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Effects of Chloramphenicol Acetyl Transferase Gene Deletion From pMOB3 on the Growth Inhibition of *Escherichia coli* C584 in M9 Minimal Media

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***Escherichia coli* C584, a pMOB3-containing strain similar to *E. coli* DH5α, was observed to be unable to grow in M9 minimal media supplemented with glycerol and thiamine. *E. coli* DH5α will thrive in M9 minimal media, but the identical *E. coli* C584 strain containing the pMOB3 plasmid grows poorly. This experiment was conducted to examine the potential role of the chloramphenicol acetyl transferase (CAT) gene in pMOB3 in the growth inhibition in M9 minimal media. The CAT gene was removed from pMOB3 and this modified plasmid, named pLGL, was transformed into DH5α. There was observable growth of these transformants in M9 minimal media, which suggested that the CAT gene was responsible for growth inhibition in M9 minimal media.**

Certain plasmids have been found to express genes that reduce the reproductive fitness of *Escherichia coli*, especially in minimal media. Such plasmids include pKH47, which inhibits *E. coli* growth in M9 minimal media including succinate, by expressing the *tet* gene for tetracycline resistance (10). This same gene *tet* gene in pBR322 hinders growth of *E. coli* in glucose-limited M9 minimal media (3). More recently, Kestell *et al.* (2) observed that pMOB3, in *E. coli* strain C584, prevented growth in M9 minimal media supplemented with 0.2% glycerol and 0.5 µg/ml thiamine, whereas a closely related strain lacking this plasmid, *E. coli* DH5α, flourished. They tested this observation by transforming DH5α cells with pMOB3 and found that the resulting transformants lost the ability to grow in M9 minimal media with glycerol and thiamine (2). Their study suggested that some gene(s) encoded in pMOB3 influenced the growth of C584 and transformed DH5α in M9 minimal media (2).

pMOB3, a plasmid derived from pBR322 and pUC19, was first developed to allow the efficient exchange of alleles, in order to study the effects of plasmid-borne mutations on bacterial hosts, particularly in *Pseudomonas aeruginosa* (8). The mobilization (MOB) cassette contained on this plasmid allows successful interspecies transfer of pMOB3 via conjugation, and therefore permits its use as an *Escherichia-Pseudomonas* shuttle vector (8). pMOB3 also contains *B. subtilis*-derived *sacB* and *sacR* genes as counter-selection markers, where gram-negative cells carrying pMOB3 will not grow in sucrose-containing media (8). In addition, this plasmid encodes genes for kanamycin and chloramphenicol resistance, where the latter gene is flanked by *Bam*H1 restriction endonuclease sites (8).

With the knowledge that antibiotic resistant genes can result in growth inhibition in minimal media (3, 10) we have chosen to delete the chloramphenicol acetyl transferase (CAT) gene in pMOB3, which is responsible for chloramphenicol resistance in C584. We hypothesize that the removal of the CAT gene in pMOB3 will allow DH5α cells, transformed with the modified plasmid, named pLGL, to gain the ability to grow in M9 minimal media.

Our research has led us to believe that the absence of the CAT gene in pMOB3 has allowed DH5α cells, transformed with pLGL, to grow in M9 minimal media.

METHODS AND MATERIALS

Strains. Two *E. coli* strains, DH5α and C584, were used in this experiment. Transformation was performed on electroporated DH5α cells (*endA, hsdR17, supE44, thi-1, recA1, gyrA, relA1, Δ(lacZYA-argF), U169, deoR, [ϕ80dlacΔ(lacZ)], M15, thi, pro, hsdR, recA, Tra*; 9). The pMOB3 plasmid was isolated from the C584 strain (*kanamycin-resistant, chloramphenicol-resistant, sacB, oriT*; 8) and restriction digested. Both strains were obtained from Dr. Bill Ramey.

