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The Effects of Incubation with Ampicillin and Tetracycline on the Expression of the *bla* and *tetA* genes of pBR322

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The plasmid pBR322 contains the genes *bla* and *tetA,* which encode resistance to ampicillin and tetracycline. *Escherichia coli* DH5α cells containing pBR322 were grown in concentrations of 50, 100, 150 and 200 µg/ml of ampicillin or 10, 15, 30 and 45 µg/ml of tetracycline. The cells were lysed and their proteins separated into cytosolic, periplasmic, membrane and secreted fractions. SDS-PAGE was carried out on the isolated fractions and the band intensities of 31 kDa and 46 kDa proteins (corresponding to the *bla* and *tetA* gene products respectively) were analyzed. It was found that both *bla* and *tetA* have basal levels of expression, which increase in response to the antibiotics to which they encode resistance – although not necessarily in a linear fashion. In addition, it was discovered that membrane stability and membrane potential also play important roles in how resistance proteins from pBR322 are expressed in response to antibiotic treatment.

The plasmid pBR322 is a commonly used cloning vector encoding both ampicillin and tetracycline resistance as selectable markers. Ampicillin is a bactericidal antibiotic that inhibits bacterial cell wall synthesis by binding to peptidoglycan-synthesizing enzymes (10). The pBR322 *bla* gene confers resistance to ampicillin via the enzyme β-lactamase, a 31 kDa protein (www.bmcd.gov:8080/cgi-bin/query/bmcd/schema/molecule?MO_ID=MOIC). The β-lactamase is secreted into the periplasm where it binds and hydrolyzes the β-lactam ring in penicillin, rendering the drug inactive (10).

Tetracycline, a bacteriostatic antibiotic, prevents attachment of aminoacyl-tRNA's to the ribosomal acceptor site, inhibiting bacterial protein synthesis (3). Originally derived from pSC101, the tetracycline resistance gene cassette of pBR322 contains the *tetA* gene (8) which encodes for a membrane-bound efflux pump approximately 46 kDa in size. TetA exports tetracycline from the cell as a proton antiporter (5), reducing the intracellular drug concentration and protecting cellular ribosomes (3).

Previous experiments investigated the effects of ampicillin and tetracycline treatments on *Escherichia coli* DH5α cells transformed with pBR322 (2,6). These experiments showed significant differences in colony counts and plasmid copy numbers between the two antibiotic treatments. Transformed DH5α cells grown on ampicillin plates showed higher initial numbers of colonies than identical samples grown on tetracycline plates (6). Increases in the concentration of ampicillin and tetracycline in cultures caused increased plasmid copy number in pBR322-transformed *E. coli* DH5α cells, but even higher plasmid copy number in cells grown on ampicillin. Chong *et. al.* proposed that the difference in colony count was due to differential expression of the *bla* and *tetA* genes from the pBR322 plasmid, suggesting that *bla* may be expressed more than *tetA* when grown on ampicillin (2). They continued to hypothesize that plasmid copy number may be a mechanism used by the cell to regulate expression of the plasmid genes. Thus, in ampicillin-treated growth conditions, plasmid copy number increases more than under tetracycline conditions. The authors assumed that the increased plasmid number corresponded to increased protein expression. However, this line of reasoning ignores the effects of transcriptional and translational control that are omnipresent in bacterial cells and complicate a direct correlation between protein expression and gene copy numbers.

Our research focused on elucidating the relationship between protein expression and antibiotic levels in growth media. Instead of looking at gene copy number, which may not accurately reflect protein expression, we looked directly at protein levels by analyzing band intensities of cell lysates ran on SDS-PAGE gels. Using different levels of ampicillin and tetracycline, we hoped to determine whether differential expression of the resistance genes actually occurs. An adjunctive experimental goal was to determine whether protein expression (not just plasmid copy number) increases with antibiotic concentration. In the process of monitoring protein expression, we also hoped to ascertain what cellular location the expressed proteins were accumulating in.

