The Journal of Antibiotics (2019) 72:225–236
https://doi.org/10.1038/s41429-019-0149-0

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ARTICLE

Doxycycline inhibits pre-rRNA processing and mature rRNA formation in *E. coli*

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Received: 4 December 2018 / Revised: 11 January 2019 / Accepted: 22 January 2019 / Published online: 8 February 2019

Abstract

In bacteria, RNase III cleaves the initial long primary ribosomal RNA transcripts/precursors (pre-rRNAs), thereby releasing the pre-16S and pre-23S rRNAs for maturation. This cleavage is specified by the double-stranded secondary structures flanking the mature rRNAs, and not necessarily by the nucleotide sequences. Inhibition of this cleavage would lead to a build-up of pre-rRNA molecules. Doxycycline has earlier been shown to bind synthetic double-stranded RNAs and inhibit their cleavage by RNase III. Since bacterial rRNA processing is primarily dependent on RNase III cleavage (which is inhibited by doxycycline), doxycycline could therefore inhibit the normal processing of bacterial rRNA. In this study, the effect of doxycycline on bacterial rRNA processing was investigated by analyzing the amounts of various rRNAs in growing *Escherichia coli* cells treated with doxycycline. The results showed a doxycycline dose-dependent decrease in mature 16S and 23S rRNAs, concurrent with an accumulation of the initial rRNA transcripts and long precursors. Morphologically, treated cells were elongated at low drug concentrations, while nucleoid degeneration indicative of cell death occurred at higher drug concentrations. These observations suggest that doxycycline inhibits the cleavage and processing of bacterial rRNA transcripts/precursors, leading to impaired formation of mature rRNAs, and the consequent inhibition of protein synthesis for which the tetracycline group of antibiotics are renowned. Since rRNA structure and processing pathway is conserved among bacterial species, this mechanism may account for the broad spectrum of antibiotic activity and selective microbial protein synthesis inhibition of doxycycline and the tetracyclines.

Introduction

Doxycycline is a member of the broad spectrum group of antibiotics known as the tetracyclines. The tetracyclines are known to inhibit bacterial protein synthesis by binding to the 16S ribosomal RNA (rRNA) and inhibiting the binding of aminoacyl-tRNA to the mRNA-ribosome complex [1–4]. However, their activity against other microbes which do not possess the 16S rRNA such as viruses, protozoa, and helminths has raised further questions as to the exact mechanism of action. In addition, despite conservation of ribosome structure and function between bacteria and host cells, the tetracyclines are sufficiently selective that the protein synthesis machinery of the host organism remains relatively unaffected. Despite their long history of usage as therapeutic agents, the mechanism(s) by which the tetracyclines achieve their wide range of effects and selectively inhibit microbial protein synthesis is not yet fully understood.

Even though binding interactions with both the 16S and 23S rRNAs had earlier been indicated for the tetracyclines [5], an in vitro study to correlate ribosomal subunit activity with drug binding suggested that inhibition of tRNA binding to the A-site is solely due to tetracycline cross-linked to the strong binding site on the 30S ribosomal subunit [6]. Hence, subsequent investigations on the mechanism of action of the tetracyclines and their interaction with ribosomal RNA concentrated on the 16S rRNA of the 30S ribosomal subunit [7]. Nevertheless, a recent study has shown that the tetracyclines (doxycycline and minocycline) bind to various synthetic double-stranded RNAs of random base sequence and inhibit their cleavage by RNase III in vitro [8]. This could imply that the double-stranded

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secondary structures that frequently occur in cellular RNAs may be more crucial for the binding of the tetracyclines to RNA than the specific base pairs; and that the mechanism of action of the tetracyclines may be linked to the effect of the drugs on the processing of such cellular RNAs. If this is correlated in vivo, it could offer insights into the mechanism that underlie the activity of the tetracyclines against a wide range of pathogens, as well as in other non-infectious therapeutic indications of the drugs.

Ribosomal RNAs constitute about 95% of total cellular RNA in *E. coli* [9]. They form the active sites of the ribosomes for decoding the message of the mRNA, as well as perform enzymatic functions in the translation process [10]. The rRNAs of prokaryotes are co-transcribed from an operon (Fig. 1), and *E. coli* has 7 copies of rRNA operons in the chromosome [11]. RNase III then cleaves the nascent rRNA transcripts at the double-stranded stem regions that flank the mature 16S and 23S ribosomal RNA sequences to release the pre-16S and pre-23S rRNAs for further maturation [11–13]. In wild-type cells, RNase III cleavage is very rapid, and occurs concurrently with transcription. Hence, only a very small amount of the long primary transcript (1–2% of total rRNA) is reported to be detectable in *E. coli* [14]. However, in RNase III-deficient strains, the 30S pre-rRNA accumulates [15]. In this study, the effects of doxycycline on the processing of bacterial ribosomal RNAs were investigated and correlated with their antibiotic activity in growing *E. coli* cells, with a view to elucidate the molecular mechanism of their antimicrobial activity.

Materials and methods

Probes and primers used in this study are listed in Table 1, and obtained from Sigma® Aldrich. Doxycycline was also purchased from Sigma® Aldrich. Nylon membranes were purchased from Roche (Roche # 11209299001). Hybridization probe labelling and detection was done using AlkPhos Direct™ labelling and detection System with ECF™ from GE Healthcare Life Sciences (RPN3692).

All procedures (sample collection, RNA extraction, gel electrophoresis, transfer to nylon membranes and northern blot hybridization) were performed at least in replicates of three independent experiments.

Total RNA extraction

An overnight culture of *E. coli* strain K-12 grown in LB broth was diluted 20-fold with fresh medium and incubated at 37 °C in a Stuart orbital incubator S1500 with shaking for 1 h to ensure growth is activated. The culture was divided into aliquots to which were added 0–200 µM doxycycline (0–96 µg ml^{-1}) and incubated at 37 °C with shaking (180 rpm). Optical density (OD) of cultures was measured at 550 nm using Biotek Powerwave XS universal spectrometer. Two millilitres of culture samples were taken from each treatment group at the indicated time points (0–240 min). Nucleic acid decay was stopped in collected samples by immediate transfer of the samples to a cold microcentrifuge tube (on ice) containing 200 µl ethanol and 20 µl phenol [16]. The bacterial cells were harvested by centrifuging at 8000 rpm for 1 min, and lysed by re-suspending the pellet in 400 µl of Sigma B cell lysis reagent (Sigma® Aldrich). Total RNA was harvested by the phenol-chloroform extraction method with ethanol precipitation [17]. Four-hundred microlitres of phenol (Sigma) was added to the samples and the tube vortexed vigorously for at least 2 min in total. Four-hundred microlitres of RNase-free water and 400 µl of chloroform (Sigma) was then added and vortexed vigorously for another 2 min. The tube was then centrifuged at 12,000 rpm for 15 min and 600 µl of the aqueous phase transferred to a fresh 2 ml tube. Sixty microlitres of 3 M sodium acetate and 1.4 ml ethanol was added, mixed and held on ice for 10 min, and centrifuged at 10,000 rpm in a microcentrifuge for 15 min. The supernatant was removed, and the pellet rinsed with 70% ethanol and dried. The resultant nucleic acid pellets were resuspended in 1× TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA) and stored at −20 °C until further use. The nucleic acid concentration of the extracts was quantified using NanoDrop ND-1000 spectrophotometer, and scored as an average of at least three readings.

