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The Effects of Ampicillin versus Tetracycline in the Selection of pBR322 Transformants

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***Escherichia coli* DH5α cells were transformed with pBR322 plasmids, which encode both ampicillin and tetracycline resistance genes. After 45- and 60-minute recovery periods, the transformed cells were plated on three types of selective plates, containing 1) ampicillin only, 2) tetracycline only, and 3) ampicillin plus tetracycline. It was found that tetracycline selection from these recovery periods resulted in a lower level of transformant survival than ampicillin selection. However, after the transformants were allowed to recover for several generations, their survival rates were similar on ampicillin plates and on tetracycline plates.**

The pBR322 plasmid is a commonly used cloning vector that contains both the ampicillin and tetracycline resistance genes as selectable markers. *Escherichia coli* cells transformed with pBR322 plasmids have a selective advantage in antibiotic-treated environments. Results from a previous experiment indicate that different selective pressures affect the survival of transformed cells, as considerably more transformants survived ampicillin-only selection than ampicillin-tetracycline selection (1). Many possible reasons can explain this difference. Firstly, the mechanisms of ampicillin resistance and tetracycline resistance encoded on the pBR322 plasmid are very different. Ampicillin resistance on the pBR322 plasmid encodes the enzyme β-lactamase, which is secreted to the periplasm and acts by binding and inactivating the ampicillin molecule (http://wine1.sb.fsu.edu/bch5425/lec13/lect13.htm). Tetracycline resistance, on the other hand, involves the production of a transmembrane efflux pump to pump out tetracycline molecules that have entered the cell (http://wine1.sb.fsu.edu/bch5425/lec13/lect13.htm). The difference of the mechanisms may contribute to the discrepancy in transformant survival rate. Secondly, the difference in rates of expression of these resistance genes may affect survival. Furthermore, the combination of antibiotics may have an additive effect on lowering *E. coli* growth.

The first part of our experiment was aimed to elucidate whether the survival rate of pBR322-transformed cells is diminished by the combination of tetracycline and ampicillin or by tetracycline alone. To determine whether there are different rates of resistance expression, the second part of our experiment examined whether the effects of antibiotic pressures on transformants were consistent after many generations of growth.

MATERIALS AND METHODS

Plasmid Isolation

E. coli DH5α cells containing plasmid pBR322 were grown to mid-exponential growth in Luria media broth containing ampicillin (50 μg/mL). The culture was then treated overnight with chloramphenicol (100 μg/mL) to inhibit growth and amplify the pBR322 plasmid. Cells were collected by centrifugation at 10,000 rpm (in IEC 819 rotor), and suspended in lysozyme breakage buffer. The cell suspension was then treated with sodium dodecyl sulphate (2% SDS) and sodium hydroxide (0.4 M NaOH). Acidified salt buffer was used to precipitate chromosomal DNA. After centrifugation at 9,500 rpm (in IEC 819 rotor), the supernatant was washed twice with phenol to remove residual protein. The plasmid suspension was separated from the phenol by centrifugation at 7,200 rpm (in IEC 819 rotor). Ethanol was used to precipitate plasmid DNA and to remove residual phenol and salt. After centrifugation at 8,500 rpm (in IEC 819 rotor), the plasmid pellet was resuspended in Tris-EDTA buffer, then treated with ribonuclease and chloroform in a dialysis bag. The suspension was dialyzed overnight in Tris buffer.

DNA Concentration

Isolated plasmid DNA was diluted 1-in-40 and 1-in-80 in final volume of 1 mL of Tris-EDTA buffer. Absorbance of the two dilutions at 260, 270 and 280 nm were determined using a double beam spectrophotometer. The concentrations of DNA in undiluted samples were calculated from absorbance units by using the conversion factor that one A_{260} unit is equal to 50μg/mL of DNA.

