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Deoxyribonucleic acid replication time in *Mycobacterium tuberculosis* H37 Rv

K. T. Hiriyanna * and T. Ramakrishnan

Microbiology and Cell Biology Laboratory and ICMR Unit on Genetics and Cell Biology, Indian Institute of Science, Bangalore 560012, India

Abstract. The DNA increment method, designed for measuring the increment in the amount of DNA after inhibition of initiation of fresh rounds of replication initiation was employed to measure the rate of deoxyribonucleic acid (DNA) chain growth in *Mycobacterium tuberculosis* H37Rv growing in Youman and Karlson's medium at 37 °C with a generation time of 24 h and also in relatively fast growing species like *Mycobacterium smegmatis* and *Escherichia coli*. From the results obtained, the time required for a DNA replication fork to traverse the chromosome from origin to terminus (C period) was calculated. The chain elongation rates of DNA of the three organisms was determined from the C period and the known genome sizes assuming that all these genomes have a single replication origin and bi-directional replication fork. The rate for *M. tuberculosis* was 3,200 nucleotides per min about 11 times slower than that of *M. smegmatis* and about 13–18 times slower than that of *E. coli*.

Key words: DNA Replication – *Mycobacterium tuberculosis* – Replication time

Mycobacterium tuberculosis, H37 Rv, the causative organism of tuberculosis in man, has an extremely slow growth rate in *in vitro* cultures. On the premise that the slow growth of the organism reflects a retarded rate of macromolecular synthesis and a consequent step down of its metabolic machinery, we investigated its rate of ribonucleic acid chain growth. The results indicated that the rate of RNA chain growth in *M. tuberculosis* is 10 times lower than that in *Escherichia coli*. However, this does not wholly explain the rather larger difference in the growth rates between the two organisms. This prompted us to undertake studies on DNA replication, since it has been shown in other well studied organisms that this process is rather intimately regulated and coordinated with cell growth.

The time required for a DNA replication fork to traverse the chromosome from origin to terminus – the C period – is a fundamental parameter of bacterial growth. We have carried out a comparative study of this parameter in *M. tuberculosis* and the relatively fast growing species like *M. smegmatis* and *E. coli* by making use of the DNA increment method, along with the data evaluation procedure of Bremer and Churchward (1977).

Offprint requests to: T. Ramakrishnan

* *Present address:* Department of Biology, Johns Hopkins University, Baltimore, MD 21218, USA

Abbreviations. DNA, deoxyribonucleic acid; td, delay in initiation; OD, optical density; CAM, chloramphenicol; RIF, rifampicin

Materials and methods

Bacterial strains. Mycobacterium tuberculosis H37 Rv strain No. 7416 was obtained from the National Collection of Type Cultures (England) and maintained by regular sub-culture on Petrick's solid medium (Gradwohl 1948). The virulence of the organism was confirmed by infecting mice.

M. smegmatis (SN2) was obtained from Institute for Experimental Biology and Medicine, Borstel, FRG.

Escherichia coli strains K12 and B/r (thy$^-$) were obtained from Dr. S. P. Champe, Rutgers State University, New Brunswick, NJ, USA.

Growth measurements of bacteria. The bacterial strains were grown as suspension cultures in the medium of Youmans and Karlson (1947). Growth was monitored as optical density (absorbance) at 540 nm in a Shimadzu double beam spectrophotometer, as wet weight and as cell number. Cell counts were done in a hemocytometer. Since both *M. tuberculosis* and *M. smegmatis* show considerable clumping, the samples were mildly disrupted using a Dounce homogenizer under judiciously chosen conditions (0.4% Tween 80, 0.5% formaldehyde and low speed) before counting under the microscope. Viable cell counting was done for *M. smegmatis* and *E. coli* by plating the dilutions on nutrient agar. Growth rate constants (K) and generation time (τ) in the log phase cultures were estimated using the standard techniques to measure mass doubling time.

