Journal of Experimental Microbiology and Immunology (JEMI) Vol. 3:87-95
Copyright © April 2003, M&I UBC

# The Effects of Ampicillin versus Tetracycline on the Plasmid Copy Numbers of pBR322

**MEI CHONG, RICKY LEUNG, CARMEN WONG, AND ALEX YUEN**

*Department of Microbiology and Immunology, UBC*

**The pBR322 plasmid contains genes encoding for tetracycline and ampicillin resistance. *Escherichia coli* DH5α cells with pBR322 plasmid were grown and plated on selective plates containing 1) ampicillin only and 2) tetracycline only with varying antibiotic concentrations of half strength, three-fourth strength and full strength (15 µg/mL tetracycline and 50 µg/mL ampicillin for full strength). Colonies from the plates were grown overnight in Luria Broth (LB) and the cells were used for plasmid isolation. Gel electrophoresis was done on the isolated plasmids and pixel counts in the digital gel images were analyzed by Kodak 1D to determine the intensity of the bands on the gel. It was found that ampicillin selection led to a higher digital pixel counts and copy numbers of plasmids per cell than tetracycline selection. Furthermore, there seemed to be a correlation that there was an increase in plasmid copy numbers with an increasing antibiotic concentration.**

---

The pBR322 plasmid is a cloning vector containing both ampicillin and tetracycline resistance genes as selectable markers, and it is a widely used vector in microbial genetic experiments in research laboratories. Ampicillin resistance gene encodes for the enzyme β-lactamase. This secreted enzyme binds and inactivates the ampicillin molecule by catalyzing action of hydrolysis of the β-lactam ring of the ampicillin (13). Tetracycline resistance gene encodes a transmembrane efflux pump that pumps out tetracycline molecules that have entered the cell (15). *E. coli* DH5α cells containing pBR322 have a selective advantage in these antibiotic-containing environments.

Previous experimental results found by Law *et al.* showed that the initial numbers of colonies formed on ampicillin plates were higher than tetracycline plates after *E. coli* DH5α cells were transformed with pBR322 plasmid (10). A number of factors were considered that could contribute to these results. One possible factor could be the level of resistance gene expression in *E. coli* DH5α cells, where there could be a higher expression of ampicillin resistance gene than the tetracycline resistance gene, thus resulting in higher number of cells grown on the ampicillin plates. This factor was considered because another experiment showed that increasing the copy numbers of the plasmid that carries chloramphenicol resistance gene enhanced bacterial efficiency at gaining drug resistance (8).

This experiment investigated the pBR322 plasmid copy numbers in *E. coli* DH5α cells grown on ampicillin and tetracycline treated environments to see whether there are differences in resistance gene expression. A higher plasmid copy number is assumed to correlate with higher gene expression. Furthermore, these cells were treated with different strengths of antibiotics to observe whether the pBR322 plasmid copy numbers in *E. coli* DH5α cells was enhanced by the increasing antibiotic strengths.

## MATERIALS AND METHODS

Three different approaches were used to test the hypothesis. The first method included isolation of the pBR322 plasmid from *E. coli* DH5α carrying the plasmid followed by transformation of the plasmid into *E. coli* DH5α with no plasmids. In the second approach, plasmid DNA from *E. coli* DH5α cells with pBR322 grown on ampicillin and tetracycline plates were isolated using a breakage buffer for rapid cell lysis. Finally a plasmid miniprep was used to isolate pBR322 from the *E. coli* DH5α with pBR322 grown in Luria broth containing ampicillin and tetracycline and the samples were electrophoresed on an agarose gel.

**Plasmid Isolation for Transformation by the Hanahan Protocol (Sambrook, 1989):** The pBR322 plasmid was isolated from *E. coli* DH5α cells growing at mid-exponential phase. Cells were centrifuged at 8,500 x g using the IEC 818 rotor and then resuspended in lysozyme breakage buffer. Sodium hydroxide (0.4 M NaOH) and sodium dodecyl sulphate (2% SDS) was added to the cell suspension. The cell suspension was then treated with acidified salt buffer to precipitate the chromosomal DNA. The sample was then centrifuged at 7,700 x g (IEC 818 rotor) and was washed with phenol twice to remove residual proteins. The plasmid suspension was centrifuged at 4,400 x g (IEC 818 rotor) to separate it from the phenol layer. The plasmid was then precipitated with ethanol and then centrifuged at 6,200 x g (IEC 818 rotor). The plasmid pellet was resuspended in tris-ethylenediamine tetraacetic acid (Tris-EDTA) buffer and was treated with ribonuclease. This plasmid sample was then placed into a dialysis bag with the addition of chloroform and was dialyzed overnight in EDTA buffer.

**Concentration of DNA:** The plasmid sample was diluted 1-in-25 and 1-in-50 at a final volume of 1 mL of Tris-EDTA buffer. The absorbance was read using the double beam spectrophotometer (Ultrospec 3000 UV/Visible Spectrophotometer, Biochrom) at wavelengths of 260, 270 and 280 nm. By using the conversion factor that one $A_{260}$ unit is equal to 50 µg/mL of DNA, the DNA concentration in the undiluted sample was determined.

