Introduction

The processivity of DNA replication requires a 5′→3′ replicative helicase - DnaB in *Escherichia coli* - to unwind double-stranded DNA in front of the polymerase and ensure the processivity of DNA synthesis. In *Escherichia coli*, the helicase loader DnaC as well as factors involved in the formation of the open complex during the initiation of replication and primosomal proteins during the reactivation of arrested replication forks are required to recruit and deposit the replicative helicase onto single-stranded DNA prior to the formation of the replisome. dnaC2 is a thermosensitive allele of the gene specifying the helicase loader; at non-permissive temperature replication cannot initiate, but most ongoing rounds of replication continues through to completion (18% of dnaC2 cells fail to complete replication at non-permissive temperature). An assumption, which may be drawn from this observation, is that only a few replication forks are arrested under normal growth conditions. This assumption, however, is at odds with the severe and deleterious phenotypes associated with a null mutant of priA, the gene encoding a helicase implicated in the reactivation of arrested replication forks. We developed an assay that involves an abrupt inactivation of rounds of synchronized replication in a large population of cells, in order to evaluate the ability of dnaC2 cells to reactivate arrested replication forks at non-permissive temperature. We compared the rate at which arrested replication forks accumulated in dnaC2 priA+ and dnaC2 priA2 cells and observed that this rate was lower in dnaC2 priA+ cells. We conclude that while replication cannot initiate in a dnaC2 mutant at non-permissive temperature, a class of arrested replication forks (PriA-dependent and DnaC-independent) are reactivated within these cells.
cells. We chose to inactivate RF with Novobiocin, a drug that inhibits type II topoisomerases and mainly Gyrase [14], after establishing that priA2 cells were hypersensitive to Novobiocin. Gyrase eliminates the positive supercoils that accumulate in front of the RF and introduces negative supercoils ensuring the progression of the polymerase [15]. The accumulation of positive supercoils in front of the RF, when Gyrase is inhibited, hails the progression of the polymerase and eventually inactivates RF. We measured by flow cytometry the accumulation of inactivated RF in different genetic backgrounds and found that dnaC2 priA2 cells accumulated 2.5 times more arrested RF than isogenic priA+ cells at non-permissive temperature. This work led us to the identification of a new class of arrested RF - representing 60% of of them, whose reactivation depends on PriA but apparently not on DnaC activity. Implications in terms of DnaC2 activity at non-permissive temperature and in terms of the frequency of RF inactivation during normal growth are discussed.

Materials and Methods

Strains, chemicals and cultures

Strains used in this study and their genetic background are presented in Table 1. To increase the sensitivity of the cells to Novobiocin, a ΔacrA mutation was introduced in the strains used in this study [16]; AcrA is a component of an efflux pump that expels various drugs in the medium, reducing their intracellular concentration. In a ΔacrA mutant, the amount of Novobiocin required to inhibit type II Topoisomerase activity is lower than in acrA+ cells [14,17]. A priA2 mutation is characterized by a high basal level of SOS induction [18]. Therefore, a sfiA+ mutation was introduced in priA2 strains to prevent filamentation [19]. dnaC2 was P1 transduced from the original PC-2 strain [11]. A mdoB::Tn cassette [18] was first introduced within PC-2 strain [11]. A mdoB::Tn cassette [18] was first introduced within PC-2 strain [11].

Table 1. Strains and plasmids used in this study.

| Strains   | Genotype                                      | reference |
|-----------|-----------------------------------------------|-----------|
| BW25113   | lacIq mI8714 ΔlacZJW16 hisDR14 ΔaraBADAH33 Δ thrhBΔDL78 | [20]       |
| CAG18430  | mdoB::Tn10                                    | [29]       |
| CM735     | metE46, trp3, his4, thi1, galK2, lacY1 or lacZ4, md11, ara9, tsx3, lon1, rps8 or rps9, supE44 Δ           | [20]       |
| JJC1398   | AB1157 sfiA1 ΔpriA::Kn/pAM-priA               | [19]       |
| JW0452    | BW25113 acrA::Kn                             | [16]       |
| JW0941    | BW25113 sfiA::Kn                             | [16]       |
| NK9069    | CM735 dnaA46                                  | [20]       |
| PC2       | leu6 thyA47Δ dra3 str153 dnaC2 dnaT12         | [11]       |
| REP1329   | CM735 dnaC2 mdoB::Tn10                       | This study |
| REP1952   | CM735 acrA::Kn                               | This study |
| REP2139   | CM735 ΔsfiA[Δkn] ΔacrA[Δkn] ΔpriA2::Kn/pAM-priA | This study |
| REP1986   | CM735 dnaC2 mdoB::Tn10 ΔacrA::Kn              | This study |
| REP2364   | REP2139 dnaC2 mdoB::Tn10                      | This study |
| REP2370   | REP2139 dnaA46 dnaT10                        | This study |
| REP2031   | REP1952 dnaA46 dnaT10                        | This study |

