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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