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Growth inhibition of DH5α *E. coli* in M9 minimal media following transformation with the recombinant plasmid pMOB3

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The DH5α *E. coli* strain will proliferate in M9 minimal media supplemented with 0.2% glycerol and thiamine (0.5 µg/ml), however a closely related strain, C584 will not. C584 is a DH5α derivative which contains the plasmid pMOB3. The purpose of this study was to investigate whether the presence the pMOB3 plasmid or an unidentified difference between chromosomal genotypes was the cause of this growth limitation. To examine this problem, pMOB3 was extracted from C584 and cloned into DH5α cells that had demonstrated growth in the specified M9-glycerol minimal media. Uptake of the plasmid resulted in loss of the ability of the cells to proliferate in M9-glycerol media. The presence of this plasmid, or specifically its translated protein products, are somehow responsible for the repression and inhibition of a biological pathway or function that is essential for growth under such limited nutrient conditions.

Kanamycin is a protein synthesis inhibitor and functions by binding to the 30S subunit of the ribosome [1]. Kanamycin can halt the 30S initiation complex (30S-mRNA-tRNA) to block further initiation of translation. It can also affect translation that has already been initiated and cause the mRNA to be misread. The purpose an earlier experiment of ours was to investigate the possibility that kanamycin may have secondary effects on *E. coli* that extend beyond inhibition of protein synthesis. To test for this, we grew a kanamycin-resistant *E. coli* strain (C584) and a kanamycin-sensitive *E. coli* strain (DH5α) in M9 minimal media supplemented with 0.2% glycerol and thiamine (0.5 µg/ml). The strains were chosen due to their similarity: strain C584 is actually the DH5α strain containing the plasmid pMOB3. Results show that the *E. coli* strain C584 does not proliferate in this M9 minimal media while the DH5α parent strain does. This suggests one of two possibilities: either the presence of the pMOB3 plasmid is responsible for the suppression of growth under limited nutrient conditions, or the C584 stain we were provided contained a chromosomal mutation resulting in an auxotrophic requirement that is not satisfied by M9 minimal media.

Our investigation involved the isolation of the plasmid pMOB3 from C584 *E. coli* and the subsequent transformation of DH5α *E. coli* and selection on Luria Broth plates containing kanamycin. The pMOB3 plasmid confers *E. coli* resistance to both kanamycin and chloramphenicol. To verify whether the recovered cells were transformants containing the pMOB3 plasmid, they were streaked on Luria Broth plates containing chloramphenicol. In addition, plasmid DNA was isolated from candidate colonies, digested with *BamHI* restriction enzyme and run on a gel with *BamHI* digested pMOB3. Following positive verification that our transformants contained pMOB3, we grew them in M9 minimal media to test our hypothesis and determine whether the presence of pMOB3 would inhibit growth.

Our research has led us to believe that the pMOB3 plasmid results in either the inhibition of an essential protein or pathway in DH5α *E. coli*, or that the presence and maintenance of this plasmid may simply require too many resources in order to allow healthy growth in M9.

METHODS AND MATERIALS

The methods and materials employed in the plasmid isolation and transformation was a modification of the ones described in Experiment B6 of the Microbiology 421 WebCT resource database [8].

Strains. Transformations were performed on *Esherichia coli* strain DH5α *(deoR, endA1, gyrA96, hsdR17(r_k^- m_k^+), recA1, relA1, supE44, thi-1, Δ(lacZYA-argFV169) ϕ80lacZ ΔM15, F-)*. Plasmid pMOB3 was extracted from *E. coli* strain C584, which was derived from DH5α parent strain. Both strains were obtained from Dr. Bill Ramey.

Plasmids. Plasmid pMOB3 was constructed by cloning a 0.9-kb *Bam*HI fragment containing the chloramphenicol acetyl transferase (CAT) gene into the *Bam*HI site of plasmid pMOB2 [7]**.** In addition to chloramphenicol resistance conferred by the CAT gene, the plasmid also includes a kanamycin resistance marker.

Plasmid p328.5 was constructed by Dr. Kathy Dobinson by cloning a *Hin*DIII C fragment of ϕ29 into the *Hin*DIII site of plasmid pBR322 [2] to inactivate the tetracycline resistance gene. This plasmid, which confers ampicillin resistance, was used as a positive control for our transformations. Plasmid p328.5 was obtained from Dr. Bill Ramey.