Preparation of competent DH5α cells

E. coli DH5α cells lacking the pBR322 plasmid were grown overnight in Luria media broth in a shaking water bath at 37°C. The $O.D._{650}$ was taken the following day and the culture was diluted to yield a 0.2 O.D. after an additional hour of aerated incubation in the 37°C water bath. The O.D. 0.2 cells were chilled on ice for 5 minutes. Nine milliliter of the chilled culture was centrifuged for 5 minutes at 6,900 rpm in a chilled IEC 819 rotor. All subsequent manipulations were done on ice. The supernatant was removed, and the pellet was gently mixed with 0.45 mL of ice-cold 50mM $CaCl_2$ in Tris. The cells were kept on ice for an additional 30 minutes.

Transformation

0.05 µL of competent DH5α were added to 3 µg of pBR322 plasmid and chilled on ice for 10 minutes. Cells were then heat shocked for 2 minutes in a 42°C water bath. The transformation was done in triplicates in addition to a negative control lacking the plasmid DNA. Cells recovered for 50 minutes in 1.0 mL Luria broth, shaking in a 37°C water bath. The transformants were then plated on three different types of Luria plates containing either ampicillin-only (50 µg/mL), tetracycline-only (15 µg/mL), or ampicillin-tetracycline (50 µg/mL, 15 µg/mL). The plates were incubated at 37°C for 24 hours.

Extended Outgrowth

Transformants selected on ampicillin, tetracycline, and ampicillin-tetracycline were picked from antibiotic-containing agar plates. They were inoculated in Luria broth under three conditions: 1) Luria broth with ampicillin (50 µg/mL); 2) Luria broth with tetracycline (15 µg/mL); and 3) Luria broth with ampicillin and tetracycline (50 µg/mL and 15 µg/mL, respectively). The transformants were cultured for many generations with aeration at 37°C. The cultures were diluted 10^{-8} following the ten generations. The diluted cultures were plated on three different antibiotic selective plates: 1) ampicillin (50 µg/mL); 2) tetracycline (15 µg/mL); and 3) ampicillin and tetracycline (50 µg/mL and 15 µg/mL, respectively). The plates were incubated at 37°C for 24 hours.

RESULTS

Table 1 depicts the number of transformants on three different selective plates: ampicillin, tetracycline, and ampicillin plus tetracycline. It was surprising to find a very high number of transformants on all ampicillin plates. Two possible reasons could explain this finding: 1) the DH5α cells used to make competent cells were already resistant to ampicillin prior to transformation; and 2) the ampicillin in the agar plates was defective. Both explanations suggest that the ampicillin plates failed to select for pBR322 transformants.

Streaking tests were performed, and it was determined that the ampicillin used was defective. This finding explains the observation that all the ampicillin plates had confluent lawns of cells. Since the ampicillin was defective, the tetracycline plus ampicillin plates behaved like tetracycline-only plates. However, there appeared to be a slight difference between them (Table 1). This difference is insignificant when the error of standard deviation is taken into account.

Despite the defective ampicillin, some interesting results did emerge from this experiment. MacConkey plates appeared to support the growth of more colonies than Luria plates. The difference in composition of the two types media may contribute to this observation. It was also observed that longer recovery time resulted in a higher number of transformants, in both Luria agar and MacConkey agar. This may be because cells were allowed longer period to recover their membrane after heat shock.

TABLE 1. Colony counts of pBR322 plasmid transformants on various selective plates after different periods of outgrowth – Trial 1.

	Ampicillin[a]		Tetracycline		Ampicillin[a] & Tetracycline	
	45 min recovery	60 min recovery	45 min recovery	60 min recovery	45 min recovery	60 min recovery
Luria plates	TNTC	TNTC	190(43)	443(102)	198(129)	275(160)
MacConkey	TNTC	TNTC	268(3)	450(450)	374(5)	550(50)

[a] Defective ampicillin.
( ) Standard Deviations

Table 2 shows our second trial of transformation. With the use of effective ampicillin, it was apparent that tetracycline plates (15 µg/mL) selected for fewer transformants than ampicillin (50 µg/mL). A possible reason may be the presence of false positives. However, further tests are required to determine whether there are untransformed cells on the ampicillin plates.

