

The Effects of Sub-Inhibitory Levels of Chloramphenicol on pBR322 Plasmid Copy Number in *Escherichia coli* DH5 α Cells

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While humans use antibiotics at inhibitory levels to prevent microbial growth, antibiotics are normally found at sub-inhibitory concentrations in nature. The role and effects of sub-inhibitory levels of antibiotics is one of the key areas of research in microbial science today. In this experiment, the effects of sub-inhibitory levels of chloramphenicol on pBR322 plasmid copy number in *Escherichia coli* DH5 α cells were examined. *E. coli* DH5 α cells with pBR322 plasmid were grown in different concentrations of chloramphenicol ranging from 1 to 5 μ g/ml. The cells were enzymatically lysed and the DNA extracted through a phenol:chloroform:isoamyl alcohol extraction. The total bacterial DNA was run on a 0.8% agarose gel; using the ratio of genomic DNA to pBR322 DNA, the plasmid copy numbers per genome were calculated. It was found that 1 μ g/ml chloramphenicol quantities had no significant affect when compared to the control; however, 3-5 μ g/ml of chloramphenicol caused pBR322 concentrations to double. Due to assay insensitivity, there was no significant difference between the 3 μ g/ml and 5 μ g/ml samples.

Chloramphenicol is a commonly used bacteriostatic antibiotic that reversibly binds to the 50S subunit (L₁₀ protein) and inhibits peptidyl transferase activity during the translation process (11). Therefore, the chief property of chloramphenicol is the inhibition of protein synthesis. It is common practice to use chloramphenicol to amplify plasmid DNA for laboratory use (10). Inhibition of protein synthesis results in the disruption of chromosomal DNA replication because chromosomal replication is dependent on *de novo* protein synthesis (3). Replication of plasmids that contain a relaxed origin of replication are independent of protein synthesis and are, therefore, not affected by the antibiotic (3). The ColE1 origin in pBR322 is a relaxed origin dependent on RNA molecules and Poll for replication. Since Poll is not found in limiting amounts, replication of pBR322 will typically occur for 10-15 hours after protein synthesis has been halted (3). The concentrations of chloramphenicol normally used for plasmid amplification purposes (10) are inhibitory to cell growth. The common protocols call for the addition of inhibitory quantities of chloramphenicol, or spectinomycin to a culture of bacteria, after the culture has been grown to the desired optical density (OD). Following the addition of chloramphenicol, the culture is incubated for 16 hours to allow for plasmid amplification (10). Plasmid DNA is extracted after this incubation period, at copy numbers that can reach 3000 per genome, compared to the typical 24 copies in a normal culture (3).

In this experiment, different sub-inhibitory concentrations of chloramphenicol were added to

bacterial cultures prior to overnight incubation. Following growth in the presence of the antibiotic, yield of plasmid DNA was analyzed and compared. It was hypothesized that this modification to the standard procedure would increase time efficiency, due to the decrease in amount of times the cells need to be grown and would allow plasmid amplification without completely inhibiting cell growth. It was expected that the cultures with sub-inhibitory concentrations of chloramphenicol would have a higher yield of plasmid copy number per genome than the control samples.

MATERIALS AND METHODS

Strains and Media: *Escherichia coli* DH5 α cells with the plasmid pBR322 were supplied from the UBC Department of Microbiology and were subsequently used throughout the experiment. Luria broth media (10 g tryptone, 5 g yeast extract, 2 g glucose and 10 g NaCl per litre of medium) was used for the growth all cultures.

Determination of Sub-Inhibitory Levels of Chloramphenicol: Ten millilitres of Luria broth was inoculated with *E. coli* DH5 α containing pBR322 plasmid and grown overnight at 37°C in a shaking water bath. Ninety-five millilitres of Luria broth was then inoculated with 5 ml of the overnight culture and incubated for 3 hr in a 37°C shaking water bath. Six, 50 ml Erlenmeyer flasks were filled with 16 ml of the 3 hr culture and chloramphenicol was added in order to obtain cultures with 0, 2, 4, 8, 12 and 20 μ g/ml concentrations of chloramphenicol respectively. The cultures were grown in a 37°C shaking water bath and their optical density at 680nm (OD₆₈₀) was measured every 20 min until the cultures entered stationary phase. OD₆₈₀ measurements were made using the Spectronic 20D (Milton Roy Company, USA).