Confirmation of DH5α Growth in M9 Minimal Media. Approximately 10 µl of a saturated culture of strain DH5α was inoculated into 25 ml M9 minimal media supplemented with 0.2% glycerol and 0.5 µg/ml thiamine. The cultures were incubated at 37°C for 24 h.

Plasmid pMOB3 Isolation. Approximately 10 µl of a saturated culture of strain C584 was inoculated into 25 ml Luria broth and incubated at 37°C for 20 h. The liquid cultures were transferred to 30 ml Oakridge tubes and centrifuged at 10,000 rpm for 6 min. The supernatant was decanted, and the cell pellet resuspended with 1 ml breakage buffer (50 mM glucose, 25 mM Tris pH 8.0, 10 mM EDTA, 5 mg/ml lysozyme). The cells were transferred to 12 ml Oakridge tubes and left at room temperature for 5 min. Then, 2 ml of detergent solution (0.2 M NaOH, 1% SDS) was added. The tube was inverted five times and placed on ice for 5 min. Subsequently, 1.5 ml of acidified salt buffer (3 M KOAc, 11.5% glacial acetic acid) was added, and the tube placed on ice for 10 min. The mixture was then centrifuged at 9,500 rpm for 10 min. Using a Pasteur pipette, the supernatant was transferred to new centrifuge tubes. Then, 4 ml of phenol/chloroform/isoamyl alcohol (25:24:1) was added, the tube inverted several times, and centrifuged at 7,500 rpm for 5 min. The phenol/chloroform/isoamyl alcohol wash was performed three times. After the final wash, 6 ml of cold 100% ethanol was added to the supernatant. The tube was inverted several times, and left at room temperature for 5 min. The mixture was then centrifuged at 8,500 rpm for 10 min., and the supernatant decanted. The ethanol wash was performed twice. The pellet was then resuspended with 500 µl TE, and transferred to 1.5 ml Eppendorf tubes. To remove contaminating RNA, 10 µl of 10-mg/ml RNAse was added. The samples were placed at 4˚C overnight. The following day, the plasmid DNA was reprecipitated by adding 200 µl acidified salt buffer and 500 µl 100% ethanol. The tubes were vortexed, and then centrifuged at 14,000 rpm for 5 min. After decanting the supernatant, the pellet was washed again with 1 ml 100% ethanol, and resuspended with 500 µl TE. Concentration of the DNA was determined spectrophotometrically.

Competent Cells. Approximately 10 µl of a saturated culture of strain DH5α was inoculated into 25 ml Luria broth and incubated at 37˚C overnight. The following day, some of the culture was diluted with Luria broth to a final volume of 15 ml and an OD_{650} of 0.18, incubated at 37˚C for 30 min. to an OD_{650} of approximately 0.22, and placed on ice for 10 min. Two 6 ml samples of the chilled culture was transferred to 12 ml Oakridge tubes and centrifuged at 7,000 rpm for 5 min. After decanting the supernatant, 0.9 ml cold $CaCl_2$ solution (50 mM $CaCl_2$, 10 mM Tris pH 8.0) was added to each cell pellet. The cells were resuspended by vortexing for 5 s and placed on ice for at least 30 min.

Transformation. Approximately 20 µg of plasmid pMOB3 and 10 µg of plasmid p328.5 were transferred to 1.5 ml Eppendorf tubes, and increased to a final volume of 50 µl using TE. Then, 50 µl of competent DH5α cells was added, gently mixed, and placed on ice for 30 min. The cells were placed in a 42°C water bath for 2 min, and returned to ice for 1 min. Then, 1 ml of Luria broth was added and the cells incubated at 37°C for 1 h to recover. The plasmid pMOB3 transformation mixture was inoculated onto LB agar containing 25 µg/ml chloramphenicol or 20-µg/ml kanamycin. The positive control plasmid p328.5 transformation mixture was inoculated onto LB agar containing 50 µg/ml ampicillin. All plates were incubated at 37°C. Transformants appeared after 20 h.