| Plasmids      | Description                                    | reference |
|---------------|------------------------------------------------|-----------|
| pAM-priA      | Plasmid, whose replication is under the control of Pmin carrying a WT copy of priA. | [19]       |

doi:10.1371/journal.pone.0033613.t001
Figure 1. Experimental design. A – Synchronization procedure. A dilution of an overnight culture of dnaC2 or dnaA46 cells was grown during 3 generations at 30°C until exponential phase (exp) and then incubated at 40°C for 90 minutes (syncro) to synchronize the cells with respect to the initiation of replication. Replication was initiated by an abrupt downshift of the temperature of the culture to 30°C. 6 minutes after the temperature downshift, the cells were brought back to 40°C for 90 minutes to synchronize the cells with respect to replication initiation [21]. Replication was initiated by shifting abruptly the temperature from 40°C to 30°C. After 6 minutes of incubation at a permissive temperature for the initiation of replication (30°C), the cells were brought back to 40°C. 40 minutes after initiation of replication, cells with active RF contain around two genomes.

The fraction of cells with active RF at t0 was calculated as 
\[ \rho_{t0} = 1 - \left( a_{t0}/a_0 \right) \]. In order to assess the kinetics of RF inactivation in response to Novobiocin, we calculated the ratio \( \rho_{t\Delta t}/\rho_{t0} \) for each time point and plotted the log value of these ratios over the time of incubation of Novobiocin at an inhibiting concentration. \( \rho_{t0} \) represents the proportion of cells with active RF at t0 that were not incubated with Novobiocin. The function \[ \text{Ln}(\rho_{t\Delta t}/\rho_{t0}) = f(t) \] is a straight line, whose slope (\( -k \)) identifies the rate at which inactivated RF accumulate when the cells are incubated with Novobiocin. The fraction of RF reactivated in dnaA46 and in dnaC2 cells at non-permissive temperature was calculated as 
\[ 1 - \left( k_{\text{priA}} / k_{\text{priA2}} \right) \].

Synchronization procedure

Overnight cultures of dnaC2 or dnaA46 cells were diluted in fresh MMA Glucose medium and grown at permissive temperature (30°C) for 3 generations (doubling time of 90 min) until reaching log phase (OD550 of 0.1). The cultures were then transferred into a shaking water bath preset at 40°C for 90 minutes to synchronize the cells with respect to replication initiation [21]. Replication was initiated by shifting abruptly the cultures to 30°C for 6 minutes. This operation was performed by the addition of an equal volume of 20°C-prewarmed fresh medium to the culture, followed by the incubation of the culture at 30°C in a shaking water bath. 6 minutes after the temperature downshift, the cells were reincubated at 40°C to prevent the initiation of additional rounds of replication (Figure 1A).

Results

Assessing the fraction of cells with active RF

The appreciation of the ability of dnaC2 cells to reactivate arrested RF requires a precise evaluation of the proportion of cells with active RF in a given population, which is made possible by flow cytometry. The incubation of exponentially growing dnaA46 cells – carrying a thermosensitive allele of the gene encoding the initiator protein - or dnaC2 cells at a non-permissive temperature blocks the initiation of replication but neither ongoing rounds of replication, which continue through to completion, nor cell division. During an incubation of 90 minutes at a non-permissive temperature of 40°C, a population of dnaC2 or of dnaA46 cells is progressively enriched in cells containing a single genome (Figure 1A and 1B). After 90 minutes of such a treatment, most...
cells contain one genome (cells with two or more genomes amount to a mere 10 to 15%); the cells are synchronized with respect to replication initiation (Figure 1B). At this stage, a short (6 minutes) and abrupt downshift of temperature to 30°C of cultures of dnaC2 and dnaA46 cells pre-incubated for 90 minutes at 40°C - results in a synchronous initiation of replication in a large proportion of cells. The quantity of DNA (followed over FL3) in cells that initiated replication shifts over time from 1 to 2 genomes (Figure 1C). 40 minutes after the temperature downshift (t40), which corresponds to the period of time required to complete a round of replication (data not shown), dnaC2 and dnaA46 cells that initiate replication contain around 2 genomes. These cells are easily identifiable as a peak on DNA histograms (Figure 1D).