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MATERIALS AND METHODS

Growth Conditions and Bacterial Strains. *Escherichia coli* DH5α with and without plasmid pBR322 were used to inoculate 20 ml of Luria broth and left to grow overnight in a 37˚C shaking water bath. For each control treatment, 1 ml of the *E. coli* DH5α (without plasmid) overnight culture was used to inoculate 20 ml of fresh Luria broth with either 50 μg/ml, 100 μg/ml, 150 μg/ml or 200 μg/ml of ampicillin or 10 μg/ml, 15 μg/ml, 30 μg/ml, or 45 μg/ml of tetracycline. The optical densities (OD_{660}) of the freshly inoculated cultures were taken, then incubated for four hours in a 37˚C shaking water bath. One ml of *E.coli* DH5α with pBR322 overnight culture was used to inoculate 20 ml of fresh Luria broth with either 50 μg/ml, 100 μg/ml, or 200 μg/ml of ampicillin or 10 μg/ml, 15 μg/ml, 30 μg/ml or 45 μg/ml of tetracycline (totaling 7 cultures/treatments). As with the control cultures, the OD_{660} was taken immediately following inoculation and then incubated for four hours in a 37ºC shaking water bath.

***Escherichia coli* DH5α Cell Lysis.** Optical density readings (OD_{660}) were taken of each culture following four hours of incubation. One mL of each culture was prepared with appropriate dilutions to obtain equivalent culture concentrations (as determined by the optical density readings) for cell lysis. The cell lysis protocol followed Sambrook instructions developed for determining the subcellular localization of phoA fusion proteins (11). The protocol enables separation of cells into cytosolic, periplasmic, membrane and secreted fractions. Samples were centrifuged as previously done. For all except the 20 minute spin, centrifugations were performed at room temperature, as opposed to 4ºC as recommended (11). Protein samples were resuspended in 0.1 M Tris-Cl (pH 8.0) and frozen at -20ºC until they were required for gel electrophoresis.

Bradford Assay (adapted from Bradford, 1976). One ml each of diluted protein sample or chicken egg albumin at final concentrations of 0, 5, 10, 20, 30, 45, 60 and 80 μg/ml were combined with 2 ml of Bradford dye reagent (BioRad) (11). The absorbance at 595nm was read following vortexing and a subsequent 10 minute wait. The concentration of protein in each sample was determined by comparing the A_{595} of the sample to that of the standard curve prepared from the chicken egg albumin results.

SDS-PAGE. Polyacrylamide gels were prepared at either 10% or 15% acrylamide solution according to Sambrook *et al.* (11) Molecular weight standards were ran in the gels beside the samples. The gels were run at approximately 200 volts for 45 minutes. After staining with Silver Staining Plus Kit (BioRad), the gel image was captured using the GelDoc system. Analysis of the gel images, using Kodak 1D Imaging Software, determined the molecular weight and mean pixel intensity of the 31 kDa and 46 kDa protein bands of interest. The gels were dried (BioRad Gel Dryer Model 583) for a permanent record.

RESULTS

Obstacles in SDS-PAGE visualisation of cell lysis samples. Repeated attempts to run cell lysis samples on 15% SDS-PAGE gels, suggested by Sambrook *et. al*. (11) as appropriate for the size of our proteins of interest, produced insufficient band separation. Switching to 10% SDS-PAGE gels greatly improved band visualisation. In addition, confusion generated by extraneous bands in the high molecular weight ladder was alleviated by running an additional molecular marker (low molecular weight ladder). These extra points of reference facilitated the approximation of the molecular weight of the gel bands.

***Escherichia coli* DH5α control strain does not have ampicillin resistance, but lysis reveals a 31 kDa band.** Optical density measurements confirmed the absence of the pBR322 plasmid in this strain, as the control strains was unable to grow in the presence of antibiotics (Table 1). However, lysis of the control strain and SDS-PAGE visualisation revealed a band at 31 kDa, suggesting significant cellular protein background at this size. Intriguingly, this 31 kDa band in the control is not present in the tetracycline-treated samples, an observation more obvious in the gel image than the band intensity analysis suggests.