Restriction Digestion of pMOB3. The pMOB3 plasmid contains both chloramphenicol and kanamycin resistance markers. The chloramphenicol resistance gene, a 0.9 kb *Bam*H1 fragment, encoded chloramphenicol acetyl transferase (CAT) in the plasmid. The plasmid was digested singly with *Bam*H1 (GIBcoBRL), *Eco*R1 (GIBcoBRL) and as a double digest with *Bam*H1 and *Eco*R1, to confirm that *Bam*H1 specifically removes the CAT gene from pMOB3.

Confirmation of Growth. DH5α strain was inoculated into 6 ml of Luria-Bertani (LB) broth, and into 6 ml of M9 minimal media supplemented with 0.2% glycerol and 0.5 µg/ml thiamine, for verification of growth. *E. coli* C584 strain was inoculated into 6 ml of LB media for confirmation of growth as well. This strain was also inoculated into 6 ml of M9 minimal media supplemented with 0.2% glycerol, 0.5 µg/ml thiamine, 25 µg/ml chloramphenicol and 20 µg/ml kanamycin for confirmation of non-growth. All cultures were incubated at 37ºC with shaking for 24 h.

Preparation of DH5α Electrocompetent cells. Approximately 5 ml of an overnight DH5α culture was inoculated into 200 ml of fresh LB media and incubated at 37ºC until an OD_{600} of 0.586 was reached. The liquid culture was chilled in an ice water bath for 20 min. The chilled cultures were transferred to 50 ml chilled Oakridge tubes and centrifuged at 4000 g for 15 min at 4ºC. Then, the supernatant was decanted and the cell pellet was washed twice by resuspension in 50 ml of ice cold water followed by centrifugation for 15 min. After the second centrifugation, the pellet was washed in 20 ml of ice cold 10% glycerol and centrifuged again at 4000 g for 15 min. The supernatant was decanted and the pellet was resuspended in 1 ml ice cold 10% glycerol. The suspension was aliquoted into 25 ice chilled microfuge tubes, each with 40 µl culture, and stored at -80ºC.

Isolation of pMOB3 Plasmid and *Bam*H1 Digestion. C584 culture was grown in 250 ml LB with 25 µg/ml chloramphenicol and 20 µg/ml kanamycin, overnight at 37ºC with shaking. pMOB3 plasmid was isolated using the Concert High Purity Maxiprep DNA purification system (GIBco), following the manufacturer's instruction. The concentration of DNA was determined spectrophotometrically. One microgram of the isolated pMOB3 plasmid was digested with the restriction enzyme, *Bam*H1 (GIBcoBRL), to remove the CAT gene. The digestion was done in a total volume of 200 µl and incubated at 37ºC for 1 h.

DNA Extraction and Ligation. The 200 µl *Bam*H1 digested pMOB3 plasmid was fractionated on a 1% agarose gel. The 7.1 kb band was excised and extracted with the QIAEX II agarose gel extraction kit (QIAGEN), following the manufacturer's instruction. Concentration of extracted DNA was determined spectrophotometrically. Ligation was performed on the extracted DNA in a total volume of 20 µl, using T4 ligase (Invitrogen). Three ligation reactions were prepared with 2, 4 and 8 µl of extracted DNA. The ligation reactions were incubated on ice for 12 h. Successful ligations produced the modified pMOB3 plasmid, named pLGL.

Transformation. One, 3 and 5 µl of each of the ligation reactions were added to the 40 µl DH5α electrocompetent cells and mixed gently. All samples were incubated on ice for approximately 5 min and were electroporated at 1.8 V. Following electroporation, 1 ml of LB broth was immediately added to the cells followed by incubation at 37ºC with shaking for 1 h. At the same time, pMOB3 was transformed into DH5α as a positive control. The transformation mixtures were centrifuged briefly and the pellets were resuspended to a final volume of 100 µl in LB media. All of the sample suspensions were plated on LB agar containing 20 µg/ml kanamycin. The positive control cell suspension was plated on LB agar containing 20 µg/ml kanamycin and 25 µg/ml chloramphenicol. All plates were incubated at 37ºC for 20 h.