Table 2 also indicated that when ampicillin and tetracycline were combined, an additive effect was observed. Ampicillin plus tetracycline plates contained the fewest number of transformants in this experiment. This result was expected, since the presence of both tetracycline and ampicillin could exert more stress on the cells.

TABLE 2. Colony counts of pBR322 plasmid transformants after 66 minutes of outgrowth – Trial 2.

	Ampicillin	Tetracycline	Ampicillin & Tetracycline
Luria plates	101(47)	54(32)	27(24)

( ) Standard Deviations

TABLE 3. Colony counts of pBR322 plasmid transformants after outgrowth. FPD at 10^{-8}.

Original Plate	Antibiotics in Outgrowth	Selection Plates		
		Day I[b]: Ampicillin	Day I[b]: Tetracycline	Day II: Ampicillin & Tetracycline
Ampicillin[a] & Tetracycline	Ampicillin	10(2)	12(6)	13(7)
Tetracycline		5(2)	7(7)	43(47)
Ampicillin[a] & Tetracycline	Tetracycline	41(4)	23(10)	76(19)
Tetracycline		108(14)	47(29)	19(4)
Ampicillin[a] & Tetracycline	Ampicillin & Tetracycline	1(0)	0(0)	0(0)
Tetracycline		3(1)	3(1)	10((7)

[a] Defective ampicillin.
[b] Plates from Day I of Extended Outgrowth Experiment. Cells were plated on ampicillin and tetracycline plates on Day II for comparison purposes. However, the numbers were evidently higher, therefore they were not included in the table. The results were not used because there were too few plates.
( ) Standard Deviation

In the outgrowth experiment, the transformed cells were grown in broth under one of the three antibiotic selective conditions (ampicillin, tetracycline, and ampicillin plus tetracycline) for ten generations to allow maximum recovery before plating onto selective plates. The results are shown in Table 3. It is evident that after extended outgrowth, the numbers of transformants on ampicillin and tetracycline plates were more or less similar compared to the number of transformants after 45 or 60 minutes of recovery. It is important to realize that cells selected on tetracycline can give numbers greater than the numbers selected on ampicillin containing plates. This suggests that the initial low transformation efficiency under tetracycline selection was not a long-term effect. Tetracycline

appears to select fewer cells than ampicillin after 45 minutes of recovery. However, after ten generations, tetracycline and ampicillin yield similar transformation efficiencies.

In the case of the transformants that were growing in the presence of tetracycline in the outgrowth, the discrepancy observed previously between ampicillin and tetracycline plates is still evident to some extent. An explanation of this may be the bactericidal and bacteristatic nature of the two antibiotics. It is possible that in the absence of bactericidal ampicillin, some non-tetracycline resistant cells were persisting inactively in the culture during outgrowth. At the same time, they may have developed a spontaneous chromosomal mutation for ampicillin resistance. Therefore, when the cells were removed from tetracycline-containing broth to new selective plates, the ampicillin-resistant mutant cells, which were unable to develop into colonies on tetracycline plates, gave rise to colonies on ampicillin plates.

It should be noted that higher numbers of transformants were seen on ampicillin plus tetracycline plates after many generations, although these plates were previously found to contain the least number of transformants (Table 2). This is likely due to the fact that transformants were plated on ampicillin-tetracycline plates one day after the actual transfromation. Although the transformants were store in 4°C, some growth likely still occurred. Another peculiar point in our data is the colony count for the ampicillin plus tetracycline plate inoculated with the tetracycline-outgrown cells. It does not follow the general trend of increase in delayed inoculation as seen under the other selective conditions. We believe this is the result of experimental error.