TABLE 1. Cell Turbidity (OD_{660}) of *E. coli* DH5α cells with or without pBR322 plasmid grown in Luria broth with Ampicillin or Tetracycline

Turbidity	*E. coli* DH5α (-) pBR322[a]				*E. coli* DH5α (+) pBR322[a]			
	Ampicillin (μg/mL)		Tetracycline (μg/mL)		Ampicillin (μg/mL)		Tetracycline (μg/mL)	
	50	200	10	45	50	200	10	45
Initial OD_{660}	0.14	0.13	0.12	0.13	0.12	0.12	0.12	0.11
Final OD_{660}	0.05	0.03	0.14	0.13	1.00	1.10	0.80	0.15
Δ OD_{660}[b]	-0.09	-0.10	0.02	0.00	0.88	0.98	0.68	0.04

[a] The inoculum was grown in Luria broth with various antibiotic strengths for 4 hours at 37 °C.
[b] Change in turbidity was derived from subtracting Initial OD_{660} from Final OD_{660}

DH5α containing pBR322 grows better in ampicillin than tetracycline. Optical density readings of the experimental cultures immediately following inoculation with overnight cultures, and readings after 4 hours incubation, revealed decreases or static culture densities in the tetracycline treatments (Table 1). Additionally, greater growth was observed in the ampicillin-treated cultures as compared to the tetracycline-treated cultures. This confirms previous findings of Law *et. al*. (16).