**Competent DH5α cells for the Hanahan Protocol (Sambrook, 1989):** *E. coli* DH5α cells lacking the pBR322 plasmid were grown overnight in Luria Broth at 37°C in a shaking water bath. When the culture had reached a reading of 0.3 optical density at 660 nm wavelength ($O.D._{660}$), the cells were chilled on ice for 5 minutes. Nine milliliters of the culture was centrifuged at 4,100 x g in a chilled IEC 818 rotor for 5 minutes. All subsequent manipulations were done on ice. The supernatant was removed and 0.45 mL of ice cold 50 mM $CaCl_2$ in Tris was added to the pellet. The cells were then placed on ice for 30 minutes.

**Transformation of *E. coli* DH5α Cells with pBR322:** Fifty microliters of competent cells were added to 3 µg of pBR322 plasmid and placed on ice for 20 minutes. The cells were then subjected to heat shock for 2 minutes in a 42°C water bath. The transformation was carried out in triplicates along with a negative control lacking the plasmid DNA. Cells were recovered in 1 mL of Luria Broth for 60 minutes in a 37°C shaking water bath. After recovery, the cells were placed on ice for 30 seconds and the transformants were plated onto two different types of Luria agar media containing either ampicillin (50 µg/mL) or tetracycline (15 µg/mL). The plates were incubated at 37°C for 24 hours. For the second and third transformation trials, the recovery period was extended to 90 minutes.

**Rapid Disruption of Colony Plasmid Isolation (Barnes, 1977):** *E. coli* DH5α with pBR322 plasmid were grown on Luria agar media for cell enumeration. Cells from these plates were streaked for isolated colonies onto Luria media containing different antibiotic strengths of either ampicillin (50 µg/mL, 37.5 µg/mL, 25 µg/mL) or tetracycline (15 µg/mL, 11.25 µg/mL, 7.5 µg/mL). Cells were also streaked on a control plate with no antibiotics. When cells reached a large colony diameter (2-3 mm), digital images of the Luria agar plates were scanned using the Epson Perfection 1650 Scanner to find the size of the desired colony.

A colony of 2-3 mm diameter from each plate were transferred to a microfuge tube and treated with breakage buffer (50 mM NaOH, 0.5% SDS, 5 mM EDTA and 0.025% Bromophenol Blue). After the incubation of the samples at 68°C for 60 minutes, glycerol was added to a final concentration of 5%. Sample buffer was added to the contents and were ran on a 0.8% agarose gel with ethidium bromide.

**Plasmid Isolation Using Concert High Purity Plasmid Purification Systems**

**Cell Cultures:** *E. coli* DH5α cells with pBR322 plasmid from different antibiotic strength plates of ampicillin and tetracycline (as stated above) were inoculated into 30 mL of Luria broth media containing the respective antibiotic type and strength. Cells were grown overnight to obtain turbid cultures. $O.D._{660}$ measurements were made before the plasmid isolation using the Spectronic 20D (Milton Roy Company).

**Miniprep Protocol:** The protocol was performed following the miniprep procedure as outlined by Concert Life Technologies. Equilibrium buffer (600 mM NaCl, 100 mM sodium acetate pH 5.0, 0.15% Triton X-100) was added to the columns and was allowed to drain by gravity. Fifteen milliliters of cells were harvested and spun to pellet using the IEC 819 rotor at 4,100 x g. The pellet was treated with cell suspension buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA) first, then cell lysis solution (200 mM NaOH, 1% SDS) was added and incubated for 5 minutes. Neutralization buffer (3.1 M potassium acetate pH 5.5) was mixed to the sample and centrifuged at 12,000 x g for 10 minutes at room temperature. The supernatant was transferred to the columns and allowed to drain through. The columns were then washed with wash buffer twice (800 mM NaCl, 100 mM sodium acetate pH 5.0) and the DNA in the column was eluted by addition of the elution buffer (1.25 M NaCl, 100 mM Tris-HCl pH 8.5). Absolute isopropanol was mixed in and the sample was centrifuged at 12,000 x g for 30 minutes at 4°C to precipitate the plasmid DNA. The plasmid pellet was washed with 70% ethanol and centrifuged at 12,000 x g for 5 minutes at 4°C. After air-drying the pellet for 10 minutes, the plasmid pellet was dissolved in 50 µL of Tris-EDTA buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA). Sample buffer was added and the samples were electrophoresed on a 0.8% agarose gel with 1 mg/mL ethidium bromide for 1.5 hours at 100 V. A linear DNA ladder from Invitrogen and a GIBCO supercoiled DNA ladder was added into the agarose gel for electrophoresis. The gel image was then captured using the Alpha Imager (Alpha Innotech Corporation).

**Quantification of Plasmids:** The concentration of the plasmid samples obtained from the miniprep protocol was determined by measuring the absorbances using the Ultrospec 3000 spectrophotometer at wavelengths of 260 and 280 nm. The samples were diluted 3 in 100 at a final volume of 1 mL with sterile distilled water. By using the conversion factor that one $A_{260}$ unit is equal to 50 µg/mL of DNA, the DNA concentration in the undiluted sample was determined.

## RESULTS

**Plasmid Isolation for Transformation:** The pBR322 plasmids from DH5α cells isolated in the phenol extraction method had an $A_{260}/A_{280}$ absorbance ratio value of 1.88 and 1.84 corresponding to a concentration of 15.8 µg/mL and 8.30 µg/mL in 1/25 and 1/50 dilutions respectively from the original isolate. The $A_{270}$ readings were lower than the $A_{260}$ readings as well and this indicates the isolated plasmid from our first approach was pure.