Cell Preparation: Ten millilitres of Luria broth was inoculated with *E. coli* DH5 α containing pBR322 plasmid and incubated overnight, at 37°C in a shaking water bath. Subsequently, 200 ml of Luria broth was inoculated with the overnight culture at ratio of 50:1. The resultant culture was then equally divided between 4 flasks.

Chloramphenicol was added to obtain cultures containing chloramphenicol concentrations of 0, 1, 3 and 5 µg/ml; these cultures were incubated for 18 hours at 37°C in a shaking water bath. The turbidity of the 18 hour culture was measured at harvest. Volumes of culture equal to one millilitre of a 4 OD₆₈₀ culture (corresponding to 1.5 × 10⁹ cells), were transferred to sterile microcentrifuge tubes and spun for 30 seconds at 10,000 rpm in order to pellet the cells. The supernatants were then discarded. The pellets were used for cell lysis and subsequent total bacterial DNA isolation.

Bacterial DNA Isolation and Purification: DNA isolation and purification was performed as previously described (9). Each pellet equivalent to cells in 1 ml of a 4 OD₆₈₀ culture was resuspended in 400 µl of TE buffer (50 mM Tris/50 mM EDTA, pH 8.0) with gentle vortexing. Eight microlitres of 50 mg/ml lysozyme in TE, pH 8, was added and the mixture was incubated for 30 mins at 37°C. Four microlitres of 10% SDS and 8 µl of 15 mg/ml proteinase K were added and incubation continued for 30 mins at 50°C. The proteinase K was then heat-inactivated at 75°C for 10 min. Subsequently, 2 µl of 10 mg/ml RNase solution was added and the mixture was incubated for 30 mins at 37°C. Afterwards, 425 µl of phenol:chloroform:isoamyl alcohol (25:24:1) was added and the sample was vortexed vigorously and intermittently for 20 s. Tubes were then spun at 4°C for 5 min at 10,000 rpm and the upper aqueous phase was transferred to a new microcentrifuge tube, using a wide-opening pipette tip (normal tip cut with a razor blade approximately 5mm from the tip). The phenol:chloroform:isoamyl alcohol purification step was repeated and the upper aqueous phase was again transferred to a new microcentrifuge tube using a wide-opening pipette tip. Afterwards, the sample was centrifuged for 5 min at 10,000 rpm in a 4°C environment. The resultant supernatant contained the purified total bacterial DNA and was transferred to a fresh microcentrifuge tube. DNA is ready for use and does not require an ethanol precipitation to concentrate.

Agarose Gel Electrophoresis and Imaging: TE buffer (50 mM Tris/50 mM EDTA, pH 8.0) was used to make serial two-fold dilutions of the purified bacterial DNA samples (2, 4, 8, 16, 32, 64, 128 and 256-fold). Twenty microlitres of the undiluted and each of the diluted samples were mixed with 4 µl of 6X TBE gel loading dye (0.5 g bromophenol blue/0.5 g xylene cyanol/30 ml glycerol/170 ml dH₂O) and was run through 0.8% UltraPure™ agarose gel (Invitrogen, Canada, cat # 15510-019). Note that agarose gel selection is critical to the sufficient separation of genomic and plasmid DNA (9). Additionally, 200 ng of 1 Kb Plus DNA Ladder™ (Invitrogen, Canada, cat. # 10787-018), as well as 100 ng of pBR322 was loaded into each gel to serve as molecular standards. The gels were run in 0.5X TBE buffer (2.7 g Tris-base, 1.37 g Boric acid and 1 ml 0.5M EDTA pH 8.0 in 1 L distilled water) at 100 V until the bromophenol blue was approximately 2 cm from the bottom of the gel. An ethidium bromide bath (0.2 µg/ml) was used to stain the gels for 20 min. Images of the gels were taken using AlphaImager (Alpha Innotech, USA). The intensity (integrated area under the peaks) of the bands was determined by the 1D-Multi (Line Densitometry) program provided in the AlphaImager software package (Fig. 2. and 3.).