It is noteworthy that the peak of replicating cells is broader in a dnaC2 background than in a dnaA46 background (Figure 1D). The peak of dnaC2 replicating cells overlaps partially with that of cells containing one genome while the peaks of dnaA46 cells with one and with two genomes are relatively narrow and well separated (Figure 1D). From cell to cell, the quantity of DNA replicated in dnaC2 cells during 40 minutes is more variable than in dnaA46 cells. Assuming that the elongation rate of the DNA polymerase is not altered in a dnaA46 and in a dnaC2 background, the broader shape of the peaks observed with dnaC2 cells at t40 likely reflects the fact that within this genetic background, rounds of replication were arrested and not reactivated at non-permissive temperature. Our data are therefore consistent with previously reported results (Maisnier-Patin et al, 2001), but still do not refute the possibility that some arrested RF be reactivated in dnaC2 cells at non-permissive temperature.

To shed light on this matter, we developed a method allowing us to measure the fraction of cells with active RF within a population.

Synchronized cells that initiated and completed a round of replication accumulate within the peak at 2 genomes per cell (Figure 2), while those in which replication did not initiate accumulate within the peak at one genome per cell. Cells in which 1 or 2 RF were arrested - before completion of replication - and not reactivated accumulate in the valley between 1 and 2 genomes per cell.

The drift of the peak on DNA histograms of cells harvested before, and 10 minutes after, the temperature downshift is almost negligible (Figure 1B). Therefore, cells in which both RF were arrested within 10 minutes after the initiation of replication and not reactivated, accumulate with those that did not initiate replication. Hence, and in order to assess the ability of dnaC2 cells to reactivate arrested RFs, a large proportion of RF was transiently inactivated soon after initiation of replication in a synchronized population of cells. Then, the cells were brought back to growth conditions permissive with respect to replication. Under such experimental conditions, cells in which replication did not initiate and those in which both RF were arrested - during the inactivation procedure - and not reactivated, accumulate within the peak at one genome (Materials and Methods). We turned to the analysis of cytograms, in which the DNA content (given by the FL3) is plotted over the FSC, which gives a rough estimate of cell mass, to delineate more precisely the fraction of cells with one genome (Figure 2). Given this starting situation, the proportion of cells undergoing replication under a given condition (p0) can be extracted from the fraction of cells with one genome at t0 (a0) and t40 (a40) (Materials and Methods).