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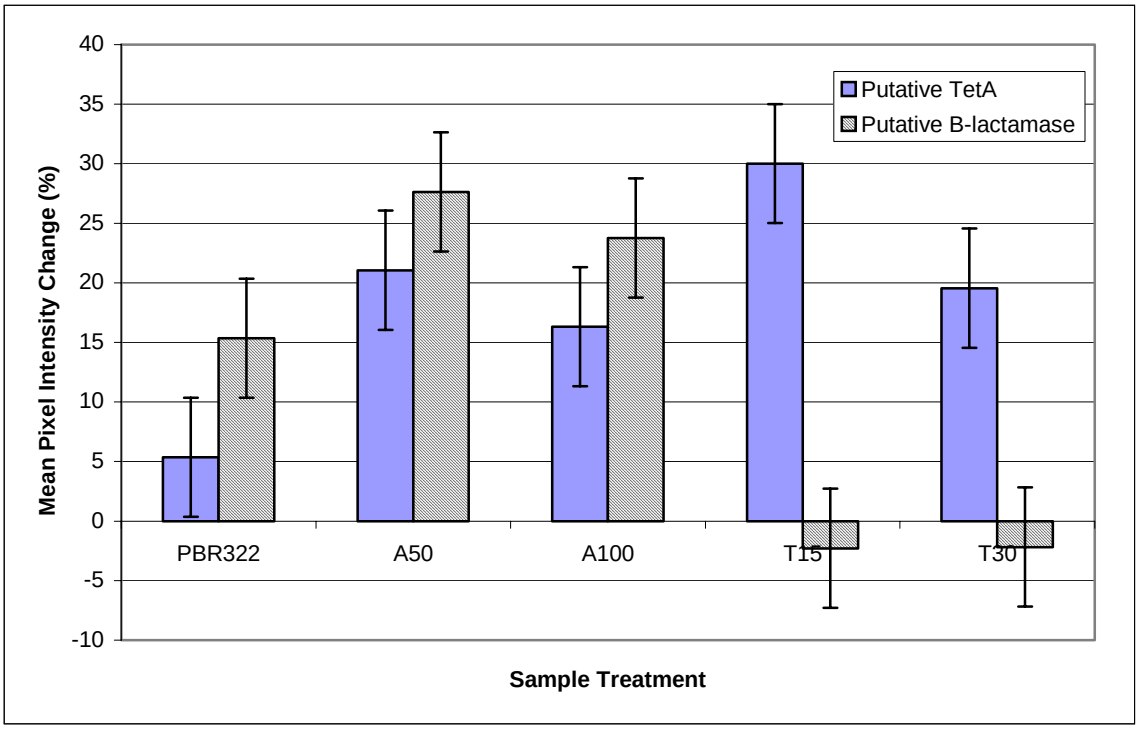
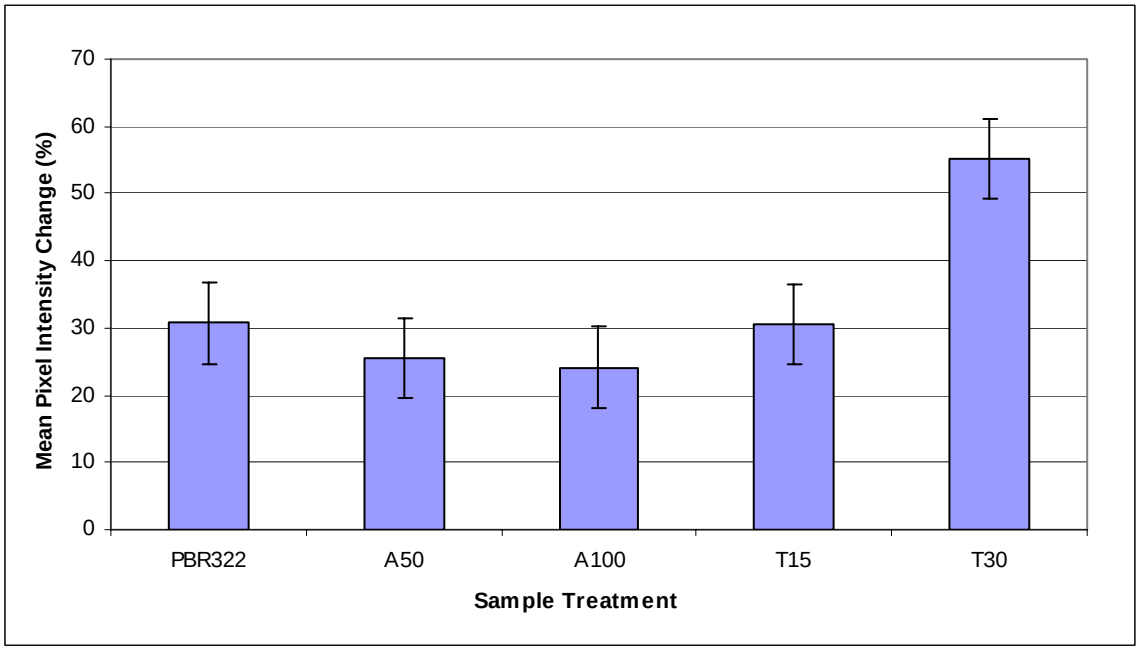
Protein levels differentially responsive to antibiotic treatments (Fig. 1). TetA and TEM-1 β-lactamase proteins are present at significant levels in at least one of the periplasmic or cytosolic fractions of the DH5α pBR322 controls (no antibiotics). The DH5α pBR322 TetA and TEM-1 β-lactamase protein levels respond to differences in antibiotic treatments. The level of TEM-1 β-lactamase increases under ampicillin treatment, but under tetracycline treatment the level decreases or approximates the levels found in strains without the plasmid. TetA increases in at least one of the fractions under treatment by either antibiotic, but increases more with tetracycline. While there is less TetA in the 30 µg/ml tetracycline (tet30) treatment as compared to the 15 µg/ml tetracycline (tet15) treatment in the periplasmic fraction, there is a significantly larger increase (approximately doubled) for tet30 treatment as compared to all other plasmid-containing samples in the membrane fraction.



A.



B.