Data Reduction: The integrated plasmid and genomic DNA peak area values were entered into an excel worksheet in order to calculate plasmid copy number (table I) as previously described (9). The dilution factors were multiplied to the corresponding peak area values to determine the adjusted genomic and plasmid DNA peak areas at the first dilution line. The percentage difference (% diff) between the adjusted values for each dilution and the adjusted value for each previous dilution was determined using the following formula (9):

$$\% \text{ diff} = \frac{\text{Adjusted Peak Area} - \text{Adjusted Previous Peak Area}}{\text{Adjusted Previous Peak Area}} \times 100\%$$

Consecutive values that differed by less than 20% were considered to be in the linear range (9). Only adjusted values within the linear range were entered in the Data Used line and these were

then averaged. The average value for the genomic DNA and plasmid DNA were entered into the Total gDNA¹ and Total pDNA² lines respectively. From this, the plasmid copy number was calculated using the following formula:

$$\text{Plasmid copy number per genome} = \frac{\text{Size of chromosomal DNA (4640kbp)} \times \text{Total pDNA}}{\text{Size of plasmid DNA (4361bp)} \times \text{Total gDNA}}$$

TABLE 1. Representation of Plasmid Copy Number Calculations form with integrated scan data obtained from the Alphamager 1D-Multi (Line Densitometry) program (Alpha Innotech, USA). Data shown is DNA isolated from a culture of *E. coli* DH5α with pBR322 plasmid grown in Luria broth containing 3µg/ml of chloramphenicol. No gDNA or pDNA was detected below a one in four dilution.

Dilution Factor	1	2	4
gDNA area	498784	261878	126394
adjusted area	498784	523756	505576
%diff		0.047679	-0.03596
Data used	498784	523756	505576
Average gDNA area	509372		
pDNA area	38052	19500	0
Adjusted area	38052	39000	0
%diff		0.024308	
Data used	38052	39000	
Average pDNA area	38526		
Total gDNA	509372		
Total pDNA	38526		
PCN ³	80.4731		

Assay Recovery and Visualization: Using pure pBR322 plasmid, internal controls were constructed to determine the efficiency of DNA extraction and the interference of genomic DNA with the visualization of pBR322 from the agarose gels, as previously described (9). To determine extraction efficiency, one control sample was divided into two and the standard DNA extraction was performed (9). To one sample, 50 ng of pBR322 was added before DNA extraction; to the other sample, 50 ng of pBR322 was added after DNA extraction. Using the following formula, the efficiency of extraction was determined:

$$\frac{\text{Sample with 50 ng spiked before extraction}}{\text{Sample with 50 ng spiked after extraction}} \times 100\% = \text{extraction efficiency (\%)}$$

To determine interference from genomic DNA, a control was divided in two, with one being spiked with 10 ng of pBR322 DNA. These two samples were run on the gel along with a 10 ng pBR322 lane. By taking the back spike and subtracting the control, then dividing by the 10 ng lane value, we determined the interference due to genomic DNA.

¹ gDNA – genomic DNA

² pDNA – plasmid DNA

³ PCN – plasmid copy number per genome

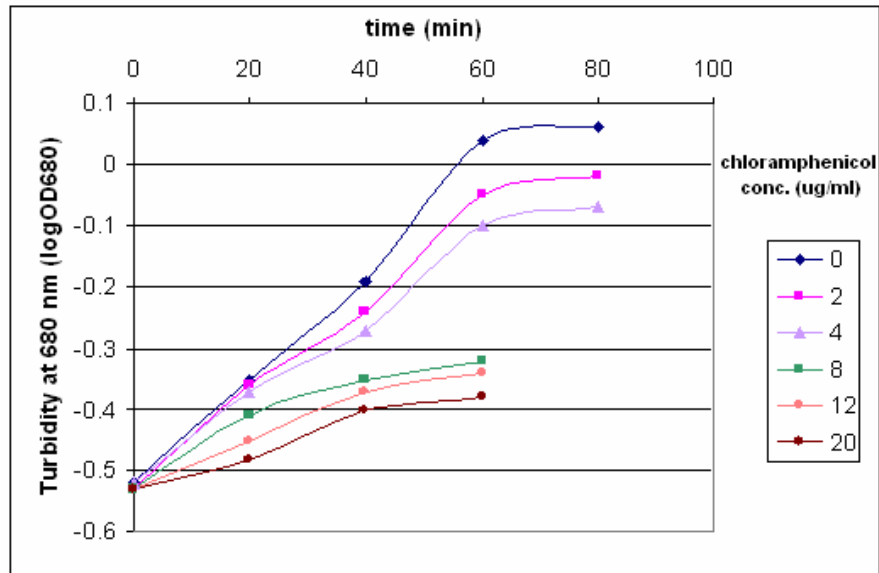


FIG. 1 Growth curve of *E. coli* DH5 α containing pBR322 plasmid growth in Luria Broth with various concentrations of chloramphenicol, at 37°C in a shaking water bath. Sub-inhibitory levels were found to be between 2 and 4 $\mu\text{g/ml}$.