Northern blot analysis of mature rRNA from *E. coli* total RNA extract

Ribosomal RNA was separated from the total RNA extracts by agarose gel electrophoresis. Five microliters of total RNA extracts from each sample was loaded in 1% agarose gels containing 1× MOPS buffer. For denaturing gel electrophoresis, the RNA samples were incubated with 3× volume of formaldehyde loading dye (Ambion) at 65 °C for 10 min before loading. Electrophoresis was carried out at 90 V for 40 min in 1× MOPS buffer and gels were stained with 1X EtBr or SYBR® Gold (Invitrogen™). Images were taken with SynGene G:Box camera using GeneSnap software. The rRNA was subsequently transferred unto positively charged nylon membranes by gravity and capillary action. The gel was soaked in 20× saline-sodium citrate (SSC) buffer for 30 min, placed right side up on the nylon membrane without trapping air bubbles in-between and covered on both sides with Whatman filter paper that had been cut to size and soaked in 20× SSC buffer. The gel was wrapped around the edges

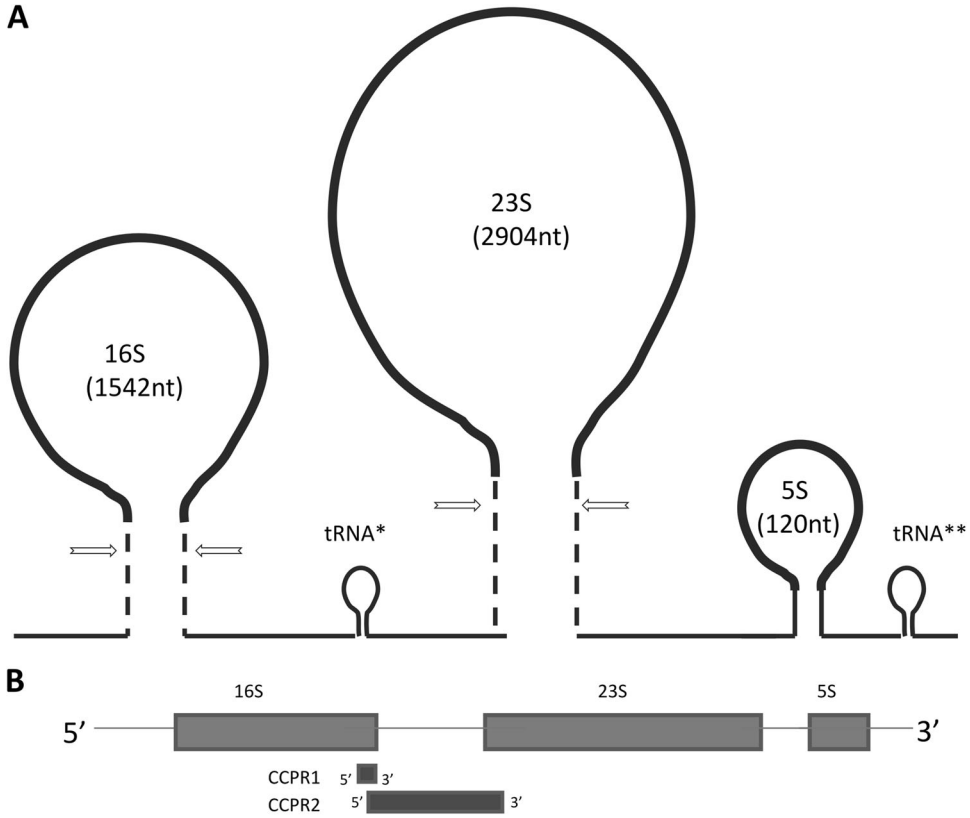

Fig. 1 Schematic representation of the primary transcript of ribosomal RNA of *E. coli*. **a** Mature rRNA sequences are indicated as bold line loops, and dsRNA within the precursor sequences represented by the stems (not drawn to scale). Arrows indicate proposed regions of double-stranded primary transcript RNase III cleavage sites, where cleavage releases the pre-16S and pre-23S rRNAs for further maturation to produce mature rRNAs. The * and ** symbols indicate the number of tRNA molecules within the operon at the indicated sites. * = 1–2, ** = 0–4. In addition, the *rrnD* in *E. coli* has two genes encoding 5S rRNA. **b** Target position of the hybridization probes in relation to the mature ribosomal RNAs in the long primary rRNA transcript of *E. coli*

Table 1 Hybridization probes used for pre-rRNA northern blotting

Probe/primer	Target description[a]	Length	Sequence	Reference
ECR2	Mature 16S rRNA	28	5′-gtccccctctttggtcttgcgacgttat-3'	[38]
ECPR2	3′ pre-16S rRNA tail (rrnA, -D, -G, -H)	30	5′-gtgtgagcactgcaaagtacgcttctttaa-3'	[38]
CCPR1	Pre-16S rRNA (3′ rrnA, -H; 5′ -D, -G)	50	5′-cctgtagaggttttactgctcattttcatcagacaatctgtgtgagcact-3′	This work
CCPR2	Pre-rRNA (3′ end of 16S to 5′ end of 23S)	457	[a]	This work
CCPR2 forward primer	3′ end of 16S rRNA	22	5′-cacctccttaccttaaagaagc-3′	This work
CCPR2 reverse primer	5′ end of 23S rRNA	19	5′-tcgcttaacctcacaaccc-3′	This work

[a]See Fig. 1b for illustration of region of complementarity with target pre-rRNA

with parafilm to prevent drying and ensure that transfer proceeds only through the gel. About 2 cm of paper towel was also cut to size, soaked in 10× SSC buffer and placed on top of the filter paper. A small flat weight was then placed on top of the stack which was allowed to stand overnight. After overnight transfer, the membrane was rinsed with 2× SSC buffer and air-dried. RNA was cross-linked to the membrane by UV illumination for 1–1.5 min using SynGene G:Box transilluminator. Membranes were then stained with 2% methylene blue and de-stained in 2% SSC buffer to check for mature rRNA bands (visible to the naked eye). Images were taken using SynGene G:Box image camera. Membranes were subsequently stored dry at 4 °C until used for hybridization.