Confirmation of Successful Transformation. To confirm successful introduction of plasmid pMOB3 into the DH5α cells, six colonies were randomly selected from the chloramphenicol or kanamycin-containing LB agar plates, inoculated into 6 ml Luria broth, and incubated at 37°C overnight. Also, 10 µl of a saturated culture of strain DH5α was inoculated as a negative control. The following day, plasmid DNA was extracted from the cells by using the Promega Wizard Plus Minipreps DNA Purification System, and resuspended to a volume of 50 µl. To verify the identity of the plasmid, 15 µl of each sample was digested for 1 h with 10 U of *Bam*HI (Invitrogen) in a final volume of 30 µl, and fractionated on a 1.0% agarose gel

Test for Transformant Growth in M9 Minimal Media. The six chloramphenicol or kanamycin-resistant colonies were inoculated in 25 ml M9 minimal media supplemented with 0.2% glycerol and 0.5 µg/ml thiamine. The cultures were incubated at 37˚C for 24 h

RESULTS

The alkaline lysis protocol yielded very pure plasmid pMOB3 DNA from C584, at a concentration of 4.0 and 5.6 µg/µl in the two trials. Since the plasmid DNA was resuspended in 500 ul TE, we isolated 2.0 mg and 2.8 mg of DNA in the two trials.

The first attempt at transformation of DH5α with the isolated plasmid pMOB3 DNA was unsuccessful in generating any transformants.

The second attempt resulted in the selection of a single putative transformant. We isolated plasmid DNA from this single colony, digested it with BamH1 and resolved the sample using agarose gel electrophoresis. Analysis of the gel (Figure 1) revealed a single band representing a 7.1 kb fragment of DNA without the expected 0.9 kb fragment accompaniment, which would have shown decisively pMOB3 presence in the transformed colony. We suspect that the absence of the characteristic band is a direct result of a limiting concentration of plasmid DNA present in the sample. We conclude that we did indeed generate a successful transformant, however, in order to be absolutely certain a third attempt was necessary to reproduce our results and aid in the calculation of transformation frequencies.