RESULTS

Determination of Sub-Inhibitory Levels of Chloramphenicol: The sub-inhibitory concentration of chloramphenicol was found to be between 2 and 4 $\mu\text{g/ml}$ (Fig. 1.).

Optimisation of Plasmid Copy Number Per Cell Using Sub-Inhibitory Levels of Chloramphenicol: *E. coli* DH5 α with pBR322 plasmid cells were grown in Luria broth overnight with various concentrations of chloramphenicol (0, 1, 3 and 5 $\mu\text{g/ml}$). Following overnight growth, total DNA isolations were performed and were run on a 0.8% agarose gel to detect the plasmid and genomic DNA. The results in figure 2 show adequate resolution to independently measure the genomic and plasmid DNA (Fig. 3.). Using the data reduction techniques outlined in the materials and methods section, the plasmid copy number per genome for the cultures grown in 0, 1, 3 and 5 $\mu\text{g/ml}$ of chloramphenicol were: 42, 48, 110 and 104 pBR322 plasmids respectively (Fig. 4.). Values for the 1, 3 and 5 $\mu\text{g/ml}$ samples are based on the average of the two duplicates for each concentration. The 0 $\mu\text{g/ml}$ sample PCN count is based on the average of 8 extractions. Results indicate a definite increase in plasmid copy number with increasing sub-inhibitory concentrations of chloramphenicol (Fig. 4.). Both the samples containing 3 and 5 $\mu\text{g/ml}$ concentrations of chloramphenicol were significantly higher (2 times), in terms of plasmid copy number, than the sample containing only 1 $\mu\text{g/ml}$ of chloramphenicol. However, there was no significant difference in plasmid copy

number between the control and the sample with 1 $\mu\text{g/ml}$. This could be attributed to the minimal quantifiable difference in plasmid copy number between cells being 7. There was also no significant difference between the 3 and 5 $\mu\text{g/ml}$ samples. The 3-5 $\mu\text{g/ml}$ range of chloramphenicol, may represent the optimal level at which plasmid production can occur without growth inhibition.

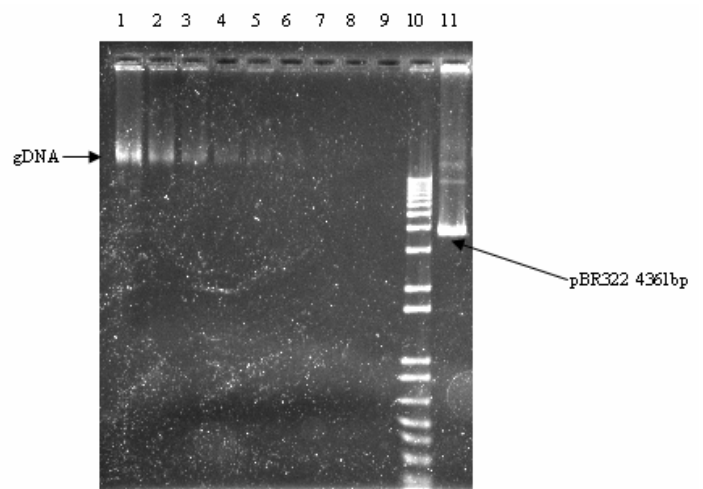


FIG. 2 An image of gel electrophoresis on agarose, showing adequate resolution of pDNA and gDNA. The samples were run on 0.8% agarose at 100 volts for 1.5 hours. Lanes 1-9 contain serial 2-fold dilutions (from undiluted to 256-fold) of the DNA isolated from a culture of *E. coli* DH5 α with pBR322 plasmid grown at 37°C in Luria broth with 3 $\mu\text{g/ml}$ chloramphenicol. Lane 10 contains the 1 kb plus DNA ladder and Lane 11 contains pure pBR322 plasmid. The gDNA and pDNA designations refer to genomic and plasmid DNA,

respectively. In this picture, plasmid DNA does not appear to be visible; however, 1D-Multi Line Densitometry performed on this picture allows for adequate visualization (Fig.3.).