Probe design

CCPR1 was designed to target a sequence 21 base pairs from the 3′ end of *E. coli* K-12 16S rRNA, and purchased from Sigma. CCPR2 was designed to target the sequence between 10 bp downstream of K-12 16S rRNA and 10 bp upstream of 23S rRNA, including the intergenic sequences (Fig. 1b). BLAST search (Basic Local Alignment Search Tool, NCBI) indicated no similarity to any other *E. coli*

gene segment apart from the ribosomal RNA. CCPR2 was synthesized by PCR amplification using the primers shown on Table 1. The amplicon size was verified by matching the band of the PCR product on agarose gel electrophoresis with the band of DNA ladder of the expected size.

Nucleic acid hybridization for detection of pre-rRNAs

The probe was labelled with alkaline phosphatase using AlkPhos Direct™ labelling and detection System with ECF™ (GE Healthcare Life Sciences) following the manufacturer's instructions. For this experiment, a 10-fold dilution of overnight culture of *E. coli* K12 cells was grown to exponential phase for about 1.5 h in LB broth (to ensure a high cell harvest/rRNA yield) before adding doxycycline at the specified concentrations. The cultures were then incubated for only 20 min to minimize the differential inhibitory effects of the antibiotic concentrations on culture growth (OD) and concentration of the RNA extracted. Hence, samples were collected at 20 min incubation time. The total RNA was extracted and separated by gel electrophoresis, then transferred onto nylon membranes. Membrane blots of the total RNA extracts were equilibrated in hybridization buffer for 15 min before overnight hybridization with the labelled probe in a hybridization oven at 42 °C. Membranes were washed at 42 °C with the primary wash buffer, and at room temperature with the secondary wash buffer. Detection reagent was applied to the membranes, which were then wrapped with cling film and incubated overnight in the dark to enhance development of the fluorescence signal. Blots were scanned and images were taken using SynGene G:Box camera.

Fluorescence microscopy

E. coli strain K-12 was grown in LB broth containing 0–200 μM doxycycline at 37 °C for 20 min. Cells were harvested by centrifuging and washed twice with 2× volume of 1× PBS. They were stained using DAPI (to examine the nucleoid morphology) by adding 1× volume of 1 μM DAPI (Sigma) and incubating in the dark for about 5 min. Samples were then mounted on glass slides with cover slips. Cell morphology was examined with Leica DM4000B fluorescence microscope using DAPI filter. Images were captured with DC500 camera using Leica IM500 software programme.

Data analysis

All RNA band intensities were quantified using the image analysis software, GeneTools from SynGene (Cambridge, UK). Statistical analysis was done using GraphPad Prisms 7.02. Means were compared using repeated measure ANOVA or paired *t*-test (as appropriate), while dose-response effects were analysed using non-linear regression fitted for direct (non-normalized) response. Statistical significance was considered at 95% confidence interval ($P \leq 0.05$ significance level).

Results

Doxycycline reduces the amounts of mature ribosomal RNA in vivo

In view of earlier reports that doxycycline inhibits RNase III degradation/cleavage of double-stranded RNA in vitro [8], the effect of doxycycline on RNase III-dependent dsRNA cleavage/processing pathways in vivo was investigated. The most important and generalized RNase III-dependent processing pathway in bacteria cells with respect to protein synthesis is the processing of ribosomal RNA. RNase III cleavage is the rate limiting step for the formation of mature rRNAs which is necessary for protein synthesis in growing bacteria cells. To assess the effect of doxycycline on this processing pathway in vivo, total RNA was harvested from doxycycline-treated and untreated *E. coli* K-12 cells and analysed by native agarose gel electrophoresis. The intensities of the mature ribosomal RNA bands in the cells growing in the presence of 100 μM doxycycline over a given time was compared with those of cells growing in the absence of the drug. The results show a significant ($P = 0.0046$ for 23S and 0.0091 for 16S rRNA) and progressive reduction ($r = -0.7365$ for 23S and -0.8126 for 16S rRNA) in the band intensities of mature ribosomal RNAs over time in the cells growing with doxycycline, in contrast to those growing without the drug, which showed a progressive increase in rRNA band intensities that peaked at about 210 min (Fig. 2). In the sample containing doxycycline, there was a sharp drop in the band intensities of the 16S and 23S ribosomal RNAs at 20 min, which subsequently increased slightly between 40 and 90 min before ultimately fading away. When gels of samples containing other concentrations of doxycycline (2–200 μM) over time were analysed, it was observed that this sharp drop in the band intensities of the 16S and 23S rRNAs at 20 min only occurred at higher doxycycline concentrations (100–200 μM); but at lower doxycycline concentrations (0–50 μM), the decrease is gradual and steady. These results suggest that doxycycline inhibits the formation of mature rRNAs, although the inhibition of bacterial growth in the presence of the drug may contribute to this effect.

To determine whether the observed depletion of 16S and 23S rRNAs in doxycycline-treated samples was caused by inhibited rRNA formation due to processing rather than