**Transformation of *E. coli* DH5α Cells with pBR322:** The first transformation yielded too numerous to count (TNTC) plates on all plates (data not shown). The TNTC plates from the first transformation were a result of insufficient antibiotics added to the Luria broth agar (only 0.1 mL of 150 µg/mL of tetracycline and 0.1 mL of 500 µg/mL ampicillin were spread onto the plates). The amount was only 5% of what should be spread onto the agar, assuming each plate had approximately 20 mL of agar. The failure of the first transformation was not due to degradation of the isolated plasmids. The second and third trials of transformation had no growth on the antibiotic plates, except for a few colonies of contaminants on the ampicillin plates, and the control plates that contained no antibiotics had confluent lawns of growth. The second and third transformation did not yield transformed colonies because the plasmids isolated became defective during the storage process. DNA bases and deoxyribose sugars are susceptible to degradation by free radical oxidation (6). The addition of EDTA and ethanol to preserve plasmids should decrease the rate of free radical oxidation at 30°C (6). In our preservation method, we solely added EDTA to the plasmids without ethanol and they were frozen at –20°C for storage. Because there is no shear stress inside a solid (1), this method of plasmid storage with EDTA should be sufficient for preservation. EDTA will chelate metal ions and prevent them from associating closely with DNA and thus preventing site-specific formation of hydroxyl radicals adjacent to the double helix (11). However, since the pBR322 plasmid DNA did not show a band on Figure 1, the plasmid was likely degraded during the storage process. This explains why our transformation procedure

failed. The degradation of plasmids was a result of repeated freezing and thawing of the plasmids during the experiment (1).

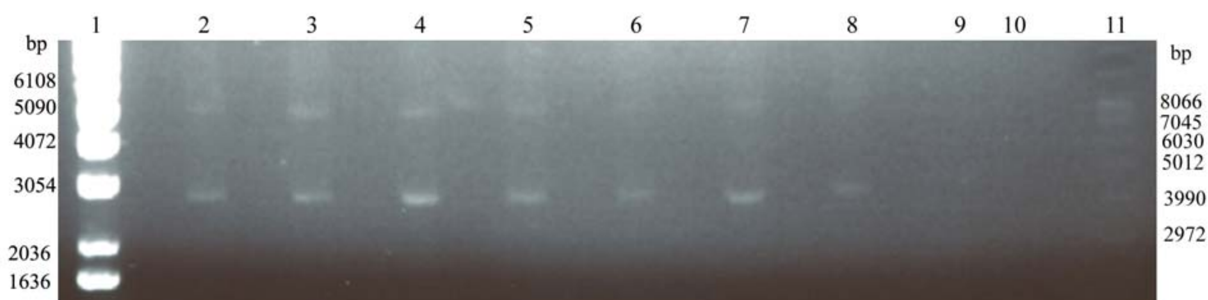


**FIG. 1.** The electrophoresis of pBR322 samples isolated from *Escherichia coli* grown on different antibiotic strengths of ampicillin and tetracycline. The samples were electrophoresed on an 0.8% agarose gel. Lane 1 contains the Invitrogen 1Kb DNA Ladder; lane 2 is the control sample; lanes 3-5 contain pBR322 from ampicillin selection at concentrations of 50 μg/mL, 37.5 μg/mL and 25 μg/mL respectively; lanes 6-8 contain pBR322 from tetracycline selection at concentrations of 15 μg/mL, 11.25 μg/mL and 7.5 μg/mL respectively; lanes 9-10 contain pBR322 isolated for transformation as undiluted and 1:25 dilution respectively; lane 11 contains the Gibco supercoiled DNA ladder. Note that plasmid samples in lanes 2-8 were isolated using the miniprep protocol whereas plasmid samples in lanes 9-10 were isolated using the phenol extraction method described in the plasmid isolation of the transformation section.

**Rapid Disruption of Colonies for Plasmid Isolation:** Since the stored plasmids from the beginning of the experiment were defective, another approach developed by Barnes (3) in 1977 (as stated in methods) was used to lyse DH5α cells with pBR322 plasmid that had been grown overnight on antibiotic plates. Gel electrophoresis of the cell content did not yield any visible bands except for the DNA ladder that was loaded (data not shown). The parameters in the method developed by Barnes in 1977 were not stringent enough to purify the pBR322 plasmids. When the cells are lysed, its contents are prone to many environmental factors that would degrade the plasmids.

**Plasmid Isolation Using Concert High Purity Plasmid Purification Systems:** Figure 1 showed the presence of two bands for every sample. The mean intensity in pixel counts (FIG. 2) from the electrophoresed plasmid was based on the lower of the two molecular weight bands, which represented the supercoiled 4.2 Kb pBR322, corresponding to the size of the supercoiled DNA ladder. The supercoiled form of the plasmid will travel further in the gel compared to the other forms of the plasmid. The higher molecular weight bands were probably the open form structure of pBR322. The open form structure could be due to a single-stranded nick on the supercoiled form. The linear form of the plasmid should be found corresponding to around 4.2 Kb of the linear DNA ladder which is absent in this gel.