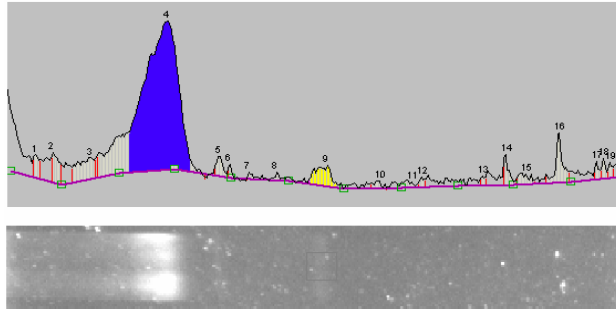


FIG. 3 Image scanning of undiluted DNA isolated from a culture of *E. coli* DH5 α with pBR322 plasmid, grown in Luria broth with 3 μ g/ml chloramphenicol, using the program AlphaImager 1D-Multi (Line Densitometry) by Alpha Innotech. Peak 4 represents the genomic DNA with an area of 498784 densitometry units and peak 9 represents pBR322 with an area of 38052 densitometry units. Ratios of the two areas were used to calculate the plasmid copy per genome. Plasmid DNA was visible using line densitometry that was not readily visible through total gel pictures (Fig. 2.).

Accuracy/Recovery: To determine recovery of plasmid and the accuracy of the visualization procedure, pure pBR322 plasmid DNA was used to spike samples, one before DNA isolation (front spike) and one before gel loading (back spike). Percent recovery in the DNA isolation was found to be 50%. Additionally, the inclusion of genomic DNA with plasmid DNA when running agarose gels was found to increase plasmid visualization by 15% (Fig. 5.).

Assay Sensitivity: The assay sensitivity was correlated to the minimum quantifiable PCN. The minimum quantifiable PCN was calculated as the average of the 8 controls' PCN divided by the average of the maximum dilution factors in the control at which the plasmid DNA area value was still in the linear range. The minimum quantifiable PCN for this assay was 7 plasmids.

DISCUSSION

The experiment tested sub-inhibitory concentrations of chloramphenicol on pBR322 plasmid copy number in *E. coli* DH5 α cells. In an effort to increase the plasmid copy per genome and to decrease the amount of time associated with growth to obtain these numbers, sub-inhibitory concentrations of chloramphenicol were used. The amplification of plasmids such as pBR322 in laboratory practice is commonly accomplished via the use of the chloramphenicol. This antibiotic acts on cell protein synthesis by inhibiting translation (11). Consequently, genomic DNA replication halts and relaxed replicative plasmids can still continue to replicate. Three of the key players in the regulation of replication in pBR322

are Rop (also know as Rom), RNAI and RNAII (8). All of these molecules are encoded on pBR322 and act to control plasmid replication. RNAII is the replicative primer and is necessary for initiation. RNAI is complementary to RNAII and thus can base pair; the base pairing prevents plasmid replication initiation because RNAII is sequestered and is also prevented from being processed. Thus, the ratio of RNAI to II is very important for the regulation of replication, especially in slow growing cells (1). Rop (repressor of primer) is responsible for the stabilization of the RNAI and RNAII complex, thus Rop is not necessary for plasmid regulation, but serves to down-regulates plasmid replication. Furthermore, the Rop protein is involved in the repression of transcription from the RNAII promoter, therefore again serving to decrease free primer level (1). As a consequence, it is suspected that because chloramphenicol down-regulates protein synthesis, while increasing RNA levels, the resultant lowered Rop levels and higher RNAII levels may be in part responsible for the increases in PCN observed in chloramphenicol treated cultures. In addition, while not a hindrance for long term enumeration of *E. coli* cells; sub-inhibitory chloramphenicol does cause a small increase in the generation time. While Rop levels are independent of growth rate, RNA I/II ratios are dependent on the growth rate (1). As the generation time lengthens, the amount of RNAII relative to RNAI increases, thus allowing for increases in plasmid replication.