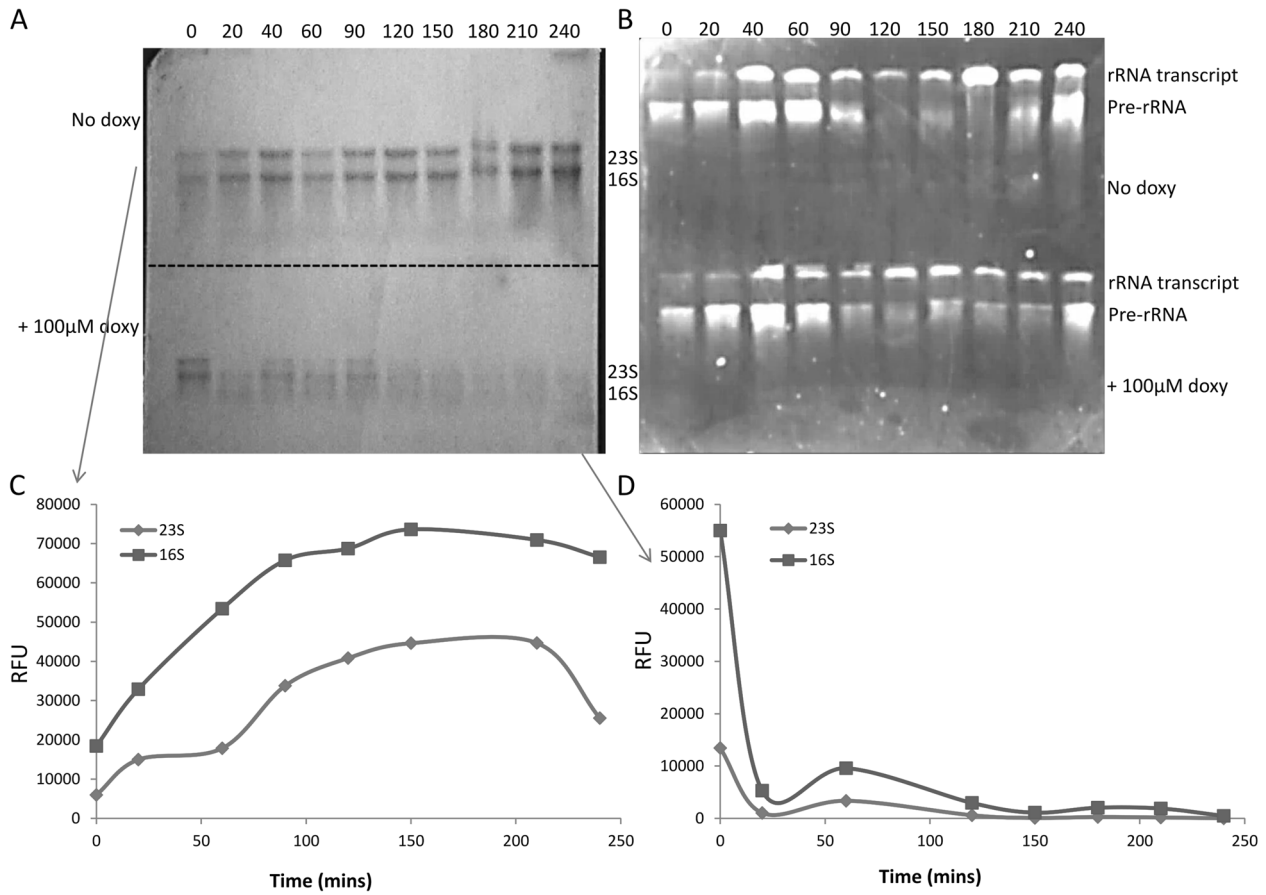

Fig. 2 Effect of doxycycline on mature rRNA amounts and rRNA sizes in growing bacteria cells over time. Northern blot membrane stained with methylene blue (**a**) of total RNA extract from *E. coli* cells growing in the absence and presence of 100 µM doxycycline at various time points during growth showing the 23S and 16S rRNAs, and the hybridized membrane blot (**b**) showing rRNA primary transcript and pre-rRNA (that indicate continued transcription of rRNA) and smearing of the pre-rRNA in the presence of doxycycline. Graphical analysis of the rRNA band intensities (**c**, **d**) show significantly decreasing amounts of 16S and 23S rRNAs with time ($P = 0.0046$, $r = -0.7365$ for 23S, and $P = 0.0091$, $r = -0.8126$ for 16S rRNA) in cells that were grown in medium containing doxycycline, when compared to the increasing amounts of the rRNAs in cells growing without the drug. RFU = relative fluorescence unit

inhibited synthesis due to growth inhibition or death of bacteria cells, the RNA samples were also analysed by northern blot hybridization to detect the pre-16S rRNA (using CCPR1 probe). If the reduction in mature rRNA band intensities seen in Fig. 2a was simply due to reduced cell numbers by the growth inhibitory activity of the drug, one would expect a commensurate and concurrent reduction in the amounts of pre-rRNA detected by northern blotting. However, northern blot hybridization assay of the samples showed the presence of long pre-rRNAs in both treated and untreated samples, with no significant difference ($P = 0.7157$) between the treated and untreated groups (Fig. 2b). There was no reduction in pre-rRNA band intensity in doxycycline-treated cells. Instead, there was smearing of the pre-rRNA bands in doxycycline-treated cells from earlier incubation time points (20–60 min), indicating the presence of variable sizes of pre-16S rRNA in all doxycycline-treated samples. This smearing only occurred at longer incubation times (120–240 min) in untreated samples. These results suggest that rRNA was still being transcribed in doxycycline-treated cells at all time points studied, but the rRNA transcripts and pre-rRNA were not adequately processed to form the mature 16S and 23S rRNAs.

Interestingly, in the course of these experiments, some samples were analysed by denaturing gel electrophoresis that was run much longer (2–4 h) to allow a better separation of the RNA bands. In these experimental conditions, we observed a difference in the profile of the long pre-rRNA bands between the samples that contain doxycycline and those without the drug. Whereas only one band was seen in the samples without the drug from about 60 min, the samples containing the drug showed an additional second band from about 210 min incubation time which represents different species/sizes of the long pre-rRNA. Since the pre-rRNA are cleavage products of the initial transcripts, the observation of different pre-rRNA sizes between doxycycline-treated and untreated cells is suggestive of impaired/abnormal cleavage or processing of the rRNA transcripts in the presence of doxycycline.

Doxycycline induces a dose-dependent inhibition of mature ribosomal RNA in growing bacteria cells

In order to further investigate the involvement of doxycycline in the observed reduction in the amounts of mature ribosomal RNA in *E. coli* K-12 cells, total RNA from cells grown in the presence of various concentrations of doxycycline was assessed. Samples collected at both 20 and 120 min of incubation time showed a dose-dependent decrease in the amounts of mature 16S and 23S rRNA with increasing amounts of doxycycline (Fig. 3). Statistical analysis showed that the concentration of doxycycline that gave a response half-way between baseline and maximal (IC_{50}) at 20 min incubation time was 8.327 μM (+/−SE 2.465, $R^2 = 0.9554$), and within the range of 4.295–17.95 μM at 95% confidence interval (CI). This increased at 120 min incubation time to 76.51 μM (+/–SE 49.6, $R^2 = 0.8947$), and within 24.05–392.6 μM at 95% CI. IC_{95} was observed at 100 μM doxycycline concentration, which had the lowest rRNA band intensity observed at both 20 and 120 min incubation times. The lower concentrations of doxycycline (2–20 μM) showed a slight increase in the intensity of mature rRNA bands at the longer incubation time (Fig. 3a, 120 min). Further analysis of samples at increasing incubation time of different doses showed that this increase in mature rRNA band intensity at lower concentrations of doxycycline was sustained over the duration of the experiments (240 min), and was highest at the lowest concentration of doxycycline used (2 μM). This suggests that sub-inhibitory concentrations of doxycycline may induce rRNA formation/synthesis with time. In addition, there was also a general increase in the total RNA concentration of doxycycline-treated samples at 120 min compared to the untreated samples (which was highest at 5 μM drug concentration; Fig. 3f), despite decreased culture growth/OD at that incubation time (Fig. 3e). However, in spite of the fact that the higher drug concentrations (50–200 μM) had higher total RNA concentration than the untreated cells at 120 min incubation time (Fig. 3e), they still showed decreased 16S and 23S rRNA band intensities in the gel (Fig. 3a). This suggests that much of the RNAs at these drug concentrations are not mature rRNA. The growth curves also showed that all drug concentrations produced similar growth inhibitory effects at 20 min incubation time, but had variable effects at longer incubation times. At 120 min incubation time, the maximum growth inhibition was achieved with 10–20 μM (Fig. 3e). These results therefore indicate that the effect of doxycycline on the formation of rRNAs in growing bacteria cells is affected by both drug dosage and incubation/treatment time, and that very low doses of doxycycline may induce rRNA transcription/formation over time.