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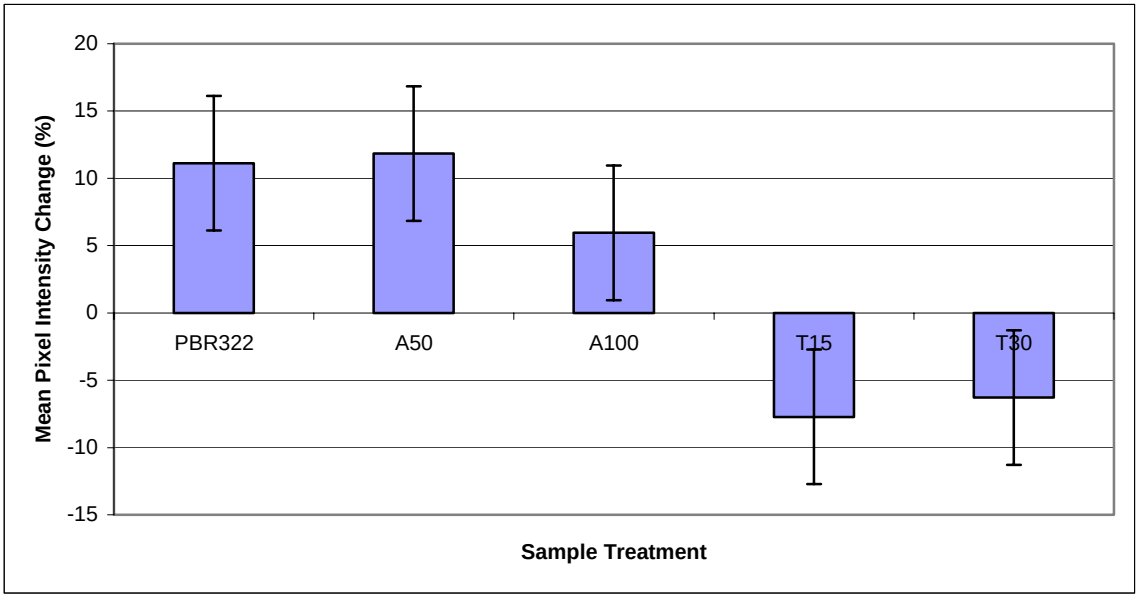

C.

Figure 1. The percentage change of the mean pixel intensity of the putative β-lactamase and TetA protein bands on a silver-stained 10% SDS-PAGE, as compared to the levels present in DH5α cells that did not contain pBR322. For all graphs 'pBR322' samples are DH5α cells with the pBR322 plasmid grown in LB media but not subjected to antibiotics, 'A' represent represents DH5α cells containing pBR322 incubated with either 50 or 100 μg/ml ampicillin for 4 hours, 'T' represents DH5α cells containing pBR322 incubated with either 15 or 30 μg/ml for 4 hours. Error was estimated to be 5%. **A.** Percentage changes of putative β-lactamase and TetA protein levels isolated from the periplasm. **B.** Percentage change of putative TetA protein level isolated from the membrane **C.** Percentage change of putative β-lactamase protein level isolated from the cytosol

Protein level responses vary with cellular location (Fig. 1): No TEM-1 β-lactamase was seen in membrane fraction, and similar patterns of intensity were observed in cytosolic and periplasmic fractions. In the cytosolic fraction, differences in TetA band intensities between DH5α with pBR322 control and ampicillin and tetracycline treated samples were insignificant. While membrane fraction intensities for the DH5α with pBR322 control, ampicillin treatments and tet15 increased equivocally, the increase doubled in the tet30 treatment. In the periplasmic fraction, TetA levels increased from DH5α with pBR322 levels, with the greatest TetA band intensity occurring in the tet15 sample and slightly lower levels in the tet30 sample.