Figures 2 and 3 illustrates the plasmid copy numbers per cell but based on different methods of quantification. Figure 2 used pixel counts while Figure 3 used absorbance values. In Figure 3, the plasmid copy number per cell for the control sample was 175; for tetracycline it was 211, 338, 395 in respect to increasing relative strength; for ampicillin it was 283, 432 in respect to the 0.50 and 0.75 strength of antibiotics; the full strength ampicillin sample was lost (1.00 indicates full strength antibiotics).

From the band intensities shown on Figure 1 and the digital pixel counts of the picture of the agarose gel (Fig. 2), DH5α cells grown in LB with ampicillin appears to have higher plasmid copy numbers than the ones grown in LB with tetracycline since the plasmids from ampicillin selected cells appear to have brighter bands and higher pixel counts than the tetracycline selected cells on a relative basis. At the same time, there was an increase in relative intensities of bands as antibiotic strengths for both ampicillin and tetracycline selection increased from 0.50 to 0.75 strength. When the quantity of DNA in the preparation of plasmid was measured by absorbance at 260 nm wavelength ($A_{260}$), a similar trend was observed (Fig. 3). There was no decrease between 0.75 and 1.00 strength antibiotic treatment but there was clearly an increase between the 0.25 and 0.50 strength antibiotic treatments. Furthermore, Figure 2 and Figure 3 showed that the control sample had a significantly lower amount of plasmids compared to the antibiotic-treated samples.

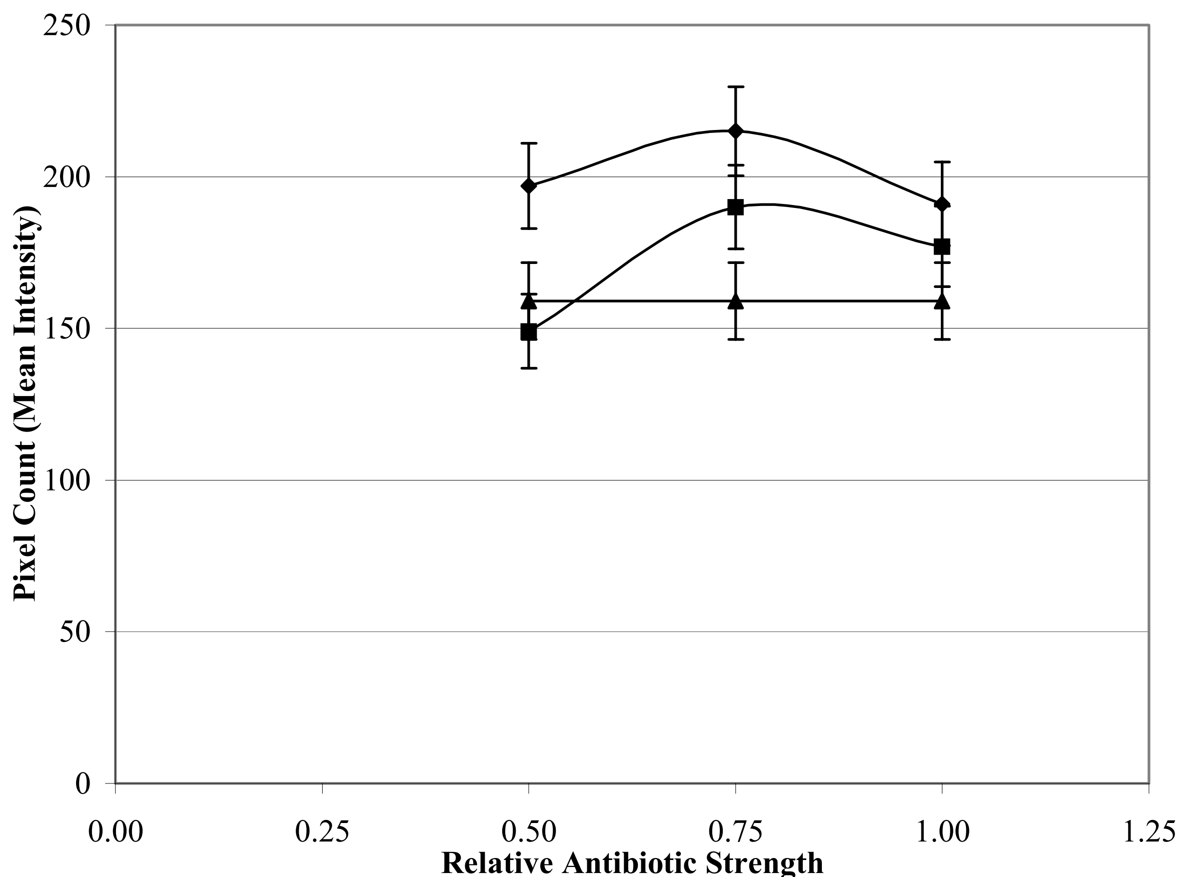


**FIG. 2.** Pixel counts of electrophoresed pBR322 plasmid with various antibiotic strength in *Escherichia coli*. The band intensities on the agarose gel (Fig. 1) were measured using the Kodak 1D software to determine relative amounts of pBR322 plasmid DNA in correlation with antibiotic strength. Mean intensity values for the supercoiled samples were used (1.00 indicates full strength antibiotics). Symbols: ♦, cell samples grown on LB containing ampicillin; ■, cell samples grown on LB containing tetracycline; ▲, control cell sample grown on LB.