Confirmation of Successful Transformants. To confirm the successful introduction of pMOB3 plasmid without the CAT gene into DH5α electroporated cells, 31 colonies were selected from the plates with putative transformants. Each selected colony was streaked onto both LB agar with 20µg/ml kanamycin and LB agar with 25 µg/ml chloramphenicol. The plates were incubated at 37ºC for 20 h. Three colonies, present on LB agar with kanamycin but not LB agar with chloramphenicol, were randomly selected and inoculated into 5 ml of LB media containing 20 µg/ml kanamycin. The culture was incubated at 37ºC with shaking overnight. On the following day, plasmid DNA was extracted from each transformant overnight culture by using the Concert Rapid Minipreps DNA purification system (GIBco), following the manufacturer's instruction. Concentration of DNA plasmid was determined spectrophotometrically. To verify the identity of the plasmid, approximately 1 µg of the DNA plasmid, in a final volume of 20 µl, was digested with *Bam*H1 for 1 h at 37ºC. Subsequently, the digestion was fractionated on a 1.0% agarose gel and compared to an Invitrogen 1 kb DNA extension ladder (Cat. No. 1051-012).

Test for Transformant Growth in M9 Minimal Media. The three kanamycin resistant (but not chloramphenicol resistant) transformant colonies were streaked onto M9 minimal agar plates supplemented with 0.5 µg/ml thiamine and 20 µg/ml kanamycin and onto M9 minimal agar plates supplemented with 0.5 µg/ml thiamine and 25 µg/ml chloramphenicol. The plates were incubated at 37ºC for 72 h.

RESULTS

Plasmid DNA (pMOB3) was first isolated from *E. coli* C584, using a maxiprep kit, yielding a DNA concentration of 1460 µg/ml. Next, the pMOB3 was digested with *Bam*HI restriction enzyme. Since the 1 kb ladder did not appear in Fig. 1, the pMOB3 restriction map described by Schweizer (8) was assumed to be correct and the *Bam*H1 digest in lane 6, which gave a larger and smaller band, were assumed to be 7.1 kb and 0.9 kb respectively. The 0.9 kb piece harboured the CAT gene, conferring chloramphenicol resistance. The digest was electrophoresed on 1% agarose gel and the 7.1 kb band was excised; this DNA extraction process was done in two separate trials. In the first trial, the extracted DNA was solublized in Tris-EDTA (TE) buffer [10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA], yielding 43 µg/ml DNA, and with distilled H_2O the second trial, giving 85 µg/ml DNA. Both trials produced a 7.1 kb band when run on 1% agarose gel (Fig. 1). The sticky ends of the *Bam*H1-cut extracted 7.1 kb DNA was ligated using T4 ligase, developing a relaxed circular form; this is shown in Fig. 1, lanes 7 to 11, where the ligated pLGL is seen to have a higher molecular weight than the original extracted 7.1 kb DNA (lanes 2 and 3).

Five ligation reactions were prepared, two using the TE buffer-solublized DNA and three with the distilled H_2O-solublized DNA. The same volume of each ligation reaction was added to each tube of electrocompetent DH5α, followed with electroporation. It is interesting to note that only the transformation done with the distilled H_2O-solublized DNA ligation worked to give a plateful of transformants, whereas the TE-solublized DNA ligation mixture did not result in any colonies after transformation.

Transformed cells were tested on LB media with kanamycin and LB media with chloramphenicol to confirm that the cells transformed with the 7.1 kb pLGL plasmid had lost the chloramphenicol resistance gene. Of the 29 colonies that grew on kanamycin LB plates, 22 of them did not grow on chloramphenicol LB plates. This demonstrated that these colonies possessed a plasmid that still had the kanamycin resistance gene but had lost the chloramphenicol resistance gene. Of the 22 colonies that did not grow on chloramphenicol, three colonies were randomly selected and their plasmid DNA isolated using a miniprep kit. When the isolated plasmid DNA was digested with *Bam*HI, only one band on the agarose gel was present, illustrating that the plasmid was the larger fragment from the original *Bam*H1 digest and likely lacked the CAT gene flanked by *Bam*H1 cut sites (Fig 2).

