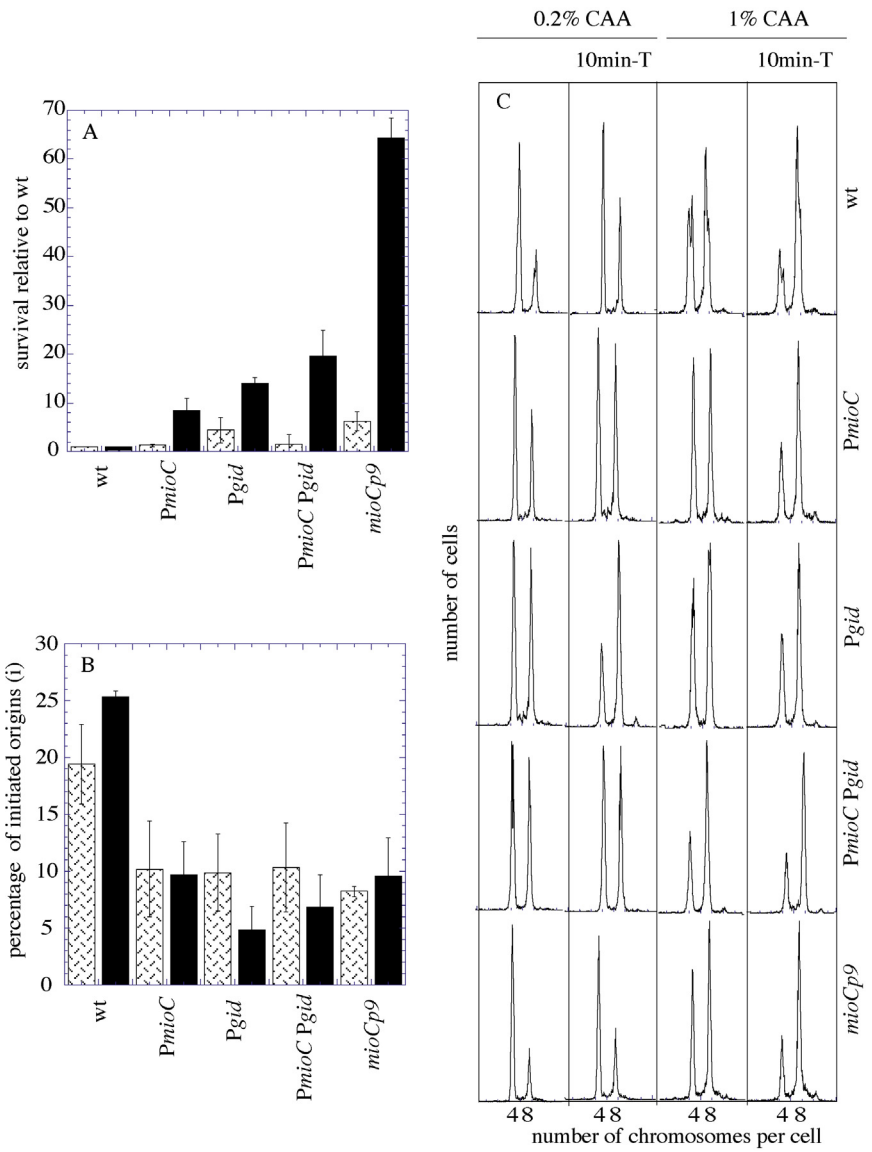

Fig. 5. Alleviation of TLD by altering the transcription around the *oriC* origin. (A) Survival after 3 h of thymine starvation of different mutants grown in MM9 medium supplemented with 0.2% (▨) or 1% (■) CAA. Values are relative to the wild type MG1693 strain. (B) Percentage of origins initiated after adding thymine to 10 min thymine-starved cells of different mutant grown in MM9 supplemented with 0.2% (▨) or 1% (■) CAA for 10 min. Error bars represent the SD from 2–4 independent experiments. (C) Flow cytometry profiles of the cultures growing in MM9 supplemented with 0.2% CAA and with 1% CAA, treated with rifampicin and cephalexin for 3 h (left panels) and thymine-starved cultures for 10 min prior addition of thymine, rifampicin and cephalexin for 3 h (right panels).