Inactivating RF with Novobiocin

We developed a procedure to generate a large quantity of arrested RF in cultures of synchronized cells to assess the capacity of dnaC2 cells to reactivate RF at non-permissive temperature. We decided to target Gyrase because PriA was shown to be essential in gyrase point mutants, revealing a high rate of RF inactivation in these strains; the mediocre activity specified by Gyrase mutant proteins and the resulting accumulation of positive supercoils in front of the polymerase was proposed to be responsible for the high rate of RF inactivation [19]. Novobiocin is a Gyrase inhibitor, Topo IV, the topoisomerase implicated in the resolution of (pre)catenated DNA that accumulates behind the RF [22], is also targeted by Novobiocin. Yet, Topo IV is much less sensitive to Novobiocin than Gyrase [14], implying that Gyrase is the primary target of Novobiocin. We verified whether the Novobiocin-induced inhibition of Gyrase could inactivate RF by assessing the sensitivity of a priA2 mutant – a mutant in which most arrested RF is not reactivated – to Novobiocin. We decided to target Gyrase because PriA was shown to be essential in gyrase point mutants, revealing a high rate of RF inactivation in these strains; the mediocre activity specified by Gyrase mutant proteins and the resulting accumulation of positive supercoils in front of the polymerase was proposed to be responsible for the high rate of RF inactivation [19]. Novobiocin is a Gyrase inhibitor, Topo IV, the topoisomerase implicated in the resolution of (pre)catenated DNA that accumulates behind the RF [22], is also targeted by Novobiocin. Yet, Topo IV is much less sensitive to Novobiocin than Gyrase [14], implying that Gyrase is the primary target of Novobiocin. We verified whether the Novobiocin-induced inhibition of Gyrase could inactivate RF by assessing the sensitivity of a priA2 mutant – a mutant in which most arrested RF is not reactivated – to Novobiocin. We decided to target Gyrase because PriA was shown to be essential in gyrase point mutants, revealing a high rate of RF inactivation in these strains; the mediocre activity specified by Gyrase mutant proteins and the resulting accumulation of positive supercoils in front of the polymerase was proposed to be responsible for the high rate of RF inactivation [19]. Novobiocin is a Gyrase inhibitor, Topo IV, the topoisomerase implicated in the resolution of (pre)catenated DNA that accumulates behind the RF [22], is also targeted by Novobiocin. Yet, Topo IV is much less sensitive to Novobiocin than Gyrase [14], implying that Gyrase is the primary target of Novobiocin. We verified whether the Novobiocin-induced inhibition of Gyrase could inactivate RF by assessing the sensitivity of a priA2 mutant – a mutant in which most arrested RF is not reactivated – to Novobiocin. We decided to target Gyrase because PriA was shown to be essential in gyrase point mutants, revealing a high rate of RF inactivation in these strains; the mediocre activity specified by Gyrase mutant proteins and the resulting accumulation of positive supercoils in front of the polymerase was proposed to be responsible for the high rate of RF inactivation [19]. Novobiocin is a Gyrase inhibitor, Topo IV, the topoisomerase implicated in the resolution of (pre)catenated DNA that accumulates behind the RF [22], is also targeted by Novobiocin.
cells with active RF was measured (Materials and Methods) for each condition (Figure 3B) and then plotted over the range of concentrations of Novobiocin tested (Figure 3C). Up to a concentration of 2 µg/ml, Novobiocin has virtually no effect on the proportion of cells with active RF (Figure 3C). In contrast, the proportion of cells with active RF was significantly reduced when the concentration of Novobiocin was at least 5 µg/ml (Figure 3C). We chose to generate arrested RF by treating cultures of cells with Novobiocin at a concentration of 10 µg/ml (an “inhibiting” concentration) because a large quantity of RF are inactivated at this concentration of drug; the fraction of cells with active RF was estimated to be less than 40% (Figure 3C). A concentration of 10 µg/ml of Novobiocin was also chosen because a 10-fold dilution brings the drug to a concentration at which the proportion of cells with active RF is merely 1.9% less than that measured in cultures of untreated cells (Figure 3C).

RF are reactivated in dnaC2 cells at non-permissive temperature

Novobiocin was added to synchronized cultures of priA+ and priA2 cells to a final concentration of 10 µg/ml, 10 minutes after replication initiation and for a length of time ranging from 1 to 10 minutes. Then the cultures were diluted to bring Novobiocin to a concentration at which the drug has an insignificant effect on Gyrase activity in dnaC2 cells (1 µg/ml, 40 minutes after initiation of replication, samples of cells were taken, fixed and processed for flow cytometry analysis (procedure summarized in Figure 4A). The experiment was performed in a dnaC2 (Figure 4B) and in a dnaC+ background (Figure 4C). In the latter case, cells were synchronized with dnaC46.