Labelling of DNA. Since wild type *M. tuberculosis* and *M. smegmatis* incorporate externally added thymine into their DNA more efficiently than thymidine (Hiriyanna and Ramakrishnan, unpublished observations), radiolabelling was carried out using ^{3}H- or ^{14}C-thymine (Bhabha Atomic Research Centre, Bombay, India). Doubling labelling was carried out by adding ^{14}C-thymine and ^{3}H-thymine at different stages of the growth. Duplicate 1 ml samples at various time points were taken in a test tube and precipitated with 2 ml of cold 10% TCA containing 0.2 M sodium pyrophosphate. The precipitated cells, which were kept for 1 or 2 h at 0 °C, were filtered through Whatman 3 MM filter discs and washed with 30 ml of 5% TCA, followed by 10 ml of 90% ethanol. The discs were then dried and counted in

Table 1. C periods and the mean generation times

	Mycobacterium tuberculosis H37Rv	*M. smegmatis* SN2	*Escherichia coli* B
Mean generation time (τ)	24 ± 2 h	180 ± 20 min	82 ± 4 min
Drug used for inhibition of initiations	CAM RIF	CAM RIF	CAM RIF
Apparent C period obtained from $\sqrt{\Gamma t}$ plotting	12.5 h 10.6 h	120 min 105 min	65 min 60 min
'td' value obtained from $\sqrt{\gamma t}$ plotting	1.5 h 0.30 h	10 min 0 min	7 min 5 min
Correct C period (apparent C − *td*)	11.0 h 10.3 h	110 min 105 min	58 min 55 min

Details of the experiment were as described in the text and Figs. 1–3

Beckman LS-100 liquid scientillation spectrometer. Suitable corrections were made whenever double labelling was used, and also for quenching due to added compounds (like rifampicin).

Measurement of DNA increment after the addition of rifampicin or chloramphenicol. DNA increment method, in which one measures the increment in the amount of DNA after inhibition of initiation of fresh rounds of replication initiation by rifampicin or chloramphenicol, was employed. The cell cultures of *M. tuberculosis* H37 Rv, *M. smegmatis* and *E. coli* B were grown to exponential phase in Youmans and Karlson's medium containing 0.05 μCi/ml of ^{14}C-thymine (sp. act. 57 mCi/mmol) and DNA synthesis was followed by taking 1 ml of the suspension at intervals and processing for counting as described above. When the OD reached a value of 1.2 for *M. tuberculosis* and *M. smegmatis* and 0.8 for *E. coli,* chloramphenicol or rifampicin were added to a final concentration of 200 μg/ml followed by the immediate addition of ^{3}H-labelled thymine (1.2 μCi/ml, sp. act. 12 Ci/mmol). Kinetics of the label incorporation were followed by taking 1 ml samples of the culture at regular intervals (every 5 min in *E. coli,* every 10 min in *M. smegmatis* and every 1 h in case of *M. tuberculosis*). Subsequent processing and counting were as described earlier.

Calculation of C period from the DNA increment data. Protein and RNA synthesis inhibitors like chloramphenicol and rifampicin have been shown to inhibit fresh DNA replication initiation (Kornberg 1980) without inhibiting the ongoing rounds of replication that continues till all the replication forks reach the termination point (Churchward and Bremer 1977). Based on the theory developed by Bremer and Churchward (1977) the apparent C period and time delay in initiation (*td*) were calculated quantitatively from the kinetics of residual DNA accumulation after inhibition of initiation.

The difference between the apparent C period and *td* is the real C period.

Theoretical curves were generated by assigning various values for C (*M. tuberculosis* 9–13 h; *M. smegmatis,* 80–100 min; *E. coli,* 40–60 min) and from experimentally determined τ for the respective species.

Calculation of DNA chain elongation rates. DNA chain elongation rates were calculated from the determined C values and the known genome sizes with the assumption that mycobacterial DNA replicates from a single origin with bidirectional replication forks. Another implicit assumption is that the rates of the moving forks are unaffected by the drug treatment.