## DISCUSSION

Ampicillin selection appears to have higher plasmid numbers than tetracycline selection as illustrated in Figures 1 and 3. Plasmid isolation of cells removed from the ampicillin and tetracycline containing broth yielded more intense bands from ampicillin selection compared to tetracycline selection as shown by the pixel counts (Fig. 2) and copy numbers (Fig. 3). This showed that plasmid numbers may alter the level of resistance gene products in the cell, which in turn may explain the different number of transformants in ampicillin and tetracycline selections as observed by Law *et al.* (10) However, it should be noted that promoter strengths also contribute to the level of resistance genes expression and that the amount of resistance gene products in the cell may not directly correlate to cell survival in the presence of antibiotics.

Given similar cell counts, tetracycline selection yielded less plasmids than ampicillin selection. This means that ampicillin selected cells needed to expend more energy to replicate plasmid for survival. An explanation of this may be due to the bactericidal and bacteriostatic nature of the two antibiotics. It is possible that since ampicillin is bactericidal, it would need to expend more energy in replicating plasmids to ensure proper transmission of plasmids to progeny. Also since ampicillin uses β-lactamases to degrade the surrounding ampicillin, as the cells grow, they will increase in volume, requiring more space and thus need to keep the regulation of the expression on the ampicillin resistance gene. On the other hand, in bacteriostatic tetracycline, a considerable amount of energy is expended by the cell to continuously expel tetracycline by an efflux pump. Maintenance of this pump will also

require a constant use of energy as the tetracycline antibiotics are never degraded. Furthermore, it has been shown in previous studies that overexpression of the *tet* gene can result in inhibition of cellular growth and even reduce cell viability (7, 16). Thus for cell survival, it may be to the cell's advantage to replicate fewer copies of pBR322 plasmid so that the gene is not overexpressed. This would account for the lower pixel counts and plasmid copy numbers as compared to ampicillin selection. This could also imply that perhaps the efflux pump is very efficient and that few transcripts are required for protection against tetracycline.

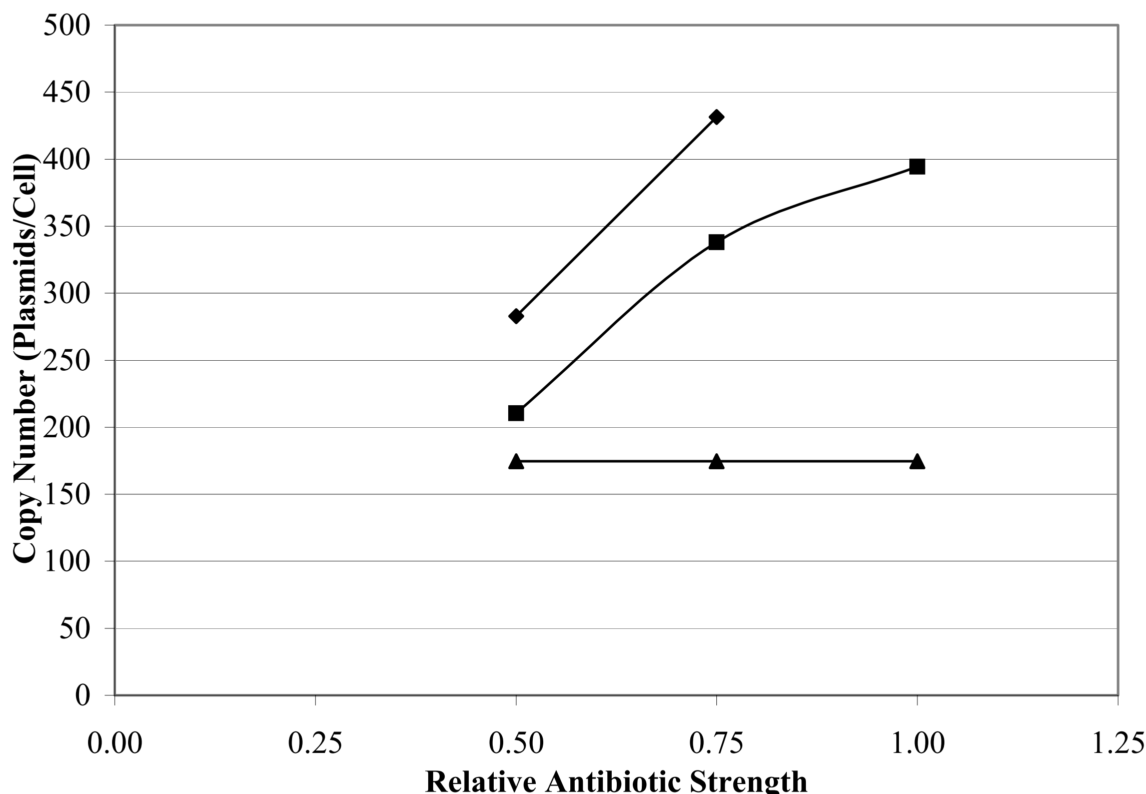


**FIG. 3.** Relationship of pBR322 plasmid copy number with different antibiotic strength in *Escherichia coli*. The values of the copy numbers were calculated based on absorbance measurements of DNA concentration and not pixel counts. Symbols: ♦, cell samples grown on LB containing ampicillin; ■, cell samples grown on LB containing tetracycline; ▲, control cell sample grown on LB.