The effects of chloramphenicol on the stringent response have interesting implications on PCN. Chloramphenicol blocks the formation of (p)ppGpp, a key alarmone involved in the stringent response. (p)ppGpp is typically formed as a result of amino acid starvation, via RelA, or by SpoT in response to carbon energy starvation (8, 11). The stringent response promotes the physiological and genetic reprogramming of the cell in response to conditions of stress that cause bacterial growth to cease (2). These modifications include a decrease in stable RNA synthesis, lowered protein synthesis and a redistribution of RNAP σ complexes. (p)ppGpp causes a decrease in the σ^{70} genes by blocking the $\beta\beta'\sigma^{70}$ interaction of RNAP with promoters in the open complex. This passively allows for higher expression of the σ^S genes. Because the stringent response is normally implicated in higher than normal plasmid levels, inhibition of this response should result in lower plasmid copy number per genome (PCN) counts (2,3). Since as little as 1 μ g/ml of chloramphenicol inhibits the formation of (p)ppGpp by RelA (8), one might expect that increasing levels of sub-inhibitory chloramphenicol should not cause an increase in PCN. In addition, a faster growth rate

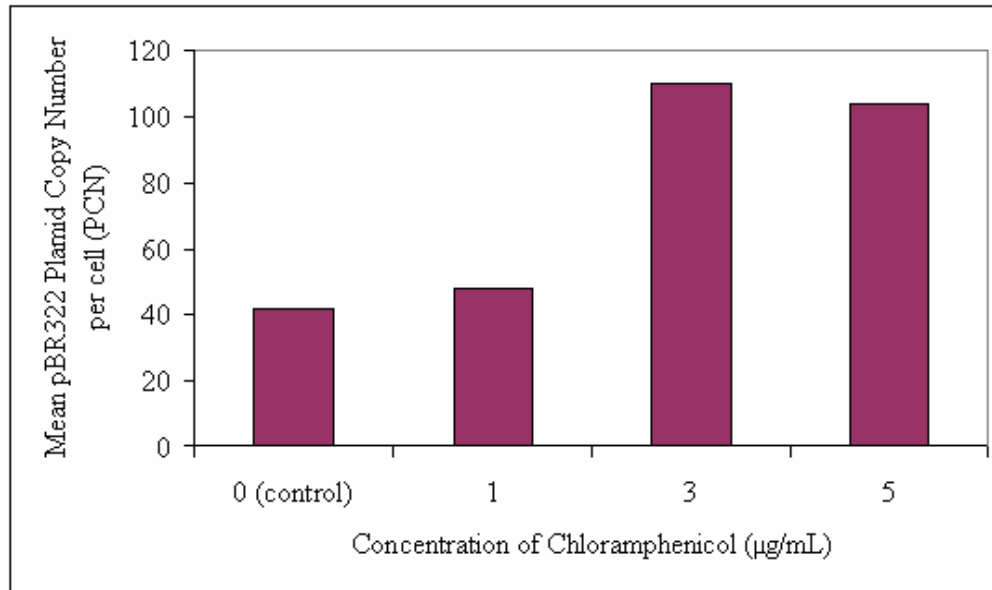


FIG. 4 The calculated average plasmid copy number per genome, when cultures of *E. coli* DH5 α with pBR322 plasmid are grown at 37°C in Luria Broth, with various concentrations of chloramphenicol. Values calculated for 1, 3, and 5 µg/ml samples are based on the average of two duplicates. To determine the assay precision, values from 8 controls consisting of *E. coli* DH5 α with pBR322 plasmid grown in Luria broth in the absence of chloramphenicol, were compared. This resulted in a mean plasmid copy number of 42 plasmids per genome and a standard deviation of 23 plasmids per genome.