When the samples were analysed by northern blot hybridization to detect pre-16S rRNA using CCPR1 probe (Fig. 3b), the intensity of the long rRNA precursors (initial transcript and pre-rRNA) increased with increasing drug concentration at 20 min incubation time, with smearing at the lower drug concentrations (2–20 μM). This indicates that more pre-16S rRNA is being retained in the long precursors with increasing drug concentration. At 120 min, all the rRNA bands (except the rRNA transcript band) were smeared. Besides smearing, the pre-rRNA bands became faint whereas the mature rRNA bands became more prominent at 120 min, especially at lower drug concentrations (2–20 μM) when compared to the 20 min samples. This indicates that the pre-rRNA was being cleaved (albeit inadequately) into smaller particles about the size of the mature 16S and 23S rRNAs with time, especially at low drug concentrations. This is consistent with the observed increase in band intensities of the 16S and 23S rRNAs at these low drug concentrations and longer incubation time in the gel image (Fig. 3a). Altogether, these results suggest that the inhibition of mature rRNA formation by doxycycline could be due to inadequate cleavage/processing of the long rRNA transcripts and pre-rRNAs.

Doxycycline induces accumulation of pre-rRNAs in growing bacteria cells

The observation that doxycycline inhibits RNase III cleavage of total RNA extracts in vitro and the formation of mature rRNAs in vivo could imply that the drug inhibits the cleavage and processing of the primary rRNA transcripts and pre-rRNA. If this is true, then doxycycline would induce accumulation of the unprocessed pre-rRNAs. This would substantiate the decrease in 16S and 23S rRNA bands as resulting from the effect of the drug on ribosomal RNA processing, rather than just a reflection of the rate of culture growth. Hence, the effect of doxycycline on the cleavage/processing of the primary ribosomal RNA transcript was further investigated in vivo by northern blot hybridization assay to assess the amounts of long primary rRNA transcripts and pre-rRNAs in growing bacteria cells treated with various concentrations of doxycycline. To minimize the growth inhibitory effect of the antibiotic and ensure good RNA yield in this experiment, bacterial cultures were initially grown to exponential phase before treatment, and thereafter, samples were harvested at 20 min incubation time to minimize differential growth in the antibiotic media (culture OD and total RNA extract concentrations were also assessed for confirmation). A probe (CCPR2) that is complementary to the spacer region between the mature 16S and 23S rRNAs (including about 10 nucleotides upstream and downstream as shown in Fig. 1) was used to detect