Plasmid copy numbers appear to depend on the presence of antibiotics. Both ampicillin and tetracycline selection yielded to higher plasmid numbers compared to the control sample as indicated by the higher mean intensity in pixel counts and plasmid copy numbers (Fig. 2 and 3). An explanation of this may be the stress caused by the antibiotics. The antibiotic conditions might select for cells with higher plasmids. It is expected that the control sample would contain fewer copies as there is no selection. And since plasmids will put a metabolic burden onto cells, if the cell does not need the plasmid, then it would not be surprising that in future cell divisions, the cell would not partition or replicate the plasmid as frequently (4,5). Hence, there is a selection for cells with fewer plasmids in the absence of antibiotics as the plasmids are not needed to survive.

The control sample had lower mean intensity in pixel counts than all the other samples except the half strength tetracycline treated sample (Fig. 2). The decrease of mean intensity in pixel counts was not significantly high and the error bars of both of these points are overlapping. This variation in the trend is negligible since it may be just minor data variation. The error might have occurred because only the pixels of the supercoiled (bottom band) plasmid in Figure 1 were counted, since the open form of the plasmid (top band) does not contribute greatly to the overall plasmid content in each lane of Figure 1. These two faulty datum points could have been a result of the method of pixel counting used, where the total amount of plasmids in each lane was not accounted for. However, the

overall trend of antibiotic treated cells have more plasmids is still feasible, since from Figure 3, the trend seems apparent. The lower plasmids per cell in the control sample in Figure 2 and 3 were expected. Plasmids impose metabolic burden for the cells because cells could grow faster with fewer plasmids. This is due the fact that plasmids also require nucleotides for replication and translation to gene products and their persistence will hinder the host cells ability to replicate (9). Therefore, the cells tended to lose plasmids if they were not under high antibiotic stress.

The plasmid count per cell shown in Figure 3 for the control sample does not agree with a study done by Atlung *et al.* They reported 11 copies of pBR322 per cell for *E. coli* grown in LB and in our experiment we obtained 175 copies per cell, which is approximately 15 times higher. This 15 fold increase of plasmid copies per cell was seen in all samples and relative to an expected value of two digit copy numbers, it is unexpectedly high. The cells are small in size and this would add to the hindrance caused by the metabolic burden of the plasmids.

**TABLE 1.** Difference in plasmid copies per cell with consideration of RNA contamination

| Sample type[a] | Number of plasmid/cell with RNA contamination | Number of plasmid/cell without RNA contamination |
|---|---|---|
| 0.75 Strength Ampicillin | 432 | 62 |
| 0.50 Strength Ampicillin | 283 | 40 |
| Full Strength Tetracycline | 395 | 56 |
| 0.75 Strength Tetracycline | 338 | 48 |
| 0.50 Strength Tetracycline | 211 | 30 |
| Control | 175 | 25 |

[a]Full strength refers to 50 µg/mL for ampicillin and 15 µg/mL for tetracycline (the full strength ampicillin sample was lost).

A possible explanation for the differences in the plasmid copy numbers per cell compared the study done by Atlung *et al.* could be that there was RNA contamination in the samples and thus resulted in an overestimation of the plasmid DNA concentration. Since the spectrophotometer used to measure the plasmid DNA concentration cannot distinguish between DNA and RNA (it only measures nucleic acid concentration), RNA contamination in the plasmid samples would result in an overestimate of plasmid concentrations. RNA contamination in our sample could be due to RNase A inactivity in the solutions provided by the miniprep as they were not stored cold before use. In a typical *E. coli* cell, there is approximately seven times as much RNA as there is DNA present (Neidhardt, Ingraham and Schaechter 12-15). Using this fact, with the RNA contamination in our plasmids, the plasmid copy number per cell presented in Figure 3 should be divided by about seven. After the correction, the resulting plasmid copy number per cell becomes a two-digit number, which makes more sense and the trends mentioned above still holds (Table 1). It seems reasonable to conclude the unusually high plasmid copy number per cell is a result of the presence of RNA.

For ampicillin and tetracycline selection, the increase of mean intensity in pixel counts was observed with increase in antibiotics strengths (Fig. 2). This indicates that increasing the strength of antibiotics introduced increasing stress in the cells. When cells were under stress, they produced more plasmids. This is also seen in the plasmid copy number counts in Figure 3. As the antibiotic strength increases, the plasmid copy number increases as well indicating that cells require the plasmid for survival since the pBR322 contains the antibiotic resistance genes. Figure 3 illustrates a near-linear relationship that increasing antibiotic strength will correlate with increase in plasmid copy numbers. However, for the ampicillin sample, there were only two data points and thus the trend of increasing copy numbers with antibiotic strength is speculated from logic and is not conclusive.