It was shown in dnaC2 cells that the inactivation of one RF does not affect the fate of the other RF present in the cell [12]. Hence, we measured the rate at which cells with two inactive RF were generated, which corresponds to the rate at which the last RF was inactivated, to estimate the rate of RF inactivation in response to Novobiocin. We followed the fraction of cells with active RF for each strain tested over the time of incubation with Novobiocin at an inhibiting concentration (Materials and Methods). \( \rho_0 \) was measured for each time point, divided by \( \rho_0^0 \) (where \( \rho_0^0 \) is the fraction of cells with active RF in the absence of Novobiocin) and the logarithm value of these ratios were plotted over the time of incubation of the cultures at an inhibiting concentration of Novobiocin (Figure 4B and 4C). For each strain, the distribution of these values fits best with a linear distribution (coefficient of determination, \( R^2 \), above 0.99), which indicates that RF inactivation in response to Novobiocin is a first-order reaction. For each series of data, the linear regression was assessed and the slope (-\( k \)) was extracted (Figure 4B and 4C). \( k \) represents the rate at which inactivated RF accumulate during the experiment. The values of \( k \) calculated in a dnaC2 priA2 (0.084±0.012 min\(^{-1}\)) and in a dnaC+ priA2 background (0.09±0.009 min\(^{-1}\)) are not significantly different, which indicates that priA2 is epistatic to dnaC2. Strikingly, the value of \( k \) calculated in a dnaC2 priA+ background (0.033±0.004 min\(^{-1}\)) is much lower than that calculated in a dnaC2 priA2 background. This result indicates that dnaC2 priA+ cells accumulate more arrested RF than dnaC2 priA2 cells in response to Novobiocin.

Discussion

The primary result of this study is that dnaC2 priA2 cells accumulate more inactivated RF at non-permissive temperature than dnaC2 priA+ cells when the cells are treated with Novobiocin, a drug that inhibits Gyrase. Two points, however, need further clarification in order to conclude definitely from these data that dnaC2 cells can reactivate arrested RF at non-permissive temperature. The first point regards the direct link that we drew between the inhibition of Gyrase and the inactivation of RF. We clarified this point by plating pra2 and pra+ cells on a medium supplemented with Novobiocin. The rationale behind this experiment was based on the requirement of the helicase activity specified by PriA and the following recruitment of the primosomal proteins for the reactivation of arrested RF [9]. Another pathway – driven by PriC and independent of PriA – was deduced from the synthetic lethality associated with a double mutant praA praC [9]. Yet, the absence of phenotype attributable to a priC single mutant led to the assumption that the PriA-driven mechanism was the
Figure 4. RF reactivation in dnaC2 cells at non-permissive temperature. A – Schematic description of the experimental procedure. Replication is initiated synchronously at t0. 10 minutes after replication initiation, Novobiocin is added to the cultures at an inhibiting concentration (dark grey) before being brought back to a permissive concentration (light grey) until the end of the experiment. A control sample, not treated with Novobiocin, (−N) was also analyzed. Samples incubated with Novobiocin (N) are identified by the time ‘i’ (in minutes) of incubation at a concentration of Novobiocin of 10 μg/ml. B – Contour plots of dnaA+ dnaC2 cells before (t0) and after initiation of replication (−N, N0, N1, N2, N5 and N10) in priA+ (top) and priA2 cells (bottom). The fraction of cells with active RF (p40) under each condition tested was normalized to that of cells that were not incubated with Novobiocin (p400), and the logarithm value of these ratios was plotted over the time of incubation with Novobiocin at
major RF reactivation pathway. Thus and despite the existence of an alternative pathway for RF reactivation, we reasoned that the inhibition of Gyrase by Novobiocin, if it results in the inactivation of RF, ought to reduce dramatically the viability of priA+ cells to low concentration of drug. We established indeed that priA2 cells are much more sensitive to Novobiocin than priA+ cells (Figure 3A) and concluded that the Novobiocin-induced inhibition of Gyrase leads to RF inactivation. The second point concerns the rate at which RF are inactivated in dnaC2 and in priA2 cells in response to Novobiocin. This point requires a clarification as well because a faster rate of RF inactivation in priA2 than in priA+ cells, or the destabilization of active RF caused by the absence of the PriA protein could alternatively explain the larger proportion of cells with arrested RF in a dnaC2 priA2 than in a dnaC2 priA+ background. This hypothesis, however, may be excluded since PriA interacts with DNA after RF inactivation and not before [9]. We may also exclude the possibility that a ∆sfiA mutation – which we introduced in priA2 cells to prevent the priA2-induced SOS response to inhibit cell division – modulates the stability of RF or the rate at which they are inactivated since we established that the rate, at which inactivated RF accumulate, was identical in dnaC2 sfiA+ and in dnaC2 sfiA− cells (data not shown). An additional point may be made with regards to the SOS response: is it possible that the SOS response, which is induced in dnaC2 cells at non-permissive temperature [23], modifies the fate of ongoing rounds of replication? In this respect, an over-stabilization of active RF in dnaC2 cells in response to the induction of the SOS response may be excluded since this response is induced in priA2 cells as well. We therefore conclude that the same quantity of RF was inactivated in dnaC2 priA− and in dnaC2 priA2 cells during the Novobiocin treatment.