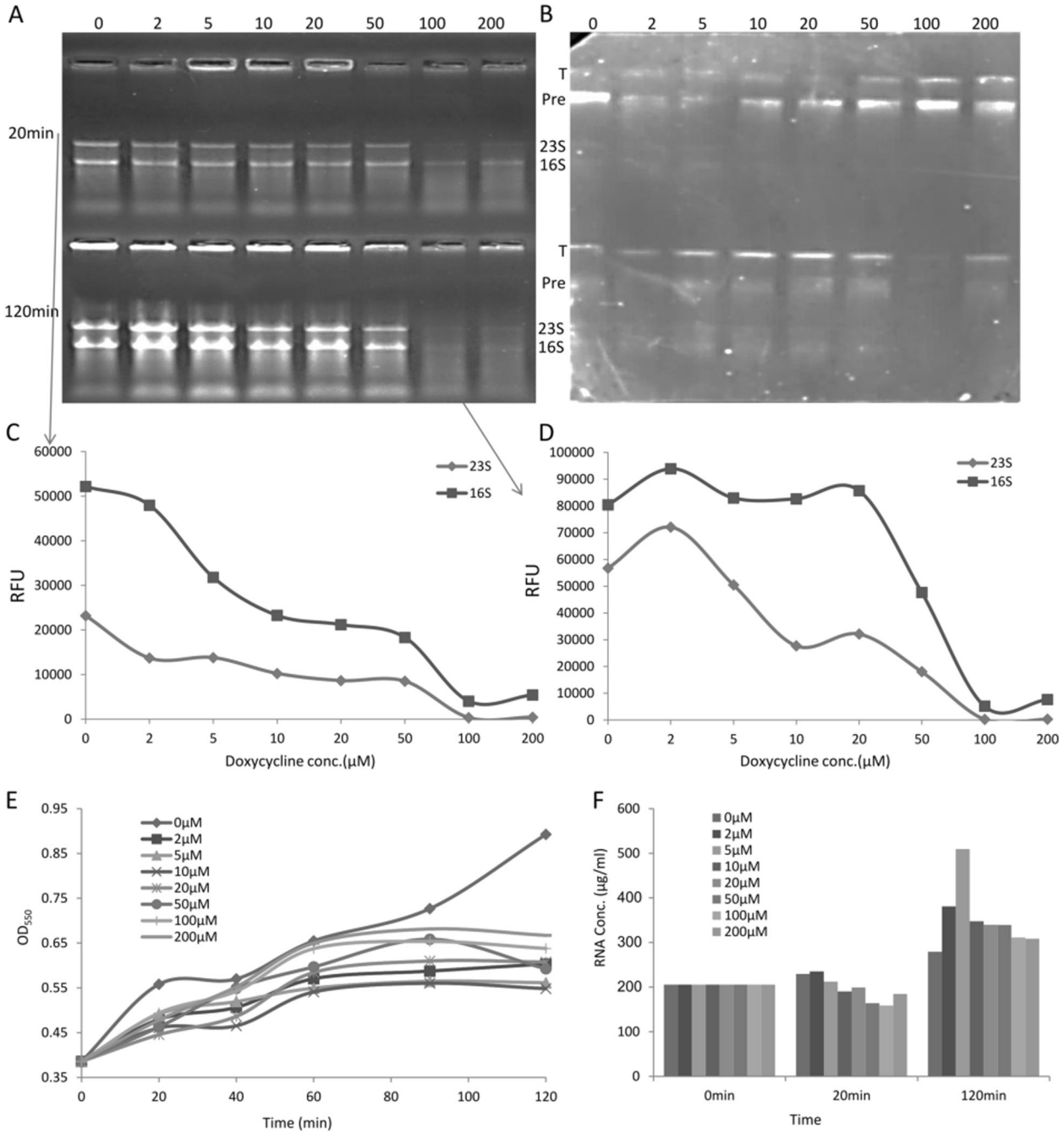

Fig. 3 Effect of increasing concentrations of doxycycline on mature rRNA formation in growing bacterial cells. EtBr-stained denaturing agarose gel image (**a**) of total RNA extracted from *E. coli* cells grown in increasing concentrations of doxycycline (0–200 μM) at 20 min and 120 min incubation periods, showing decreasing amounts of 23S and 16S ribosomal RNAs with increasing concentration of doxycycline as illustrated in the graphs (**c**, **d**), hybridized membrane blot of the gel (**b**) showing smearing of the RNA bands at 120 min, growth curve (**e**) and total RNA concentration of the samples (**f**). $IC_{50} = 8.327$ μM (+/–SE 2.465, $R^2 = 0.9554$) at 20 min incubation time and 76.51 μM (+/−SE 49.6, $R^2 = 0.8947$) at 120 min. RFU = relative fluorescence unit, T = rRNA transcript

uncleaved rRNA transcripts and long pre-rRNAs in the total RNA extracts. If rRNA processing occurs normally, this region is cleaved off by RNase III, and further processing yields mature 16S and 23S rRNAs. If RNase III cleavage is inhibited, this region is retained and would accumulate in the initial transcripts and long pre-rRNA.

Northern blot hybridization assay of the total RNA extracts from cells grown in the presence of various concentrations of doxycycline for 20 min (using the probe CCPR2) showed a dose-dependent accumulation of the initial transcripts and long pre-rRNA species, concomitant with a dose-dependent decrease in the 16S and 23S rRNA

species (Fig. 4). There were two distinct bands representing two uncleaved long rRNA precursors detected by the probe: a long initial rRNA transcript (positioned just below the wells), and a long pre-rRNA (estimated to be about 9KB in size). Linear regression analysis of culture OD showed no significant change in the culture OD ($P = 0.7745$). For total RNA concentration, 50% of the observed reduction was induced at about 192 µM doxycycline concentration (IC_{50} at $R^2 = 0.8534$). Despite the culture OD being stable at all drug concentrations, and decreasing total RNA concentrations from 50 to 200 µM drug concentrations (Fig. 4c), both the initial rRNA transcript and the pre-rRNA band intensities increased with increasing doxycycline concentration (Fig. 4d). It is interesting to note that the highest pre-rRNA band intensity occurred at doxycycline concentrations at which total RNA concentrations decreased. These observations strongly indicate that the effects of doxycycline on rRNA band intensities may be due to inhibition of rRNA transcript processing, and not essentially a reflection of culture growth inhibition by the antibiotic.

When the RNA band intensities were quantified by densitometry, statistical analysis of the results showed that 27.21 (+/−19) µM doxycycline concentration (or 8.832–104.2 µM at 95% CI, $R^2 = 0.7949$) induced 50% of the observed accumulation of pre-rRNAs (EC_{50}). On the other hand, 4.149 (+/− 1.535) µM doxycycline concentration induced 50% of the observed reduction in 16S and 23S rRNAs (2.028–8.553 µM at 95% CI, $R^2 = 0.9596$). It should be noted that the 16S and 23S rRNAs detected in this experiment are not yet fully mature (as they still contain some base sequences that are excised at maturation, which is detectable by the probe), and may differ slightly from the fully matured ones described in the previous sections. The concurrent increase in precursor rRNA species and decrease