DISCUSSION

Analysis of band intensities on SDS-PAGE gels of cell lysates reveals trends, consistencies and differences between antibiotic treatments and cellular locations. It is known that transcription of the *tetA* gene is highly regulated by the *tetR* gene product, TetR. In the absence of TetR, tetracycline binds to the operator region of the *tetA* promoter, repressing *tetA* transcription. In the cell cytoplasm, tetracycline binds the repressor protein and changes its conformation so that it can no longer bind to the *tetA* operator, allowing TetA to be produced (3). We expected *E.coli* DH5α (with plasmid pBR322) production of the TetA efflux pump to predominate over β-lactamase production in the presence of tetracycline, and β-lactamase production to predominate over TetA expression in the presence of ampicillin. Additionally, the control strain of DH5α without pBR322 should not have bands corresponding to TetA or β-lactamase, and the DH5α pBR322 without antibiotic treatment should show either no or constitutive levels of the resistance proteins. SDS-PAGE analysis of samples grown without pBR322, and with pBR322 in Luria broth, ampicillin and tetracycline revealed a 46 kDa protein in every sample (data not shown). The ubiquitous presence of this 46 kDa protein may be due to significant levels of cellular background proteins approximating this size. A 46 kDa band appeared from the sampled control strain *E.coli* DH5α (without pBR322), even though the strain did not possess the tetracycline efflux pump as it was unable to grow in the presence of tetracycline (Table 1). The higher mean intensities of the 46 kDa bands in all *E.coli* DH5α samples containing pBR322 (see Figures 1A and 1B) suggest that a constitutive, basal level of TetA production when the plasmid is

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present in cells. To determine whether the 46 kDa band we have been tracking is composed of our protein of interest, TetA, or background proteins would require Western blotting and detection with monoclonal antibodies directed against TetA. Also a 2-D gel may facilitate better resolution of proteins in the size range of TetA or β-lactamase.

We also expected increases in tetracycline concentration to increase the level of TetA expression. As seen in Figure 1B, increasing the concentration of tetracycline from 15 to 30 μg/ml resulted in a significant increase of the putative 46 kDa TetA protein in the membrane, confirming expected results. Additionally, the observation that the band intensity remained at the same level for all treatments except the large tetracycline dose suggests that the constitutive levels of TetA may be sufficient for levels of tetracycline up to a concentration between 15 and 30 μg/ml. However, while membrane TetA increased in the 30 μg/ml tetracycline treatment from levels seen in the 15 μg/ml treatment, there is less TetA in the 30 μg/ml tetracycline-treatment compared to the 15 μg/ml tetracycline-treatment in the periplasmic fraction (Figure 1A). This apparent decrease in periplasmic TetA in the 30 μg/ml tetracycline-treatment could be due to a mysterious increase in TetA's proper secretion to the membrane. TetA is not expected to be in the periplasm, as it is an inner membrane protein which should have no need to traverse the periplasmic space. TetA detection in the periplasm may have been an artifact of the lysis protocol. Some TetA may actually be from the inner membrane, if some inner membrane disruption occurred before harvesting the periplasmic fraction.

Unless a plasmid has a repressor to regulate resistance genes, the resistance genes are most often constitutively expressed – including genes encoding β-lactamases (9). Therefore, we expected that *E.coli* DH5α with plasmid pBR322 would produce the 31 kDa β-lactamase but that cells lacking pBR322 would not. SDS-PAGE analysis revealed that a 31 kDa protein was produced in all samples including those without pBR322 (data not shown). Again, this may be due to significant cellular background at this size as with the putative 46 kDa TetA protein. The use of monoclonal antibodies directed against the TEM-1 β-lactamase would aid in differentiating β-lactamase from cellular background proteins. The inability of *E.coli* DH5α without pBR322 to grow in the presence of ampicillin (Table 1) refutes any possible chromosomal mutation resulting in a 31 kDa β-lactamase, supporting the likelihood of background protein interference.

Chong *et al.* (2) showed that as the concentration of ampicillin was increased the copy number of pBR322 increased as well. Since β-lactamase is constitutively expressed this should correlate with an increase in the 31 kDa protein. However, as Figure 1A and 1C show, the periplasmic and cytosolic levels of putative β-lactamase protein actually decreases when the ampicillin concentration increases from 50 μg/ml to 100 μg/ml. The higher concentration of ampicillin could weaken the cell wall, causing release of the β-lactamase protein from both the periplasm. β-lactamase binds to penicillin binding proteins (PBP) responsible for the transpeptidation reaction in peptidoglycan synthesis. Thus, the peptidoglycan subunits' glycan chains are no longer cross-linked and the cell wall becomes progressively weaker. In addition, autolysins that digest the existing cell wall are released when ampicillin binds to PBPs (7). As the cell loses β-lactamase from the periplasm, it may transport more β-lactamase from the cytosol (thus depleting cytosolic levels).