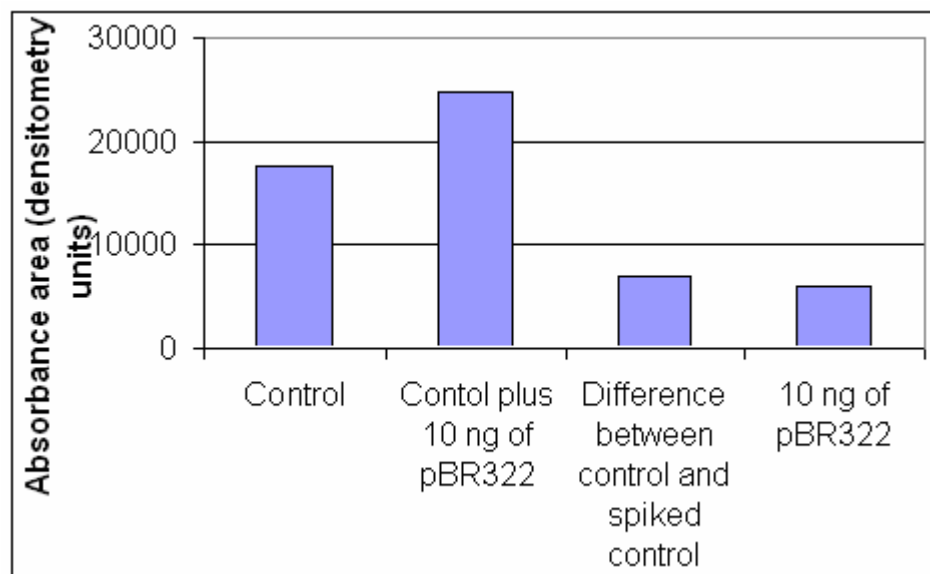


FIG. 5 The absorbance area calculated using 1D-Multi Line Densitometry by Alpha Innotech, for the assay accuracy control samples. The plasmid DNA absorbance area of the difference between the control and spiked control was 15% larger than the area of 10 ng of pBR322. Therefore, inclusion of genomic DNA with known quantities of pBR322 increases densitometry measurements by 15%.

lowers (p)ppGpp levels. Therefore, by this logic, one would expect to see pBR322 counts lowered as chloramphenicol concentrations increased. However,

the formation of (p)ppGpp lowers the levels of GTP in the cell, and thus there is less GTP available for plasmid replication. Consequently, inhibition of

(p)ppGpp formation may be beneficial to plasmid replication. As mentioned earlier, (p)ppGpp causes a decrease in σ^{70} promoters and a passive increase in other σ promoters. This does have an effect on the relative transcription rates from the replicative control genes encoded on pBR322. It has been shown that while $P_{R_{NAI}}$ is not significantly affected by levels of (p)ppGpp, the $P_{R_{NAII}}$ is stimulated by (p)ppGpp (8). Furthermore, a relaxed phenotype, associated with low (p)ppGpp is correlated with a low level of Rop, and thus a higher plasmid count (8). Therefore, most evidence dictates that lower (p)ppGpp levels produced by chloramphenicol, would decrease PCN. However, our results showed the opposite, suggesting that inhibition of protein synthesis, or another effect, is epistatic to (p)ppGpp control of pBR322 PCN.

A further effect of chloramphenicol, compounding the changes in σ affinity, may be changes to the bulk mRNA pool. Because chloramphenicol inhibits translation, a higher level of untranslated mRNA exists. Consequently, there exists more competition for the ribosomes and mRNA with higher affinity Shine-Delgarno sequences win out (8). If the mRNA of the Rop protein, or other negative regulators of plasmid replication are affected in this way, it may explain the trends we observed. Without further investigation into the ribosome affinities of the Rop protein encoding mRNA and other regulatory elements, it is impossible to tell if this is one of the driving forces behind the results we observed.

The large standard deviation found when comparing the control samples, in terms of plasmid copy number, may be due to a number of factors. For instance, fragmentation of the DNA may have occurred due to vigorous vortexing in the bacterial DNA isolation protocol (9), repeated freeze-thawing, and the use of non wide-opening pipette tips when loading the gel. This would have led to inaccuracies when determining genomic to plasmid area ratios in each lane as fragments of chromosomal DNA may have resulted in interference in the readings of plasmid DNA. As well, the high variability of the genomic DNA densitometry measurements may have resulted from this fragmentation affect. The fragmentation of plasmid DNA would decrease observed PCN, while fragmented genomic DNA would overestimate PCN. As well, variation may be due to interference of plasmid migration by genomic DNA. When running the gels, a portion of the chromosomal DNA was often left in the wells (Fig. 2.3.). As a result, plasmid DNA can get trapped by this stationary genomic DNA, leading to a substantial decrease in plasmid DNA density within the gel lanes. Moreover, due to time constraints, a number of laboratory personnel performed the extractions. Ideally, only one person should have carried out the extractions, with only one

of each pipette to ensure that the results are as consistent as possible. In addition, instead of diluting the samples above a factor of 8, which were commonly undetectable on the gel, a higher number of replicates for each of the lower dilution factors could have been performed. Perhaps the use of more controls (approximately 16) may have served to reduce the standard deviation. The use of a miniprep to perform DNA extractions may have also led to more consistent results.