We considered also the possibility that some inducible Stable DNA replication (iSDR) – induced during the SOS response [24] – be initiated in dnaC2 cells and misinterpreted as RF reactivation. For this hypothesis to be valid, however, one would have to assume that the replicative helicase can be loaded in dnaC2 cells at non-permissive temperature as well, since DnaC and PriA activities were shown to be required for the initiation of iSDR [25–26].

The reduced cfu of priA2 cells grown with Novobiocin at a concentration of 1 μg/ml indicates that RF are also inactivated at this concentration of drug (Figure 3A). Hence, one may argue that in addition to the RF that were inactivated during the incubation of the cells with Novobiocin at a concentration of 10 mg/ml, other RF were inactivated during the so-called “permissive conditions” of our experiment, i.e., after Novobiocin was diluted to a concentration of 1 μg/ml. The quantity of inactivated RF was estimated. The proportion of cells with active RF was merely reduced by 1.9 and 4.5% in dnaC2 priA+ and dnaC2 priA2 cells, respectively, after a 30 minutes incubation of the cells with Novobiocin at a concentration of 1 μg/ml (condition N0 in Figure 4). In contrast, the proportion of dnaC2 priA+ and dnaC2 priA2 cells with active RF was reduced by 30 and 60%, respectively, after a 10 minutes incubation of the cells with Novobiocin at a concentration of 10 μg/ml. Thus, and although the objection is legitimate, we considered the fraction of cells in which RF were inactivated during the incubation of the cells with Novobiocin at 1 μg/ml to be low enough to be insignificant. Hence, the calculation of the proportion of cells with active RF after 40 minutes of treatment with Novobiocin, as presented here, is appropriate and meaningful to determine whether arrested RF were or were not reactivated in dnaC2 cells at non-permissive temperature.

dnaC2 priA2 and dnaC+ priA2 cells accumulate inactivated RF at a similar rate in response to Novobiocin (0.084 and 0.09 min−1 in dnaC2 priA2 and dnaC+ priA2, respectively), which indicates that the rate at which priA2 cells accumulate inactivated RF is independent of the allele of dnaC. Under the same experimental conditions, priA− dnaC2 cells accumulate inactivated RF at a much lower rate (0.033 min−1), while inactivated RF were undetectable in priA+ dnaA46 cells. The reactivation of a fraction of arrested RF is dependent on DnaC, in agreement with previous results [12]. Altogether, these results indicate that among the arrested RF, 40% need active DnaC for their reactivation, while 60% do not. (Materials and Methods). Granted that around 18% of dnaC2 cells fail to complete replication when cultivated at non-permissive temperature (i.e., cells in which arrested RF were not reactivated) [12], and assuming that the efficiency of RF reactivation in dnaC2 cells at non-permissive temperature is the same with and without Novobiocin, we may conclude that around 45% of replications are arrested before completion, under normal growth conditions.

This study raises new questions about the activity and the function specified by DnaC2 at non-permissive temperature. Can DnaB be loaded onto DNA without the assistance of DnaC? The replicative helicase is extremely stable as a hexameric ring and DnaC is referred to as a ring breaker in E. coli because it catalyzes the opening of the ring, which is required to place the helicase onto single-stranded DNA [2]. Thus, the loading of DnaB on DNA without the help of a loader may be excluded. Is it possible, however, that another factor specify the function of loading the replicative helicase onto DNA? During the replication of the bacteriophage lambda, for example, the loading of DnaB at the origin of replication of the phage is ensured by the phage protein λP and not by DnaC [27]. Our strain is devoid of lambda [20] and the inspection of its genome (through BLAST) did not reveal the presence of close or distantly related copies of the lambda P gene. Yet, we cannot exclude that an alternative replicative helicase loading system be specified in E. coli. Since DnaC is an essential gene, however, such an alternative replicative helicase loading system ought to work specifically during the reactivation of arrested RF and not during the initiation of replication.