Next, we assessed the possibility that the partial suppression of TLD by altering transcriptional activity around *oriC* observed in Fig. 5A might be caused by a reduction in ChIC. We determined the fraction of initiated origins, *i*, accumulated during 10 min of thymine starvation in the *oriC* transcription mutants. To this end, exponential phase MG1693 cultures carrying the *mioC112, gid113*, *mioC112 gid113* and *mioCp9* alleles were grown to an OD_{550} of 0.2 in medium containing ^{3}H-*thymine*. Two aliquots were removed and treated either with 150 μg ml^{-1} rifampicin for 3 h, obtaining ΔG; or starved of thymine for 10 min and then grown with thymine and 150 μg ml^{-1} rifampicin for 3 h to obtain $\Delta G'$. The relative amount of DNA accumulated was determined by the amount of TCA-precipitated material in the samples. From this, the proportion of initiated origins that had accumulated during the 10 min of thymine starvation can be calculated (Fig. 5B; Table 1). These results show that ChIC was reduced in the *mioC112, gid113*, *mioC112 gid113* and *mioCp9* mutants grown in MM9 medium supplemented with 1% CAA compared to wild type cells. The reduced TLD in *mioC* and *gid* mutant strains strongly suggests that the transcriptional activity required for the initiation of chromosomal replication is a limiting factor for chromosome replication initiation under thymine starvation conditions. Flow cytometry profiles of the strains in MM9 supplemented with 1% of CAA show that complete chromosomal replication was achieved by any of the *mioC* or *gid* mutant cells starved of thymine for 10 min after re-adding thymine (Fig. 5C).

2D gel analysis was also performed on cells that were thymine-starved for 60 min carrying the *mioCp9* mutation which showed decreased replication initiation and greatly alleviated TLD when grown in MM9 medium supplemented with 1% CAA. Significantly, no bubble arcs and only very faint simple-Y arcs were observed after thymine starvation of the mutant strain comparable to that observed for the wild type MG1693 strain grown in rich medium (Fig. 4C).

The relevance of these results relies on the fact that TLD suppression in these mutants must be related to the alteration of the normal transcription levels around *oriC*, This correlation,

together with the broad reduction of chromosome replication initiation intermediates by these mutants, strongly implicates the transcriptional-dependent step of replication initiation as being critical for TLD, even though there is not a strong quantitative correlation between both parameters. This could be due to the different effect on the transcription activity that has been reported for these mutants. Thus, *mioCp9* is a mutation that constitutively promotes transcription from *mioC* promoter entering to *oriC*; while P*mioC* and P*gid* are defective alleles in the promoter region, i.e., no transcription is supported from these regions around *oriC*. This would imply that even though the proportion of initiated origins under thymine starvation were quite similar in all mutants (Fig. 5 B), constitutive transcription entering into *oriC* that occurs in the *mioCp9* could protect somehow *oriC* region and eventually improve the viability of the thymine-starved cells with respect to P*mioC112* and P*gid113* mutant strains.

4. Conclusions

In this work, we have shown that TLD is alleviated when the transcriptional-dependent step of chromosome replication initiation is impaired. Thus rifampicin-dependent suppression of TLD seems to be related to the inhibition of replication initiation events in thymine-starved cells. Three lines of evidence support this hypothesis. Firstly, increased ChIC in thymine-starved cells inversely correlates with cell survival and with the rifampicin concentration added during thymine starvation. Secondly, using 2D agarose gel electrophoresis, we found that *oriC* bubble, simple-Y, and double-Y replication intermediates accumulate progressively during thymine starvation. None of these DNA structures could be visualized in thymine-starved cells if initiation events were inhibited either by rifampicin addition or by thermal inactivation of DnaA. Finally, specifically altering transcription in *cis* to the *oriC* region using *mioC* and *gid* alleles, diminished ChIC and alleviated TLD under thymine starvation conditions.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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