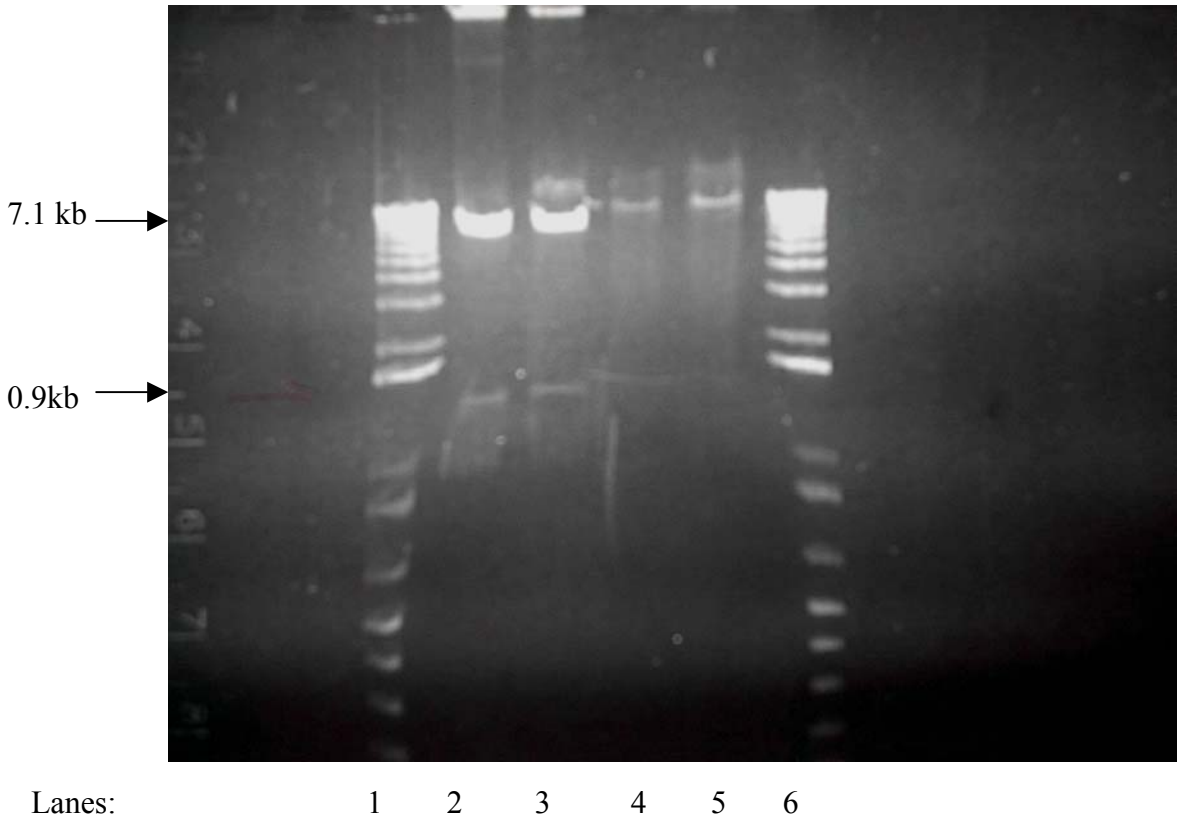

FIGURE 1. 1.0% garose gel electrophoresis of the pMOB3 plasmid and plasmid DNA isolated from DH5α cells transformed with pMOB3 following restriction enzyme digest with BamH1. The single transformant colony was isolated following the second transformation. Lanes 1 and 6: DNA ladder; lanes 2 and 3: pMOB3 +ve plasmid; lanes 4 and 5: plasmid isolated from potential transformant.

TABLE 1. $CaCl_2$ Transformation Results Observed in an *E. coli* DH5α with pMOB3 Culture

Volume of Sample Plated (ml)	Final Plated Dilution of Sample	Plated onto Luria Broth plates containing:	Number of colony forming units (CFU) observed	Concentration of cells surviving transformation (10^7 cells/ml)	Concentration transformants (transformants/ml)
.200	$2x10^{-1}$	kanamycin	15	-	[out of range]
.200	$2x10^{-1}$	kanamycin	30	-	150
.200	$2x10^{-1}$	chloramphenicol	13	-	65 ESPC*
.200	$2x10^{-1}$	chloramphenicol	18	-	90 ESPC*
.200	$2x10^{-3}$	kanamycin	0	-	-
.200	$2x10^{-3}$	kanamycin	0	-	-
.200	$2x10^{-3}$	chloramphenicol	0	-	-
.200	$2x10^{-3}$	chloramphenicol	0	-	-
.200	$2x10^{-5}$	kanamycin	0	-	-
.200	$2x10^{-5}$	kanamycin	0	-	-
.200	$2x10^{-5}$	chloramphenicol	0	-	-
.200	$2x10^{-6}$	-	247	12	-
.200	$2x10^{-6}$	-	182	9	-
.200	$2x10^{-7}$	-	22	[out of range]	-
.200	$2x10^{-7}$	-	4	[out of range]	-

*ESPC - Estimated Standard Plate Count

TABLE 2. $CaCl_2$ Transformation Results Observed in an *E. coli* DH5α with pMOB3 Culture

Volume of Sample Plated (ml)	Final Plated Dilution of Sample	Plated onto Luria Broth plates containing:	Number of colony forming units (CFU) observed	Concentration of cells surviving transformation (10^8 cells/ml)	Concentration transformants (transformants/ml)
.100	10^{-1}	kanamycin	29	-	290
.100	10^{-1}	kanamycin	31	-	310
.100	10^{-1}	chloramphenicol	8	-	80 ESPC*
.100	10^{-1}	chloramphenicol	10	-	100 ESPC*
.100	10^{-3}	kanamycin	0	-	-
.100	10^{-3}	kanamycin	2	-	[out of range]
.100	10^{-3}	chloramphenicol	0	-	-
.100	10^{-3}	chloramphenicol	0	-	-
.100	10^{-5}	kanamycin	0	-	-
.100	10^{-5}	kanamycin	0	-	-
.100	10^{-6}	-	203	2.0	-
.100	10^{-6}	-	123	1.2	-
.100	10^{-7}	-	8	[out of range]	-
.100	10^{-7}	-	27	[out of range]	-

*ESPC - Estimated Standard Plate Count

TABLE 3. $CaCl_2$ Transformation Results Observed in an *E. coli* DH5α with p328.5 Culture

Volume of Sample Plated (ml)	Final Plated Dilution of Sample	Plated onto Luria Broth plates containing:	Number of colony forming units (CFU) observed	Concentration of cells surviving transformation (10^8 cells/ml)	Concentration transformants (10^4 transformants/ml)
.100	10^{-1}	ampicillin	TNTC*	-	[not countable]
.100	10^{-1}	ampicillin	TNTC*	-	[not countable]
.100	10^{-3}	ampicillin	30	-	3.0
.100	10^{-3}	ampicillin	54	-	5.4
.100	10^{-5}	ampicillin	0	-	-
.100	10^{-5}	ampicillin	0	-	-
.100	10^{-6}	-	368	[out of range]	-
.100	10^{-6}	-	207	2.1	-
.100	10^{-7}	-	36	3.6	-
.100	10^{-7}	-	23	[out of range]	-