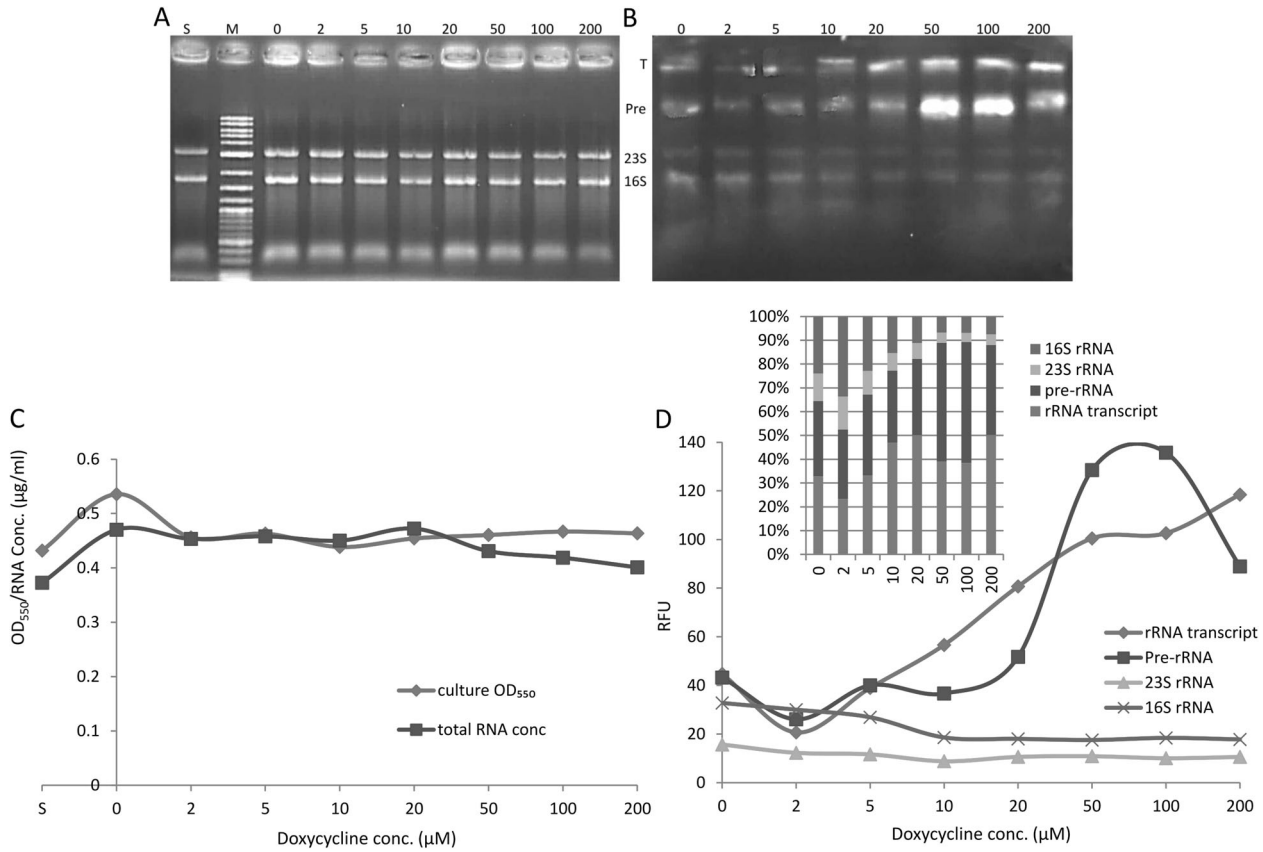

Fig. 4 Effect of increasing concentrations of doxycycline on the processing of *E. coli* rRNA in vivo. **a** EtBr-stained denaturing agarose gel of total RNA extract from *E. coli* K-12 cells grown in increasing concentrations of doxycycline. Samples were collected 20 min after treating with doxycycline, and total RNA extracted from the samples were fractionated in 1% denaturing agarose gel. S = starting culture sample at 0 min, 0 = untreated culture at 20 min, M = NEB log 2-log DNA ladder (0.1–10.0 kb). **b** Northern blot nylon membrane hybridized with pre-rRNA probe CCPR2 showing the initial rRNA transcript (T) and long pre-rRNA. **c** Graphical presentation of the optical density of cultures and the concentration of total RNA extracted from them, showing no significant change in culture OD ($P = 0.7745$), and a slight decrease in RNA conc, from 50 µM doxycycline conc. S = starting culture sample at 0 min, 0 = untreated culture at 20 min. **d** Graph of densitometric analysis of the various rRNA bands in (**b**). The blot and graph show a dose-dependent increase in the long pre-rRNAs and concurrent decrease in 16S and 23S rRNAs with increasing doxycycline concentrations. RFU = relative fluorescence unit. Inset shows percentage contribution of each rRNA species, as detected with the probe

in 16S and 23S rRNAs indicates that much of the rRNAs are increasingly present as long precursor rRNAs with increasing doxycycline concentration. This reaches a peak at the higher drug concentrations (50–200 µM) when only about 10% of the rRNA detected by the probe is in the 16S and 23S rRNA bands (Fig. 4d inset). Taken together, these results indicate that doxycycline induces accumulation of uncleaved/long rRNA precursors, while inhibiting the formation of 16S and 23S rRNAs. This implies that doxycycline inhibits the cleavage of the rRNA transcripts and pre-rRNA into the smaller 16S and 23S fragments.

Doxycycline induces bacterial cell elongation

In order to correlate the molecular observations of the effects of doxycycline on ribosomal RNAs with the effect of the drug on the whole bacteria cell in vivo, the nucleoid morphology of cells treated with increasing concentrations of doxycycline for 20 min was examined. The results show that doxycycline induces elongation of bacteria cells at low doses (≤50 µM), which is indicative of cell division inhibition. At higher drug concentrations, nucleoid degeneration was observed, which is indicative of early stages of cell death (Fig. 5). This is consistent with the observed decrease in total RNA concentration at 50–200 µM doxycycline concentration, suggesting that bacterial cell death occurs at high drug concentrations.

Discussion

The currently held 16S rRNA binding mechanism of action of the tetracyclines so far have not been sufficiently correlated with in vivo effects of the drug and their wide range of antimicrobial (not just antibacterial) activities [18]. The recently reported double-stranded RNA binding may therefore be a mechanism worth investigating to help elucidate the molecular basis of their wide range of activities [8]. If the tetracyclines bind to dsRNA and inhibit their cleavage/degradation by RNase III as previously reported [8], it could induce the accumulation of rRNA transcripts/precursors in growing bacteria cells.

The results presented here show a dose-dependent reduction of 16S and 23S rRNAs, concurrent with the accumulation of long rRNA precursors by doxycycline. Although any antibiotic that causes a reduction in bacterial growth would result in fewer cells growing in a culture medium, the observations in this study cannot be merely attributed to the growth inhibitory effects of an antibiotic. Several factors point towards a specific effect of doxycycline on mature rRNA formation rather than a reflection of the amount of cells in the culture. For instance, these effects were mostly observed at 20 min incubation time, when the effect of the drug on culture growth (OD) and total RNA concentration was minimal. Particularly, the greatest increase in pre-rRNA band intensity (Fig. 4) occurred at

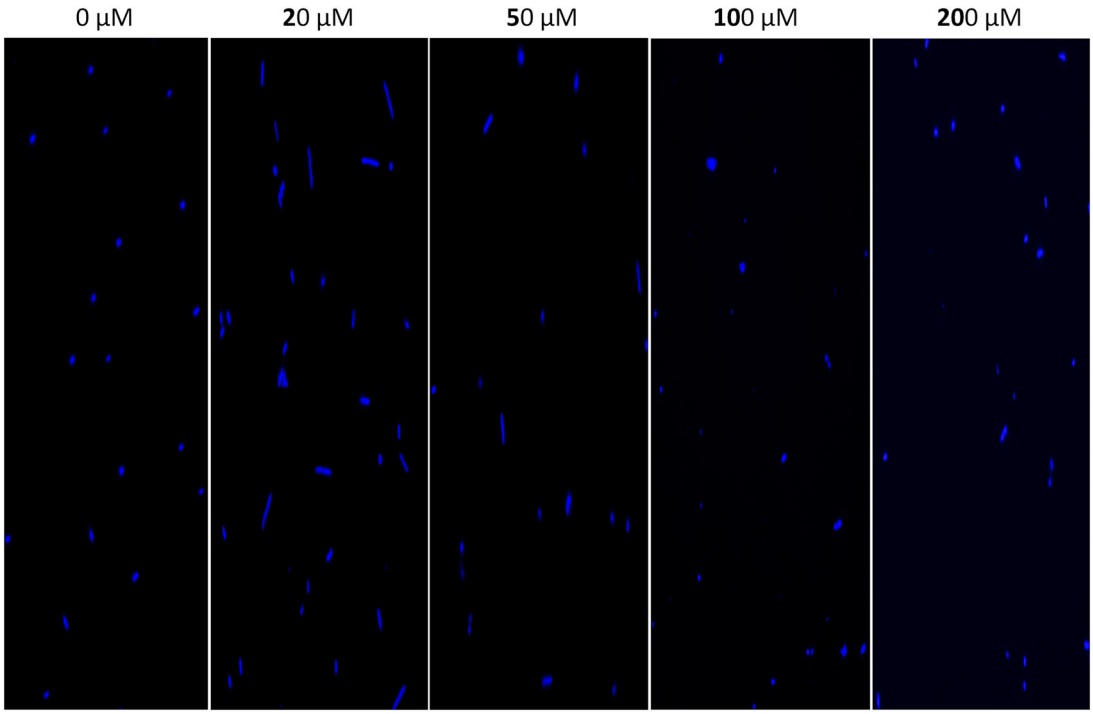

Fig. 5 Effect of doxycycline on nucleoid morphology of *E. coli*. Fluorescent microscopy images of *E. coli* K-12 cells treated with 0–200 µM doxycycline and incubated for 20 min before sample collection and processing for microscopy. Cells appear elongated at 20 and 50 µM doxycycline, with nucleoid degeneration at 100–200 µM. (×630)