Results

Growth pattern and generation time. Growth constant (K) and generation time (τ) for *Mycobacterium tuberculosis* H37 Rv, *M. smegmatis* SN2 and *Escherichia coli* in Youmans and Karlson's medium have been tabulated in Table 1. As observed earlier by Youmans and Youmans (1949), a higher inoculum (2–4% of 5 days old preculture) gives more consistent results in the case of *M. tuberculosis.* The extent of growth, as indicated by the OD values reached towards the end of the log phase of *M. smegmatis* culture was found to be 3–4-fold higher than that reached by *E. coli* and that of *M. tuberculosis* was 2–2.5-fold higher than the latter.

Increment of DNA in the presence of rifampicin or chloramphenicol. The general features of the kinetics of DNA accumulation after the addition of rifampicin or chloramphenicol (at 250 μg/ml concentration) to cultures of *M. tuberculosis* H37 Rv (Fig. 1a), *M. smegmatis* (Fig. 2a) and *E. coli* (Fig. 3a) have been analysed. The results show that the accumulation of DNA continues after the addition of the drugs and reached a plateau value after a time period characteristic of the species. For *M. tuberculosis* it is 11–12 h (Fig. 1b) for *M. smegmatis* it is 95–120 min (Fig. 2b) and for *E. coli* 60–70 min (Fig. 3b). The time delay in initiation for the three organisms is given in Figs. 1c, 2c and 3c.

C period determination. Experimental values for apparent C period calculated from $\sqrt{\Gamma t}$ curve, *td* calculated from $\sqrt{\gamma t}$ curve and corrected C periods are presented in Table 1. As can be seen from the table, with chloramphenicol as the initiation inhibitor, higher apparent C values were obtained, whereas in rifampicin treated cells the C values obtained are relatively lower. The *td* values are also correspondingly higher in chloramphenicol treated cells indicating that there is relatively longer delay in inhibition. With *M. smegmatis,* however, the *td* values are lower when compared to *M. tuberculosis.* As can be seen from the table, *M. tuberculosis* has an unusually long C period when compared to *M. smegmatis.* The C period values calculated for *E. coli* are relatively higher when compared to the C period values determined by some earlier workers (Pritchard and Zaritsky 1971) but close to the values obtained by recent workers (Churchward and Bremer 1977) for a culture growing with similar generation times. Theoretical curves with an assigned C value of 11 h corresponds most to the experimentally observed DNA increment kinetics for *M. tuberculosis.* The experimental patterns closely resembled the theoretical ones for *M. smegmatis* also, indicating that the theory of Bremer and Churchward (1977) with its assumptions holds good for *M. tuberculosis* and *M. smegmatis.*