*TNTC - Too Numerous To Count

The third attempt at transformation resulted in many putative transformants and a selection of these were tested for the presence of plasmid pMOB3 DNA. The average transformation frequency of DH5α with pMOB3 with kanamycin (Tables 1 and 2) was $1.7x10^{-6}$. The average transformation frequency of DH5α with pMOB3 with chloramphenicol (Table 1, Table 2) was $6.3x10^{-7}$. Therefore, transformants of DH5α with pMOB3 were selected more than twice as effectively with kanamycin as compared with chloramphenicol. It was also noted, that DH5α spontaneously gains ampicillin resistance at a rate of $5.0x10^{-7}$, when plated onto Luria Broth plates containing ampicillin (Table 4). Results from the second gel (Figure 2) clearly demonstrate the presence of the expected 0.9 kb fragment, which is the CAT gene (chloramphenicol acetyltransferase) that confers chloramphenicol resistance. This decisively shows that the pMOB3 plasmid is present in the selected transformants. The six positively identified transformants, along with representative C584 and DH5α *E. coli* strains were grown overnight in M9 minimal media. As expected, C584 did not grow in the medium, but DH5α flourished. All six of the DH5α transformants containing the pMOB3 failed to grow in the minimal media (Table 5).

TABLE 4. $CaCl_2$ Transformation Results Observed in an *E. coli* DH5α Control (no plasmid) Culture

Volume of Sample Plated (ml)	Final Plated Dilution of Sample	Plated onto Luria Broth plates containing:	Number of colony forming units (CFU) observed	Concentration of cells surviving transformation (10^8 cells/ml)	Concentration transformants (transformants/ml)
.100	10^{-1}	ampicillin	7	-	70 ESPC*
.100	10^{-1}	kanamycin	0	-	-
.100	10^{-1}	chloramphenicol	0	-	-
.100	10^{-6}	-	162	1.6	-
.100	10^{-6}	-	114	1.1	-
.100	10^{-7}	-	9	[out of range]	-
.100	10^{-7}	-	13	[out of range]	-

*ESPC - Estimated Standard Plate Count

TABLE 5. Observed overnight growth of specified *E. coli* cultures in M9 minimal media supplemented with 0.2% glycerol and thiamine (0.5 μg/ml).

E. coli Strain	Culture Turbidity (OD_{460})
C584 #1	0.003
C584 #2	0.003
DH5α #1	0.151
DH5α #2	0.252
Transformant #1	0.002
Transformant #2	0.004
Transformant #3	0.008
Transformant #4	0.014
Transformant #5	0.007
Transformant #6	0.009

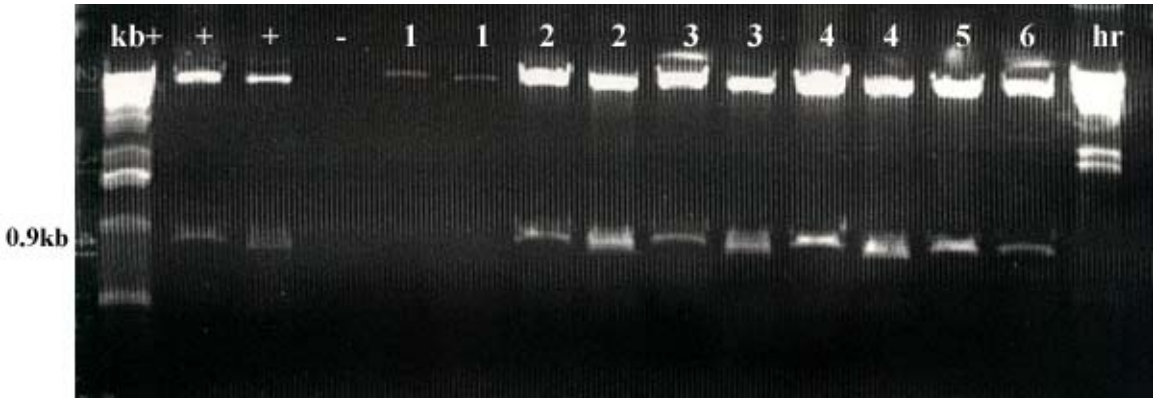

FIGURE 2. 1.0% agarose gel electrophoresis of the pMOB3 plasmid and plasmid DNA isolated from DH5α cells transformed with pMOB3 following restriction enzyme digest with BamH1. Transformant colonies 1 through 6 were isolated following the third transformation. Lane "kb+": DNA ladder; lane "+": pMOB3; lane "-": negative control; lanes 1 to 6: minipreps of the transformants.