drug concentrations at which there was a decrease in total RNA concentration (50–200 μM). Even the cell morphology changes, which are consistent with the molecular observations and earlier reports for tetracycline [19], were also observed at 20 min incubation time. Moreover, the increase in mature rRNA band intensities of low drug concentrations at longer incubation periods (120 min) when the growth inhibitory effects of the drug should have been more pronounced (Fig. 3a, d) indicate an effect on rRNA processing rather than culture growth. This is in agreement with earlier reports that Chlortetracycline induces initial stimulation of RNA synthesis especially at low concentrations, and subsequent accumulation of RNA while inhibiting protein synthesis [20, 21]. These reports suggested that the accumulated RNA species differ from both 23S and 16S rRNAs in their sedimentation properties (attributed to "incomplete precursors"), but could synthesize ribosomes during recovery from the antibiotic effects. In this study, the concurrent decrease in mature rRNAs and increase in precursor rRNAs as detected by northern blot hybridization (Fig. 4d) indicate effects on rRNA processing by doxycycline. Furthermore, the observations of smeared pre-rRNA bands at longer incubation periods which decrease in intensity as the mature rRNAs increase in intensity (Fig. 3b) also indicate effects on rRNA cleavage/processing. In view of the ability of doxycycline to inhibit RNase III degradation/cleavage of dsRNA [8], these results indicate that doxycycline inhibits the cleavage of long rRNA transcripts/precursors by RNase III; leading to the accumulation of the pre-rRNAs [15]. This initial inhibition of cleavage of the long rRNA precursors by doxycycline is subsequently relieved with time (Fig. 3), as has also been demonstrated in vitro with synthetic dsRNA [8]. A combination of this subsequent recovery from the inhibitory effects of doxycycline with time and possible alternate processing pathway which is less efficient than the RNase III cleavage pathway [15, 22], would lead to improved processing of the rRNA precursors at longer incubation periods. This could explain the observation of increased mature rRNA band intensities at longer incubation time with lower drug concentrations (Fig. 3a). The dose-dependent increase in the long rRNA precursors (Fig. 4b) seems to suggest that doxycycline also stimulates rRNA transcription. This may occur via a positive feedback mechanism, as the transcribed rRNA is not being processed to yield functional mature rRNA. Such feedback mechanisms involved in transcriptional regulation have been described in bacteria [23–25], and have recently been associated with the regulation of rRNA transcription [26]. On the other hand, it is unlikely that the inhibition of mature rRNA formation was due to inhibition of transcription. If that was so, one would expect a decrease in the initial rRNA transcript amounts. However, the reverse was the case in this study (Figs. 3b, 4b, d), indicating the possibility of a positive feedback mechanism instead. The general picture appears to be like this: As doxycycline is added, the mature rRNA decreases and the cells react to the shortage of mature rRNAs by increasing rRNA transcription. At higher doxycycline concentrations, more uncleaved/unprocessed pre-rRNA accumulate, and the cells activate/enhance alternative cleavage/processing pathways (such as by other nucleases) in an attempt to clear the accumulating pre-rRNAs.

It has been reported that although RNase III cleavage is necessary for the maturation of 23S rRNA, it is not essential for its function [27]. On the other hand, maturation of 16S rRNA could proceed in the absence of RNase III cleavage, as has been demonstrated in RNase III-deficient strain, even though such strains are known to grow slowly [28]. This is believed to be due to an alternative processing pathway in the absence of RNase III by other nucleases acting independently of RNase III [29, 30]. However, unlike the immature 23S rRNA which is functional in protein synthesis, the immature 16S rRNA is not functional in protein synthesis [28]. In this study, doxycycline was found to inhibit the amounts of both the 16S and 23S rRNAs. It is therefore possible that the non-functionality of the immature 16S rRNA, in contrast to the functionally active immature 23S rRNA, led to the previous belief that the tetracyclines exert their antibacterial action solely by binding to the 16S rRNA [6].

It is interesting to note that the inhibitory effects of doxycycline on rRNA processing were observed at the effective antibacterial concentrations of the drug. MIC of doxycycline for *E. coli* K-12 and the range of plasma concentrations following clinical therapeutic usage is ≈ 4–8 μg ml^{-1} (≈10–20 μM). However, drug concentrations in organs may reach 10–25 times that of serum [31]. Also, time-kill studies have shown that doxycycline exhibits time-dependent antibacterial effect on *E. coli* at low concentrations (2–4 times the MIC), but optimal dose-dependent killing is achieved at higher drug concentrations of about 8–16 times the MIC [32]. This complex interplay of dose and time was also observed in this study on the effect of doxycycline on mature rRNA formation (Fig. 3), and could have clinical implications for the effective use of doxycycline and other tetracycline antibiotics. Also, mutations in the 16S rRNA sequence that have been shown to confer resistance to tetracycline often occur at the double-stranded stem regions, and disrupt base pairing and formation of the secondary structures necessary for RNase III recognition and cleavage [33].

The broad spectrum of antibacterial activity of the tetracyclines can be attributed to the highly conserved nature of rRNA processing via RNase III cleavage pathway among prokaryotes. In eukaryotes, however, the processing of the ribosomal RNA involves a much more complex pathway

that is not dependent on RNase III [34]. In addition, eukaryotic rRNA processing, occurs in a protected environment (nucleolus) where ionic conditions (especially Mg^{2+}/divalent metal ion concentrations) are not ideal for doxycycline binding [8]. These differences in the processing pathway of prokaryotic and eukaryotic ribosomal RNAs could account for the selective inhibition of microbial protein synthesis, with minimal effects on eukaryotic protein synthesis [18]. The recovery from the inhibitory effects of the drug on the formation of mature ribosomal RNA with time supports the bacteriostatic mode of action of the tetracyclines.

Although the results presented here for doxycycline slightly digress from the 16S rRNA binding mechanism of action currently held for the tetracycline antibiotics, many of the underlying principles have been indicated long ago for various tetracyclines [5, 13, 19–21, 35–37]. However, those leads seem to have been largely ignored in favour of certain postulations from in vitro studies [6, 18]. Nevertheless, this work would serve as a basis for further studies with other tetracycline antibiotics in this perspective. When correlated with their effects on non-bacterial and eukaryotic rRNA processing and non-infectious disease conditions, the molecular mechanism of action of the tetracyclines would be more definitively elucidated.

Acknowledgements This work was supported by the Commonwealth Scholarship Commission in the UK.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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