The release of β-lactamase from the periplasm should be visualised as higher intensities of β-lactamase bands in the secreted fractions. However, difficulties with the gels with which the secreted fractions were analysed resulted in a lack of data on secreted protein profiles. Loading unequal protein concentrations onto the polyacrylamide gel and problems with silver staining meant that no informative results were obtained from the secreted protein fractions of the cell lysates.

Interestingly, the levels of the putative β-lactamase protein in the *E.coli* DH5α cells with pBR322 and without pBR322 in the tetracycline-treatments were approximately the same in the cytoplasmic and periplasmic fractions (see Fig. 1A and 1C). The observation that there is little change in protein expression from control levels suggests that the *bla* gene is not being expressed in the cells. However, it seems unlikely that no β-lactamase is being produced, since it has constitutive expression levels. What may be occurring is that as tetracycline concentration increases the TetA efflux pumps increase activity. TetA acts as an antiporter, trading the exiting tetracycline drug for an incoming proton. This decreases the proton gradient, reducing the positive charge on the outside of the cell (5). However, β-lactamase, among other secreted proteins, requires a proton gradient to properly orient the signal sequence on the protein (the electrochemical gradient across the membrane facilitates the transmembrane orientation of the signal sequence's charged side chains) (4). Without the necessary proton gradient, β-lactamase cannot orient in the membrane, cannot expose its cleavage site to the signal peptidase in the periplasm, and cannot be secreted into the periplasm. (4) Thus, the inability of β-lactamase to be released from the membrane may inhibit further trafficking of β-lactamase pre-protein to and through the secretory pathway. The accumulated β-lactamase pre-proteins in the cytosol and membrane may be degraded by proteases, erasing any trace of their presence in those

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cellular compartments. Similar difficulties in TetA secretion into the membrane may occur under high levels of TetA antiporter activity.

Analysis of protein expression levels and cellular localisation of TetA and β-lactamase under tetracycline and ampicillin treatments reveal differential responses to both antibiotics. Both resistance proteins have constitutive expression and increase in response to their respective antibiotics, although not necessarily in a linear fashion. Resistance protein expression may peak at certain concentrations. However, the addition of other antibiotics affects resistance protein expression in unpredictable ways. Thus, the relationship between antibiotics and proper cellular expression of resistance proteins is not simply due to the effects of plasmid gene regulation. Effects of membrane stability and membrane potential are also involved in the expression of resistance proteins from pBR322 in response to antibiotic treatment.

FUTURE EXPERIMENTS

Analysis of β-lactamase and TetA expression. A specific yet sensitive system must be used to accurately identify and quantify the expression of both β-lactamase and TetA in *E. coli*. In this experiment, proteins isolated from *E. coli* were separated based on their size using SDS-PAGE. The gels were then silver stained to detect the presence of proteins. This method identified the presence of proteins at the 31 kDa and 46 kDa levels, but it is impossible to conclude with absolute certainty that these bands represent only the *bla* and *tetA* gene products respectively. For future analysis of *tetA* and *bla* expression, a Western blot using monoclonal antibodies specific to β-lactamase and the TetA proteins could be used to specifically identify and quantify β-lactamase and TetA.

Leakage of β-lactamase. This experiment demonstrated that there is a decrease in the level of β-lactamase in the periplasm when the presence of TetA in the inner membrane is increased. As previously suggested, this is likely a result of TetA collapsing the membrane potential (5), which in turn disrupts the secretion of β-lactamase into the periplasm (4). To confirm that the TetA-induced disruption of the membrane potential inhibits the secretion of β-lactamase, membrane potentials can be measured in DH5α cells with pBR322 and compared to DH5α cells containing pBR322 with an inactived *tetA* gene. β-lactamase and TetA expression can be qualified and quantified via Western blot as described above. Results can be analyzed to search for a correlation between the membrane potential, level of TetA in the inner membrane, and the level of β-lactamase present in the periplasm.

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