DISCUSSION

This seemingly straightforward plasmid isolation and transformation turned out to be far more trying than initially anticipated. Results were slow coming and as complications arose, many modifications were made to the protocol to solve these problems. Generating transformants caused the greatest difficulty primarily because of the poor frequency of DH5α transformation with pMOB3. In order to obtain DH5α *E. coli* containing pMOB3, we performed three transformations, each time modifying the procedure slightly to improve our result.

The first attempt to transform DH5α *E. coli* with the plasmid pMOB3 was unsuccessful. The competent cells were inadvertently killed during the procedure, which led to low turbidity being observed during the recovery period and no colonies growing on the spread plates. The cause of this failure was determined to be the result of a toxic calcium chloride solution, which was used to generate the competent cells. The solution was prepared from a concentrated stock solution and we did not neutralize the pH before its use. However, the solution was determined to have a pH of 7.2 following the failed protocol, and it is believed that the concentration of $CaCl_2$ must have been too high, thus causing the death of the DH5α cells. To remedy this problem the transformation protocol was run a second time using a 50 mM $CaCl_2$ solution that was prepared from scratch and neutralized to pH 7.0. This solved the issue of cell viability and a turbid broth developed during recovery following the heat shock treatment. A plasmid DNA negative control was also added for the second (and third) transformation to determine whether the $CaCl_2$ was responsible for killing the cells, not the pMOB3 plasmid sample.

In the second transformation attempt the competent cells remained viable, but selection for transformants continued to be unsuccessful. Colonies did grow on all of the non-selective Luria Broth plates, but only one rare colony could be found over all 12 of the kanamycin containing selection plates. This lone colony was either the product of random mutation, contamination, or possibly a desired transformant containing pMOB3. To verify whether the cells from this colony did contain the pMOB3 plasmid, they were streaked on Luria Broth plates containing chloramphenicol. The pMOB3 plasmid confers *E. coli* resistance to both kanamycin and chloramphenicol. Therefore, positive growth on both plates strongly favoured the suggestion that the single selected colony was in fact a transformed isolate. To further substantiate this hypothesis, plasmid DNA was isolated from the candidate colony, digested with *BamHI* restriction enzyme and run on a gel with *BamHI* digested pMOB3. *BamHI* digestion of pMOB3 excises the chloramphenicol resistance gene to generate two DNA fragments. A 0.9kb fragment containing the resistance gene and 7.1 kb DNA fragment representing the remainder of the plasmid. Unfortunately, the banding pattern of the isolated plasmid DNA did not perfectly match the expected pattern generated by the pMOB3 control (Figure 1). The 7.1 kb fragment was present, but the 0.9 kb fragment was not visualized. It is believed that this DNA fragment was indeed present, but it was not resolved because an insufficient amount of transformant DNA was isolated and loaded onto the gel. Ethidium bromide interacts with DNA in proportion to the size of the fragments by intercalating between nitrogenous bases in the DNA. This explains why the larger 7.1 kb fragment could be identified in the sample, while the smaller fragment appears to be absent. A greater amount of pMOB3 control was loaded onto the gel resulting in increased intensity and visualization of both the control bands. Although not absolutely conclusive, the observed resistance to chloramphenicol, in addition to the results from the gel indicated that the transformation protocol was effective in generating and selecting a single transformant. This suggests that the frequency of transformation of DH5α with pMOB3 is very low. To validate this hypothesis and quantify the transformation frequency, the protocol was modified in order to increase cell yield. This modified procedure was used for the final transformation attempt and is the one documented in the Methods and Materials section of this report.