A peculiar point in our data is the pixel count for the three-fourth strength ampicillin and tetracycline (Fig. 2). These points do not follow the general trend of increase in pixel count as antibiotic strength increases. The three-fourth strength of both antibiotic treated samples had higher pixels compared to full and half strength treated samples. This could be due to the method used in preparing the three-fourth antibiotic treated plates. Since the three-fourth strength plates were prepared by addition of one-quarter of the antibiotics through spreading over the surface of the half strength agar, it is possible that the one-quarter antibiotics was not absorbed thoroughly into the agar. A portion of antibiotics stayed on the surface of the plates which increased the concentration of antibiotics and was not distributed evenly. These may account for the sudden increase of mean intensity in both three-fourth strength antibiotics treated samples with the increased antibiotic strength. Despite the increase in pixel counts, the

Copyright © April 2003, M&I UBC

plasmid numbers per cell (Fig. 3) demonstrated that the trend of increasing plasmid number with respect to antibiotic strength does appear to exist. But note in Figure 3, there is not a sudden increase in plasmids per cell in the three-fourth antibiotic strength.

The discrepancy between the pixel counts and the plasmid copy number trend is not surprising as pixel counts are a more relative estimate and more subjective calculation. If the error bars of the points in Figure 2 were taken into account, making a data point on the maximum or the minimum values in the range of some data points, a stable increasing trend can be observed, as illustrated in Figure 3. In Figure 3, it was shown that for both antibiotics, increasing the strength increases the plasmid copy numbers. The plasmid copy number of full strength ampicillin treated cells in Figure 3 was missing due to the sample being lost. However, this point is anticipated to follow the trend as well. On the other hand, it is possible that the samples examined for the absorbance method contained RNA contaminations as mentioned earlier that accounted for the abnormally high plasmid copy numbers per cell. If this was true, then this could explain the differences between Figure 2 and Figure 3. The plasmid copy numbers in Figure 3 should be lower if RNA was taken into account. It is possible that the full-strength tetracycline treatment contained more RNA contamination than the three-fourth strength sample. If this was taken into account, then the trend seen in Figure 3 should be similar to that in Figure 2. The trend in Figure 2 showed that plasmid copy numbers would increase as antibiotic concentration was increased only up to a certain point where it would decrease despite increasing antibiotic concentration. However, since we do not know the exact amount of RNA present that interfered with the results in Figure 3, we could not conclude as to the exact trend of plasmid copy numbers imposed by the antibiotic concentrations. Despite this, both Figures 2 and 3 showed an apparent trend of increase in plasmid numbers as antibiotic concentration is increased from half-strength to three-fourth strength.

The paper by Law *et al.* described lower tetracycline plate counts as compared to ampicillin (10). It is also possible that the antibiotic strength had enhanced high translation of the *tet* gene thereby causing detrimental effects to the cell since overexpression would reduce cell viability (9). It may also be possible that during transformation recovery, having weak cell membrane integrity, it would make cells more susceptible to tetracycline diffusion and thus to counter this, cells would enhance the expression of the *tet* gene and perhaps this may have led to too much expression causing some reduction in cell viability. Cells may replicate more plasmids to increase gene expression and this can be seen from the increase in plasmid copies due to induction from half strength tetracycline to full strength (15 μg/mL) tetracycline (Fig. 3). Thus the lower plate counts on tetracycline observed by Law *et al.* could be possibly due to these reasons of plasmid copy numbers (9). However, the higher copy number seen could have been a result of selection for cells with more plasmids when the cells are exposed to antibiotics.

In this experiment, it showed that ampicillin treated samples contained higher plasmid copy numbers than that of tetracycline. This could be a result of stress caused by tetracycline on the cells. Synthesizing the tetracycline efflux pump may require more energy than producing β-lactamase. Thus, tetracycline may cause stress on the cells from the efflux pump synthesis, and so there is less energy to produce plasmids, resulting in lower plasmid numbers on cells exposed to tetracycline.

The effect observed by Law *et al.* correlated to higher plasmid in the presence of ampicillin but it does not mean that higher plasmid is necessary to grow in the presence of ampicillin. Also, there seemed to be a correlation between plasmid content and exposure. Ampicillin appears to increase the copy number of plasmid more than tetracycline. The experiment presented in this paper showed that there appeared to be a linear trend with increasing copy number of plasmid with increasing antibiotic strength.

## FUTURE EXPERIMENTS

From the results of this experiment, it is possible that plasmid copy number did have an influence over transformation survival. However, it does not eliminate the possibility of that the membrane integrity of transformed cells may play a role in the differences in plate counts. Since tetracycline functions by the use of an efflux pump, this pump requires the use of the cell membrane. During transformation, the cell membrane is disrupted to facilitate DNA uptake into the cell. Therefore this is a possible explanation for the lower plate counts in tetracycline media stated by Law *et al.* To further test this observation, it would be useful to analyze the cell membrane integrity of the cells after transformation with pBR322. The experiment could be attempted by using methods to analyze the cell membrane structure after transformation to see if it is due to the lack of membrane integrity to hold onto the efflux pump, causing the inefficient pumping of the tetracycline out of the cells and leading to a higher rate of cell death due to exposure to tetracycline.

Another observation seen in the experiment was there appeared to be a trend with the plasmid number correlating to the strength of the antibiotics. From the experiment, it appears that this is possibly a linear relationship were plasmid numbers increase with increasing antibiotic strength in the media used. Thus it would be useful to test this

to see if such a linear trend does exist and if there are limitations in this trend. It may be possible that limitations in this trend could have some effect on the transformation efficiency as different antibiotic strengths may or may not inhibit or slow down cell growth. In addition, experiments can be conducted to test whether combined ampicillin and tetracycline selection have an additive effect on the copy numbers of the pBR322 plasmid.