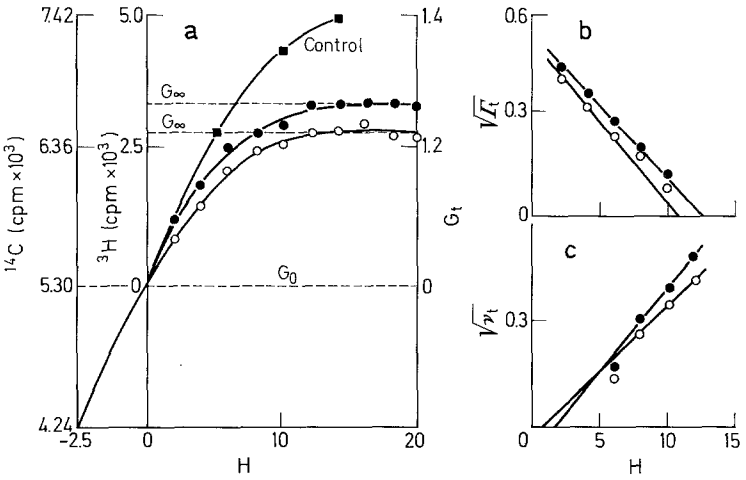

Fig. 1 a – c. DNA increment kinetics after initiation inhibition in *Myobacterium tuberculosis* H37 and $\sqrt{\Gamma t}$ and $\sqrt{\gamma t}$ plots. **a** The cells were labeled continuously with ^{14}C-thymine (0.05 μCi/ml). When the culture reaches the log phase, rifampicin and chloramphenicol were added to separate aliquots of the culture and the incubation was continued further with the immediate addition of ^{3}H-thymine (1.2 μCi/ml). Amounts of DNA present (relative value calculated by measuring ^{14}C-thymine incorporation) at the point of addition of the drug is considered to be equal to 1 = G_0. Gt and G_∞ were calculated from the kinetics as described in the text. (■——■) Control (no drug added). Both ^{3}H- and ^{14}C-thymine uptake are represented. (●——●) Chloramphenicol treated; (○——○) rifampicin treated. **b** $\sqrt{\Gamma t}$ was calculated from the values of Gt, G_0 and G_∞ as described in the materials and methods (●——●). Chloramphenicol treated; (○——○) rifampicin treated. The point where the extrapolated line cuts the horizontal time axis represents the apparent C period. **c** $\sqrt{\gamma t}$ was calculated as described in Materials and methods. ●——● Chloramphenicol treated; ○——○ rifampicin treated

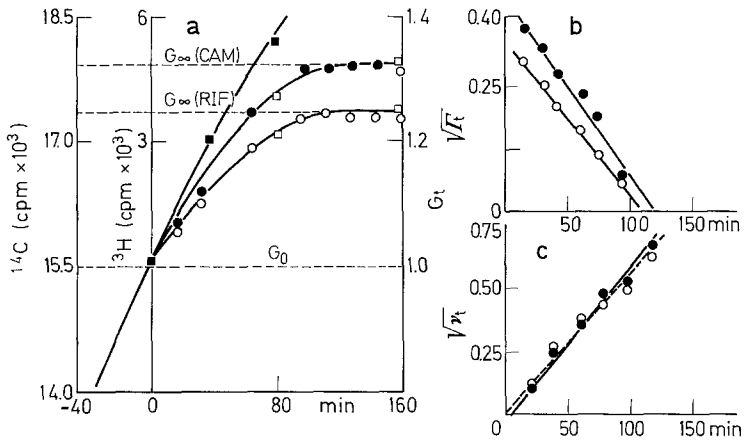

Fig. 2 a – c
DNA increment kinetics after initiation inhibition in *M. smegmatis* SN2. Experiments carried out under similar conditions as described for *M. tuberculosis* (Fig. 1). **a** Under similar conditions described in Fig. 1. (□——□) ^{14}C-thymine incorporation; ■——■ control; (●——●) chloramphenicol treated; ○——○ rifampicin treated

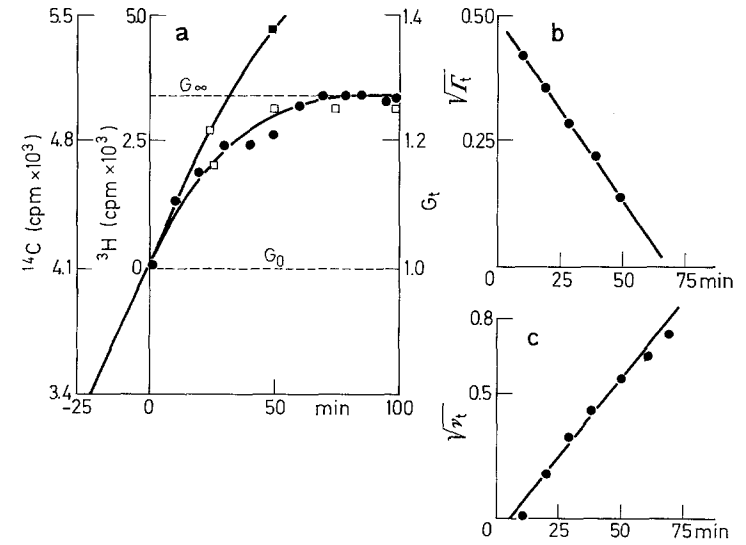

Fig. 3 a – c
DNA increment kinetics after initiation inhibition in *E. coli* B. Experiments wre carried out under similar conditions as described for Figs. 1 and 2. **a** (■——■) ^{14}C-thymine incorporation (control); (□——□) ^{14}C-thymine incorporation; (●——●) ^{3}H-thymine incorporation into chloramphenicol treated cultures. **b** and **c** Chloramphenicol treated