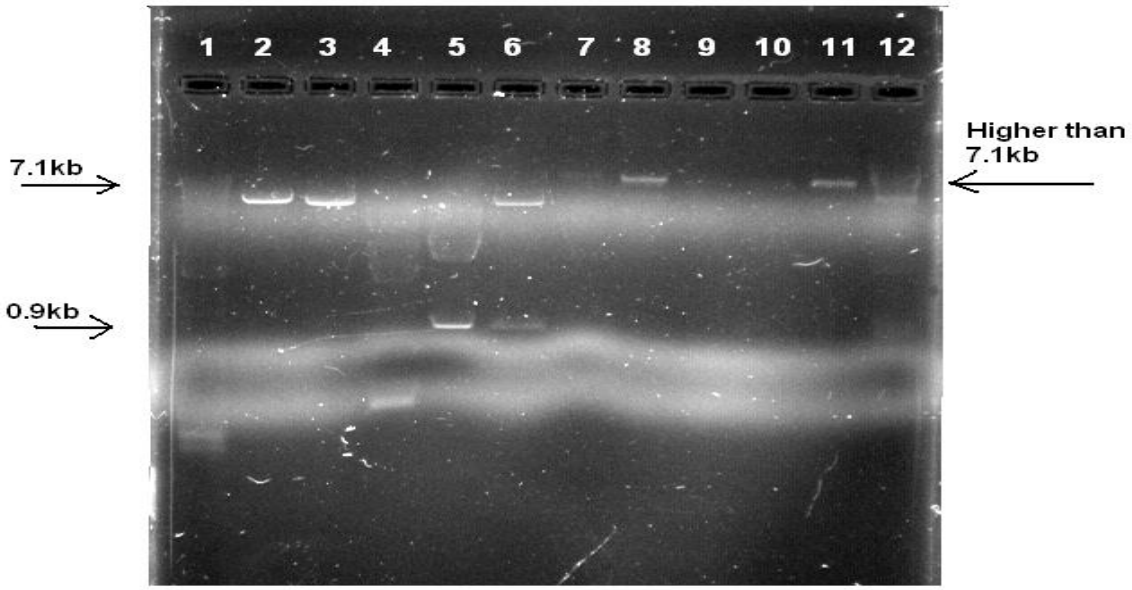

Figure 1. One percent agarose gel electrophoresis of pMOB3 restriction digestion and pLGL ligation. Lanes 1 and 12: 1 kb DNA ladder*; lane 2: gel extracted 7.1 kb pLGL in TE; lane 3: gel extracted 7.1 kb pLGL in distilled H_2O; lane 4 *Eco*R1 and *Bam*H1 digested pMOB3; lane 5: *Eco*R1 digested pMOB3; lane 6: *Bam*H1 digested pMOB3; lanes 7 to 11: products of ligation reaction with gel-extracted 7.1 kb pLGL, at different concentrations. *The ladder in lanes 1 and 12 did not show up during the gel UV exposure. Sizes were identified by looking at the sizes in the corresponding digests in Fig. 2.