The efficiency of DNA extraction of 50%, was acceptable, but fell short of the 86-98% efficiency previously observed (9). Moreover, our back spiked samples showed that running pBR322 with genomic DNA increased visualization by 15%. Moreover, problems were encountered visualizing plasmid DNA at low concentrations. This could be attributed to fragmentation of the chromosome, which may have caused an increase in the baseline that, in turn, would lead to a decrease in the plasmid DNA reading. The low visualization may have been prevented by using SeaKem® Gold agarose to prepare the gels rather than UltraPure™ agarose by Invitrogen, as previously recommended (9). Perhaps the use of a more sensitive staining technique could have also improved the sensitivity by allowing visualization of plasmid copy number below a dilution factor of 8.

In general, a number of the discrepancies encountered in the results may be due to the subjectivity in the interpretation of the data; this is due to the nature of the program used to quantify the DNA. For instance, it was often difficult to distinguish the point at which one peak ended and the other began. Genomic DNA was much easier to differentiate than plasmid DNA peaks because the former had substantially higher and broader peaks. In addition, density readings of the plasmid DNA could have been affected by background ethidium fluorescence spots. These spots intensified some of the bands, especially in the highly diluted plasmid samples (Fig. 3.).

It should be noted that when compared to typical literature, we overestimated the plasmid copy number per genome in the control samples. In the control samples, it was predicted to see a copy number of 20-24, but we actually obtained approximately 40 plasmids per genome (3). However, our large standard deviation of 23 means that direct comparison is difficult. This is most likely a result of genomic DNA fragmentation, experimenter subjectivity, as well as the possibility of gel contaminants and background spots that led to an additive effect in the peaks, as mentioned above. For our technique to be effective a very accurate quantification of plasmid DNA is needed and the integrity of the genomic DNA must be maintained (9).

Plasmids are a metabolic stress to the cell and thus, tight regulation of copy number is expected (4). Not

only do their production and maintenance create an energetic burden to the cell, but over-expression of plasmid borne genes can be toxic to the cells. pBR322 has been shown to be toxic to vegetative cells at high levels due to the effects of the *tet* gene (12). Therefore, an ideal situation would be to strike a balance between a high copy number and minimization of toxic effects and metabolic burden (6). We showed that it is possible to significantly amplify the PCN of pBR322 in *E. coli* DH5 α while still maintaining the cells ability to grow at an exponential rate.

As we had predicted, and despite the technical challenges, there was a notable difference in plasmid copy number between the cells treated with sub-inhibitory chloramphenicol and the control samples. Sub-inhibitory levels of chloramphenicol appeared to successfully allow plasmid amplification without inhibition of cell growth and therefore, could be employed in laboratories for efficient enumeration of plasmids and cells. It also raises questions as to the effectiveness of sub-inhibitory levels of antibiotics and to the role of chloramphenicol in a natural setting.

FUTURE EXPERIMENTS

The experiment raised a number of possible explanations for the trends observed; however, these hypotheses need significant experimental research to validate their role in sub-inhibitory chloramphenicol plasmid amplification. Further experiments should determine the limits of our results by testing the effects of 7 μ g/ml and 9 μ g/ml of chloramphenicol on cell growth and plasmid copy number.

Studies using Δ *relA* *E. coli* cells may help to confirm if inhibition of RelA by chloramphenicol is an important factor in exponentially growing *E. coli*. This would help support hypotheses involving (p)ppGpp levels affecting PCN. As well, studies validating the decrease in protein synthesis, and the increase of RNA levels at sub-inhibitory chloramphenicol levels, must be performed to further support the hypothesis set forth by our group.

However, before new studies are undertaken, the DNA isolation protocol (9) needs to be validated. This protocol demonstrated high sensitivity, reproducibility and accuracy, but our experimental error analysis showed this was not achieved. Consequently, efforts to improve gel visualization, DNA extraction efficiency and removal of experimenter subjectivity must be undertaken. Additionally, evaluation of fragmentation of the genomic DNA should be part of the experimental error analysis if performing another experiment using this method. Moreover, other simpler techniques for plasmid quantification should be investigated for their application to experiments similar to ours. Such techniques include: using cell concentration, calculated

though turbidity measurements and plasmid DNA quantification, calculated through plasmid isolation (alkaline lysis) and spectrophotometry, to determine PCN.

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