In the third and final transformation, many alterations were made to the protocol in order to optimize the probability that we would be able to isolate and identify DH5α transformants containing the pMOB3 plasmid. To accomplish this, the recovery time was increased by 50% to a total of 1.5 hours, both the chloramphenicol and kanamycin containing Luria Broth plates were independently used as selection media, and the volume of inoculum plated was doubled to 200 µl in one series of spread plates. The plasmid p328.5 was also used in a control transformation with DH5α to verify that the transformation protocol is effective with the DH5α *E. coli* strain. Employing these modifications, we were able to generate many transformants.

The frequency of transformation with pMOB3 was very poor compared to the control plasmid p328.5. The transformation frequency with pMOB3 was $1.7x10^{-6}$ with selection on kanamycin plates, while the transformation frequency with the p328.5 control was 100 fold greater at $1.5x10^{-4}$ with selection on ampicillin plates (Tables 1-3). To verify that the selected colonies contained the pMOB3 plasmid, a *BamHI* digest was performed on plasmid DNA isolated from the transformants. Approximately double the amount of DNA was loaded on the gel compared to what had been used in the digest following the second transformation attempt. This ensured that both DNA bands were resolved and decisively proved that we had generated transformants (Figure 2).

DH5α *E. coli* will proliferate in M9 minimal media broth containing 0.2% glycerol and thiamine (0.5 μg/ml). We found, however, that when DH5α is transformed with the pMOB3 plasmid, it loses the ability to grow under such limited nutrient conditions. This suggests that the pMOB3 plasmid results in the inhibition of an essential protein or pathway, or that the presence and maintenance of this plasmid may simply require too many resources in order to allow healthy growth in M9.

Let us assume that the reason for pMOB3 interference with *E. coli* growth in M9 minimal media is due the metabolic burden of constitutively producing kanamycin and chloramphenicol antibiotic resistance. Resistance to kanamycin can result from plasmid-encoded cytoplasmic phosphotransferases that enzymatically modify the antibiotic. Perhaps the production of the phosphotransferases is too metabolically taxing on E. coli cells grown on M9 minimal media.

Additionally, the copy number of the expression plasmid used could potentially be a contributing factor to the observed growth inhibition. The pMOB3 plasmid is a medium copy number plasmid. Overexpression of kanamycin and chloramphenicol antibiotic resistance proteins on the plasmid perhaps results in inhibition of a biochemical pathway that is essential under limited nutrient conditions. Potentially, a lower copy number plasmid would not have such an inhibitory effect. However, the fact that this plasmid is not of high copy number was a contributing factor to the observed low transformation frequency.

Heurgué-Hamard et al. [3] suggest that premature dissociation of peptidyl-tRNA from the ribosome (termed 'drop-off') is a normal accompaniment of protein synthesis, and that peptidyl-tRNA hydrolase (Pth) is essential to cell growth. Pth hydrolyzes dissociated peptidyl-tRNA allowing the tRNA to be charged by aminoacyl-tRNA synthetase and reutilized in protein synthesis. It has been shown that two sets of proteins catalyze ribosome drop-off. One set (RF3, RRF, Ef-G), is composed of factors involved in the process of ribosome recycling. The initiation factors IF1 and IF2 comprise a second set of proteins stimulating drop-off. When the frequency of drop-off exceeds the capacity of Pth to recycle the tRNAs sequestered as peptidyl-tRNA, starvation for essential tRNA isoacceptors occurs, leading to an inhibition of protein synthesis and eventually to cell death. Perhaps the antibiotic resistance genes (phosphotransferases), in the absence of antibiotic, can phosphorylate factors involved in ribosome recycling or initiation, thereby increasing their activity. This activity could catalyze premature dissociation of peptidyl-tRNA from the ribosome. If the rate of drop-off exceeded the capacity of Pth to recycle the tRNAs sequestered as peptidyl-tRNA, starvation for essential tRNA could occur, leading to an inhibition of protein synthesis and eventually to cell death. Minimal media differs from complete media in that it does not contain any supplied tRNAs and perhaps this reservoir of tRNAs present in complete media is responsible for the survival of bacteria with antibiotic resistance genes. However, cells typically do not take up tRNA from the environment other than those which are targeted for degradation to nucleotides. The absence macromolecular precursors such as nucleotides and amino acids in minimal media are a likely contributor to the growth inhibition observed under such conditions.