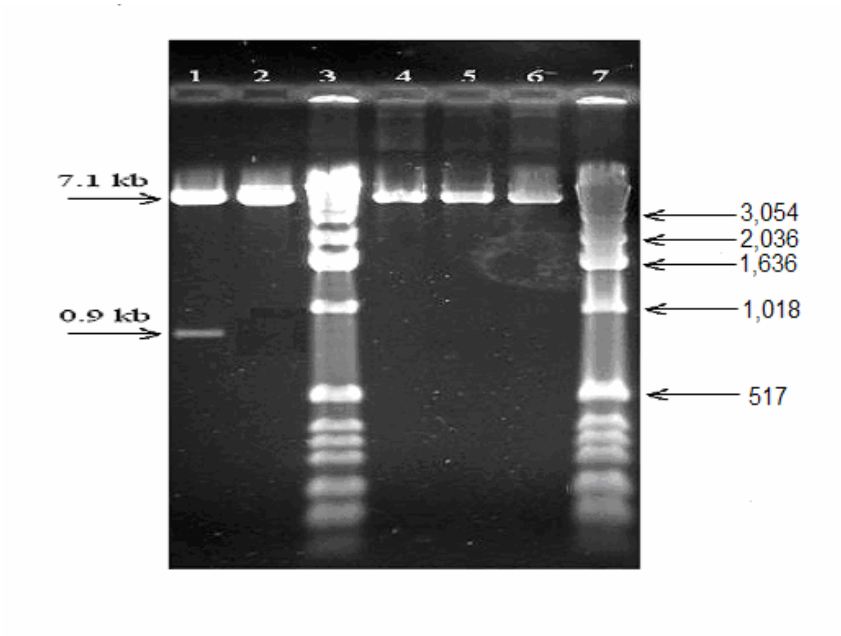

Figure 2. One percent agarose gel electrophoresis of pMOB3 digestion and pLGL digestion. Lanes 3 and 7: 1 kb DNA ladder; arrows indicate size of DNA standards in bp; Lane 1: pMOB3 digested with *Bam*H1; lane 2: gel extracted 7.1 kb of *Bam*H1 digested pMOB3; lane 4 to 6: *Bam*H1 digested plasmid of three transformant colonies.

Table 1. Relative growth properties of *E. coli* DH5α and C584 in LB media and M9 minimal media in the absence of antibiotics (A) or presence of Kanamycin (B), Chloramphenicol (C) or Kanamycin and Chloramphenicol (D)

A. No Antibiotics

Strain	Medium[a]	Growth[b]
DH5α	LB	++
C584	LB	++
DH5α + pMOB3	LB	++
DH5α + pLGL[c]	LB	++
DH5α	M9	+
C584	M9	–
DH5α + pMOB3	M9	–
DH5α + pLGL[c]	M9	++

B. Kanamycin

Strain	Medium[a]	Growth[b]
DH5α	LB	–
C584	LB	++
DH5α + pMOB3	LB	++
DH5α + pLGL[c]	LB	++
DH5α	M9	–
C584	M9	–
DH5α + pMOB3	M9	–
DH5α + pLGL[c]	M9	+

C. Chloramphenicol

Strain	Medium[a]	Growth[b]
DH5α	LB	–
C584	LB	++
DH5α + pMOB3	LB	++
DH5α + pLGL[c]	LB	–
DH5α	M9	–
C584	M9	–
DH5α + pMOB3	M9	–
DH5α + pLGL[c]	M9	–

D. Kanamycin and Chloramphenicol

Strain	Medium[a]	Growth[b]
DH5α	LB	–
C584	LB	++
DH5α + pMOB3	LB	++
DH5α + pLGL[c]	LB	–
DH5α	M9	–
C584	M9	–
DH5α + pMOB3	M9	–
DH5α + pLGL[c]	M9	–

[a] M9 minimal media was supplemented with 0.2% glycerol and 0.5 μg /ml of thiamine.
[b] Although there was contaminant growth on some plates, all growth indicated in the tables represents the growth of real constructs only.
[c] DH5α + pLGL represents all three transformant isolates, each which had given identical growth results.

These three colonies were also tested for growth on M9-glycerol plates with kanamycin and all three colonies grew, but at a rather slow rate. All three isolates gave identical growth results and are represented as DH5α + pLGL in Table 1; relative growth properties of different constructs grown in different conditions is also presented in Table 1.

When C584 and DH5α transformed with pMOB3 were plated on M9 minimal media with kanamycin and chloramphenicol, a few colonies were present after 72 h incubation, despite the fact that there should be no growth. The colonies were later confirmed to be contaminants by gram staining and microscopic observation. These contaminants were filamentous rather than rod shaped as *E. coli*. When DH5α transformed with pLGL were plated on M9 minimal media with kanamycin, a mixture of large and small colonies appeared after 72 h incubation. By gram staining and microscopic analysis, it was confirmed again, that the small colonies were filamentous contaminants, while the large colonies possessed the morphology of *E. coli*.