## ACKNOWLEDGEMENTS

We thank Dr. William Ramey, Karen Smith, Andre Comeau, Nick Chang, and our UBC Microbiology 421 classmates for their technical expertise, support, and assistance in the completion of this experiment.

## REFERENCES

1. **Ando, S., D. Putnam, D.W. Pack, and R. Langer.** 1999. PLGA microspheres containing plasmid DNA: preservation of supercoiled DNA via cryopreparation and carbohydrate stabilization. J. Pharm. Sci. **88**:126-130.
2. **Atlung, T., B.B. Christensen, and F.G. Hansen. 1999.** Role of the Rom protein in copy number control of plasmid pBR322 at different growth rates in *Escherichia coli* K-12. Plasmid **41**:110-119.
3. **Barnes, W. M. 1977.** Plasmid detection and sizing in single colony lysates. Science. **195**: 393-394.
4. **Corchero, J.L. and A. Villaverde.** 1997. Plasmid maintenance in *Escherichia coli* recombinant cultures in dramatically, steadily, and specifically influenced by features of the encoded proteins. Biotechnol. Bioeng. **58**:625-632.
5. **Diaz Ricci, J.C., and M.E. Hernandez.** 2000. Plasmid effects on *Escherichia coli* metabolism. Crit. Rev. Biotechnol. **20**:79-108.
6. **Evans, R.K., Z. Xu, K.E. Bohannon, B. Wang, M.W. Bruner, D.B. Volkin.** 2000. Evaluation of degradation pathways for plasmid DNA in pharmaceutical formulations via accelerated stability studies. J. Pharm. Sci. **89**: 76-87.
7. **Guay, G.G., and D.M. Rothstein.** 1993. Expression of the tetK gene from *Staphylococcus aureus* in *Escherichia coli*: comparison of substrate specificities of TetA(B), TetA(C), and TetK efflux proteins. Antimicrob. Agents Chemother. **37**:191-8.
8. **Guerrier-Takada C.G., R. Salavati, and S. Altman.** 1997. Phenotypic conversion of drug resistant bacteria to drug sensitivity. Proc. Natl. Acad. Sci. **94**: 8486-8472.
9. **Jones, K.L., S.W. Kim, and J.D. Keasling.** 2000. Low-copy plasmids can perform as well as or better than high-copy plasmids for metabolic engineering of bacteria. Metabol. Engin. **2**:328-338.
10. **Law, J.S., S. Lee, A. Tseng, K.W. Tsui, and N. Yu.** 2002. The effects of ampicillin versus tetracycline in the selection of pBR322 transformants. J. Exp Microbiol. Immunol. **2**:189-193.
11. **Lloyd, D. R., and D.H. Phillips.** 1999. Oxidative DNA damage mediated by copper (II), iron (II) and nickel (II) fenton reactions: evidence for site-specific mechanisms in the formation of double-strand breaks, 8-hydroxydeoxyguanosine and putative intrastrand cross-links. Mutat Res. **424**: 23-26.
12. **Neidhardt, F.C., J.L., Ingraham, and M. Schaechter.** 1990. Composition and Organization, p.12-15. *In* Physiology of the Bacterial Cell: A Molecular Approach. Sinaver Associates, Sutherland, MA.
13. **Raynor, D.** 1997. Penicillin and ampicillin. Prim. Care Update Ob Gyns. **4**:147-152.
14. **Sambrook, J., E.F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. **Schnappinger, D., and W. Hillen.** 1996. Tetracyclines: antibiotic action, uptake, and resistance mechanisms. Arch. Microbiol. **165**:359-369.
16. **Valenzuela, M.S., E.V. Ikpeazu, and K.A.I. Siddiqui.** 1996. *E. coli* growth inhibition by a high copy number derivative of plasmid pBR322. Biochem. Biophys. Res. Commun. **219**:876-883.

Copyright © April 2003, M&I UBC

**Appendix**

Plasmid Number per Cell Sample Calculation:

Ampicillin 0.75 Strength:

(DNA concentration reading from Ultrospec 3000) x (50 µL elute from Miniprep) x dilution factor = amount of DNA

27.75 µg/mL x 50 µL x (100/3) = 46.25 µg

Conversion to moles of plasmid (pBR322 Molecular Weight = $2.83*10^6$g/mol)

46.25 µg / $2.83*10^6$ µg/µmol = 16.34 µmol

16.34 µmol DNA x 1 mol / $10^6$ µmol x 6.02 x $10^{23}$ molecules / mole = 9.84 x $10^{12}$ molecules

Number of cells in culture:

1 optical density (O.D. at $A_{660}$) = $10^9$ cells/mL

(O.D. at $A_{660}$) x (dilution factor) x (conversion factor) x (15 mL) = # cells in original 15 mL of culture

0.380 $O.D._{660}$ x 4 x $10^9$ cells/mL / $O.D._{660}$ x 15 mL = 2.28 x $10^{10}$ cells in 15 mL of culture

Number of Plasmids per cell:

9.84 x $10^{12}$ molecules / 2.28 x $10^{10}$ cells = 432 plasmids / cell

**Error analysis of pixel count:**

Pixel count given by Kodak 1D = 215
$\sqrt{215} = 15$