Moreover, it has been previously found [5, 9] that high expression of a plasmid-encoded tetracycline resistance gene in E. coli reduces the ability of the cells to grow in minimum media. Valenzuela et al. [9] show the cause appears to be a decrease in membrane-bound ATPase activity, apparently brought on by decreased ATP synthase activity, although the mechanism remains a mystery. Like kanamycin and chloraphenicol, tetracycline is also a protein synthesis inhibitor. They differ in that the kanamycin resistance enzyme is a cytoplasmic phosphotransferase that enzymatically modifies the antibiotic, whereas the tetracycline resistance enzymes bind to the bacterial inner membrane and are responsible for mediating efflux of tetracycline. Therefore, while the antibiotics appear similar, the mode of action of the resistance genes is quite dissimilar. Thus, though not likely, a decrease in ATP synthase activity may be the basis for the growth inhibition observed with our pMOB3 transformants in minimal media and should be tested.

It is interesting to note that Shoham et al. [6] observed that the pCED3 plasmid constructed from pUB110 and pBR322 and also containing a kanamycin-resistance gene, interfered with growth of *Bacillus subtilis*. Unfortunately the mechanism of this inhibition was not documented.

Further studies could involve removal of the kanamycin or chloramphenicol resistance genes from pMOB3 and then transforming the resulting plasmid back into DH5α cells. The cells could then be grown in M9 minimal media in order to identify whether an antibiotic resistance gene is responsible for the observed growth inhibition. If no growth in M9 is always observed amongst the generated transformants, then another gene on the pMOB3 plasmid must responsible for the growth inhibition. To identify the pathway in DH5α that is being inhibited as a result of the presence of pMOB3, various nutrients could be added to the minimal media until growth is observed. Relief of growth inhibition by the addition of a supplementary nutrient would pinpoint the biochemical pathway that is being blocked by expression of genes on pMOB3.

REFERENCES

1. **Davies, J. E.** 1991. Aminoglycoside-Aminocyclitol Antibiotics and their Modifying Enzymes. Lorian (ed.), Antibiotics in Laboratory Medicine, 3rd ed. Williams & Wilkins, Baltimore, Md.
2. **Dobinson K. F., and G. B. Spiegelman.** May 25 1985. Nucleotide Sequence and Transcription of a Bacteriophage 29 Early Promoter. J Biol Chem. 260:5950-5905.
3. **Heurgué-Hamard V., V. Dincbas, R. H. Buckingham, and M. Ehrenberg** (2000). Origins of minigene-dependent growth inhibition in bacterial cells. EMBO J. 19:2701-2709
4. **Katayama T., and T. Nagata**. 1990. Inhibition of cell growth and stable DNA replication by overexpression of the bla gene of plasmid pBR322 in Escherichia coli. Mol. Gen. Genet. 223:353-360.
5. **Lee S. W., and G. Edlin**. 1985. Expression of tetracycline resistance in pBR322 derivatives reduces the reproductive fitness of plasmid-containing Escherichia coli. Gene. 39:173-180.
6. **Shoham Y., E. Israeli, A. L. Sonensheim, and A.L. Demain**. 1991. Inhibition of growth of Bacillus subtilis by recombinant plasmid pCED3. Arch. Microbiol. 156:204-212.
7. **Schweizer, H. P.** 1992. Allelic exchange in *Pseudomonas aeruginosa* using novel ColE1-type vectors and a family of cassettes containing a portable oriT and the counter-selectable *Bacillus subtilis* sacB marker. Mol. Microbiol.:1195-1204
8. **Ramey, W. D.** 2002. Microbiology 421: Manual of Experimental Microbiology. University of British Columbia, Vancouver, B.C.
9. **Valenzuela M.S., K. A. I. Siddiqui, and B. L. Sarkar**.1996. High Expression of Plasmid-Encoded Tetracycline Resistance Gene in E. coli Causes a Decrease in Membrane-Bound ATPase Activity. Plasmid (36): 19-25.

APPENDIX

Sample Calculations

Transformation frequency of DH5α with pMOB3 with kanamycin (Table A1)

$= 150 / 1.1 \times 10^{8} = \underline{1.4 \times 10^{-6}}$

Transformation frequency of DH5α with pMOB3 with kanamycin (Table A2)

$= 300 / 1.6 \times 10^{8} = \underline{1.9 \times 10^{-6}}$

Average transformation frequency of DH5α with pMOB3 with kanamycin = $\underline{1.7 \times 10^{-6}}$