DISCUSSION

Tetracycline selection appears to depend on the length of the recovery period. Forty-five minutes after recovery, cells are more sensitive to tetracycline than to ampicillin (Table 1). A combination of factors could contribute to this observation. One aspect that should be considered is the integrity of the cell membrane after recovery. DH5α cells were made competent by treatment with $CaCl_2$. These ions destabilize the membrane, enabling the entry of extracellular DNA during heat shock. Since the time of recovery was only 45 minutes, equivalent to approximately one generation or less, it is possible that cells were unable to fully recover from membrane permeabilization. This may have an adverse impact on tetracycline resistance. The plasmid pBR322 encodes tetracycline efflux, a transmembrane export protein. Transformants need to express the efflux pump on their cell membrane in order to survive in the presence of tetracycline. However, survival is likely to be low if cell membranes are not fully recovered and intact. A mere extension of 15 minutes in recovery time appears to produce an increase in recovered cell numbers. Colony counts approximately doubled with a 60-minute recovery time compared to 45 minutes. Since it is unlikely that the additional 15 minutes entirely accounts for doubling merely due to cell growth, we believe that the increase was partially due to cell membrane recovery.

On the other hand, cell membrane recovery may have less significance in survival in the presence of ampicillin. This may be due to the fact that ampicillin resistance gene encodes β-lactamase, which is secreted into the periplasm (http://wine1.sb.fsu.edu/bch5425/lec13/lect13.htm), thus require a less intact outer membrane.

Furthermore, tetracycline efflux has been shown to be an electroneutral antiport system that catalyzes the exchange of a tetracycline-divalent-metal-cation complex for a proton (http://biosafety.ihe.be/AR/Tetracycline/Menu_Tet). The expulsion of tetracycline by an efflux pump functions at the expense of the proton gradient across the cell membrane by using the energy via the proton motive force; whereas β-lactamase catalyses hydrolysis of the β-lactam ring of ampicillin (http://biosafety.ihe.be/AR/Tetracycline/Menu_Tet). Growth under tetracycline is therefore a more stressful condition. β-lactamase destroys ampicillin, while tetracycline molecules are unaffected by tetracycline efflux. This means that the concentration of ampicillin in the media gradually decreases, while the concentration of tetracycline remains constant. Therefore, a considerable amount of energy is expended by the cell to continuously expel tetracycline. The extra stress in addition to the constant level of tetracycline may account for the lower survival rate on tetracycline plate than on ampicillin plates.

Another factor to consider is the level of resistance gene expression in DH5α cells. It is likely that ampicillin and tetracycline resistance genes have different promoter strengths. The promoter strength may imply the level of resistance gene products in the cell, which in turn may explain the different number of transformants in ampicillin and tetracycline. Promoter strengths can be further studied by fusing reporter genes such as β-galactosidase to each promoter and comparing the amounts of expression using an enzyme assay. However, the amount of resistance gene products in the cell may not directly correlate to cell survival in the presence of antibiotics. It is plausible that the products of one resistance gene expressed at a lower level is equally efficient to another expressed at a comparably higher level.

As stated earlier, the numbers of transformants in ampicillin-only and tetracycline-only are significantly different when recovery time is short. However, this difference is not observed after many generations of growth in selective media, where transformant selection in ampicillin was comparable to that in tetracycline. This suggests that, once the cells are fully recovered and stabilized in stationary phase, the two antibiotics have similar efficiency. The difference in resistance mechanisms suggested earlier may have a lower impact, if not negligible when cells are fully recovered from heat shock. Therefore, the initial difference we observed was merely a matter of recovery period.

When both ampicillin and tetracycline are present, transformation efficiency is lower than ampicillin or tetracycline alone. This additive effect implies that ampicillin plus tetracycline confers a stronger selective pressure on the cells, subsequently lowering survival rate.

Our experiment explored the notion that tetracycline has lower transformation efficiency than ampicillin following minimal recovery time. When the recovery period is extended, ampicillin selection and tetracycline selection yield similar transformation efficiencies. Our findings support the reason why ampicillin is more frequently used than tetracycline for transformant selection in research laboratories.

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