Is DnaC systematically required to reactivate arrested RF? If DnaB were still present on arrested RF, for example, its reactivation should not require DnaC. Such a situation should not require PriA either, because the very function of PriA is to assist the loading of the replicative helicase onto DNA [9]. Thus, this hypothesis may be excluded. We may instead consider that DnaC2 is active for reloading DnaB at arrested RF and at non-permissive temperature. Yet, if DnaB can be loaded at arrested RF by DnaC2 at non-permissive temperature, why cannot DnaB be loaded at oriC by DnaC2 during the initiation of replication? The fact that different partners are involved in the recognition of the helicase complex is most certainly part of the answer. During replication initiation at oriC, the replicative helicase complex interacts directly with DnaA [6], while the replicative helicase is presented to a complex composed of primosomal proteins during the reactivation of the RF [9]. It is tempting to speculate that the aptitude of the DnaC2 mutant protein to load the replicative
helicase at non permissive temperature reflects indirectly a property acquired by the helicase loader through evolution. While RF reactivation is vital for the cell and has to be ensured by any means, the blockage of the loading of the replicative helicase during replication initiation is not deleterious and may – in addition to the already known activities regulating this stage [28] - bring an additional level of control for the cell to verify that the conditions are appropriate to initiate replication of the chromosone. Thus, it is possible that structural peculiarities of the primosomal complex, specific interactions between the primosomal complex and the helicase complex, or even an as yet unknown additional factor, facilitate specifically the loading of the helicase during RF reactivation.

References
1. Kim S, Dallmann HG, McHenry CS, Marians KJ (1996) Coupling of a replicative polymerase and helicase: a tau-DnaB interaction mediates rapid replication fork movement. Cell 84: 643–650.
2. Davey MJ, O’Donnell O (2003) Replicative helicase loaders: ring breakers and ring makers. Curr Biol 13: R94–958.
3. Galletto R, Jastrzebska MJ, Bujalowski W (2003) Interactions of the Escherichia coli DnaB Helicase hexamer with the Replication Factor the DnaC Protein. Effect of Nucleotide Cofactors and the sDNA on Protein-Protein Interactions and the Topology of the Complex. J Mol Biol 329: 441–465.
4. Makowska-Grozycka M, Kaguni JM (2010) Primase directs the release of DnaC from DnaB. Mol Cell 37: 90–101.
5. Kornberg A, Baker TA (1989) DNA Replication. Freeman, San Francisco.
6. Mott ML, Erzberger JP, Coons MM, Berger JM (2000) Structural synergy and molecular crosstalk between bacterial helicase loaders and replication initiators, Cell 135: 623–634.
7. Keyamura K, Abe Y, Hijikash M, Ueda T, Katayama T (2009) DnaA dynamics are coupled with changes in initial origin complexes leading to helicase loading, J Biol Chem 284: 25031–25050.
8. Sandler SJ (2000) Multiple genetic pathways for restarting DNA replication forks in Escherichia coli K-12. Genetics 155: 497–499.
9. Heller RC, Marians KJ (2006) Replisome assembly and the direct restart of stalled replication forks. Nat Rev Mol Cell Biol. pp 932–943.
10. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, et al. (2012) Fiji: an open-source platform for biological-image analysis. Nat Methods 9: 676–682.
11. Elmore SD, Davis IV, Wolf J, Farooq UA, Reisler R, et al. (2010) k-means++: An iterative clustering algorithm for discovering a diverse subset of representative images. In: Proceedings of the 13th International Conference on Image Analysis and Recognition. Lecture Notes in Computer Science, vol 6250. Springer, Berlin, pp 701–708.

Acknowledgments
We are thankful to Bénédicte Michel for suggestions, fruitful discussions and for providing strains. We are grateful to Linda Sperling for editing the manuscript and to Lila Ferat for technical assistance. We thank François Michel for his support.

Author Contributions
Conceived and designed the experiments: JLF. Performed the experiments: JLF BS. Analyzed the data: JLF BS. Contributed reagents/materials/analysis tools: JLF BS. Wrote the paper: JLF.