Table 2. DNA chain elongation rates

	M. tuberculosis H37Rv	*M. smegmatis* SN2	*E. coli* B	*E. coli* B/r
Molecular weight of the genome	2.6×10^9	4.5×10^9	2.8×10^9	2.8×10^9
Length of the genome in kilo base pairs	3900	6800	4300	4300
C period in minutes	620	105	55	40
Chain elongation rate (nucleotide monomer per min)	3200	32400	39000	53750

DNA chain elongation rates (velocity of the replication fork) was calculated using the data available on the molecular weights of the genomes for *M. tuberculosis* (Barksdale and Kim 1977). The procedures adopted in the calculations are described in the text

DNA chain elongation rates. The DNA chain elongation rate (that is, the mean rate at which nucleotides are added to the growing chain at the DNA replication fork in vivo by the replication complex) or the velocity of DNA replication was calculated for the organism under study using the method and assumption described under Materials and methods. As can be seen from Table 2, the rate of chain elongation in *M. tuberculosis* is about 11 times slower than that of *M. smegmatis* and about 13–18 times slower than that of *E. coli*. DNA chain elongation rates of *M. smegmatis* and *E. coli* are comparable.

Discussion

The increment in the amount of DNA in growing bacteria after inhibition of initiation of rounds of replication has been used by several workers to estimate the time C taken to replicate the bacterial chromosome (Lane and Dernhardt 1975; Maaløe and Hanawalt 1961; Pritchard and Zaritsky 1971; Zaritsky and Pritchard 1971). We have used rifampicin and chloramphenicol to effect this inhibition. However, we have taken the values of C periods determined by using rifampicin in the further calculations on chain elongation rates, since the delay in inhibition (*td*) with this drug was less than that with chloramphenicol. Further, mycobacteria and their RNA polymerases have been shown to be relatively highly sensitive to rifampicin (Barksdale and Kim 1977; Tamonoi et al. 1980; Woodley et al. 1981) and less sensitive to chloramphenicol. Rifampicin is a better candidate for yet another reason, as it has been shown both in vitro and in vivo that the RNA polymerase might be directly involved in the replication origin activation leading to inhibition (Bagdasarian et al. 1977; Projan and Wechler 1981) and that the initiation process is sensitive to rifampicin. However, a small lag (*td*) observed before inhibition of initiation in mycobacterial cells could be due to the fact that the drug is not taken up in sufficient concentration immediately.

The data evaluation method of Bremer and Churchward (1977) has been used to determine the delay and the corrected values for C period have been obtained. It is clear that the values for C period especially in the case of *Mycobacterium tuberculosis* is about 12 times higher than that of *Escherichia coli* B.

Support for the values determined by us for C period of *M. tuberculosis* comes from the recent observations of Woodley et al. (1981). They employed an entirely different method, wherein they have tried to map drug resistant markers using nitrosoguanidine sequential mutagenesis. They observed that the frequency of mutation for any drug-resistant markers doubles every 10 h, indicating the fact that the C period could be 10 h.

In the calculation of chain elongation rates using the known values for the size of the mycobacterial and *E. coli* genome, we have implicitly assumed a single replication origin and a bidirectional replicational fork. We do not yet have direct evidence for the nature of origin in mycobacteria, either from our studies or from other laboratories. However, all the bacterial genomes so far investigated, e.g., *E. coli*, *Salmonella typhimurium* and many of the plasmids and phages have been shown to possess single replication origins and bidirectional replication forks. However, the presence of more than a single replication origin has been shown in case of T7 (Tamonoi et al. 1980) and *B. subtilis* genome (Seiki et al. 1981), but it has been demonstrated that in any case only one origin (ori) is dominant and at a given time only one origin is used for replication. Unidirectional replication fork has been demonstrated for coli E1 and related plasmids. However, barring these exceptional cases, in all other instances, a single origin and bidirectional replication forks have been reported in case of prokaryotes. We feel that the value of C would not be seriously affected in either case because these experiments were done with non-synchronized exponential cultures. On this basis the DNA chain elongation rate in *M. tuberculosis* is 28 times slower than *E. coli* growing with a τ of 20–40 min and has a close correspondence to its slow growth rate.

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