DISCUSSION

A lot of problems were encountered with the gel electrophoresis. One of the biggest problems was that the 1 kb ladder would not show up in the gel picture. This happened twice and might have been due to the degradation of the 1 kb ladder DNA. This problem was solved by using another, new tube of 1 kb ladder. Another setback was that during the exposure of the ethidium bromide pre-stained gel, only faint bands appeared. This might have been due to the fact that the pre-stained agarose gel was prepared a day before use and stored in a refrigerator, overnight in 0.5X TBE buffer. During this time, ethidium bromide might have washed out of the gel, resulting in poor exposure of the gel. This problem was overcome by re-staining the gel, after electrophoresis, in an ethidium bromide-buffer wash. Another problem that we encountered was the appearance of bright lines appearing horizontally across the

gel picture, which interfered with our analysis. We suspected that this was due to excess dye in the loaded samples; upon decreasing the volume of the sample loading buffer, the problem was resolved.

There seemed to be a problem when using the ligation reaction with gel extracted DNA eluted in TE buffer; use of this ligation mixture resulted in no transformants following electroporation. From our results there seemed to be no problem with the transformation when the DNA was eluted in distilled H_2O. Most transformation protocols suggest solubilzing DNA, for electroporation, in either distilled H_2O or TE buffer. TE buffer is a low ionic strength solution and should not cause arcing, which commonly occurs in electroporation if the salt concentration is too high (1). There were no problems with arcing in our sample, but the TE-solublized DNA ligation reaction, when electroporated with the competent cells, appeared to have a shorter pulse length (time constant). A longer pulse length increases the number of transformants (1) therefore, a short pulse length may indicate problems in the electroporation process.

In our experiment, the chloramphenicol resistance gene, CAT, was deleted from pMOB3. DH5α, transformed with pLGL, regained its ability to grow in M9 minimal media with glycerol, thiamine, and kanamycin, thus supporting our hypothesis. Past reports of growth inhibition due to plasmid-borne genes point largely to genes that encode antibiotic resistance (3, 10). For this reason, we believe that the presence of the CAT gene in pMOB3 may have contributed to the growth inhibiting effects on DH5α in M9 minimal media.

It may be possible that the expression of the CAT gene in *E. coli* places the cells in energetically stressful conditions, where the cells are unable to maintain normal reproductive fitness in deprived media. This change in cell physiology may be a result of the actions or energetic needs of the constitutively expressed CAT gene (7). It is known that CAT enzymatically transfers the acetyl moiety of acetyl coenzyme A (acetyl CoA) to chloramphenicol molecules, and thus prevents covalently modified chloramphenicol from binding and inhibiting protein synthesis at ribosomes (7). Therefore, the presence of chloramphenicol may decrease the intracellular concentration of acetyl CoA available for use in downstream biosynthesis. Acetyl CoA, made from pyruvate, normally donates its acetyl group to oxaloacetate to form citrate (5). This molecule in turn, reacts with other downstream citric acid cycle components leading to the formation of various biosynthetic precursors (5); in particular, acetyl CoA is essential for the production of fatty acids, sterols, and also certain amino acids (5). As such, when growing in deprived media, the cells may not be able to replenish the pools of acetyl CoA by using exogenous nutrients and thus growth is repressed. Therefore, the removal of the CAT gene from pMOB3 should relieve growth inhibition in minimal media.

Further support for this idea is seen in a study by Potrykus and Wegrzyn (7), who found that a CAT-expressing strain of *E. coli* (CM2555) was sensitive to chloramphenicol due to decreased intracellular levels of acetyl CoA (7). More specifically, they discovered that as the levels of acetyl CoA decreased, due to the efficient acetylation of chloramphenicol by CAT, the bacteriostatic effects of the unmodified chloramphenicol was able to hinder cell growth. A related experiment observed the growth of CAT-expressing *E. coli* mutants, that lacked genes required for the production of acetyl CoA from pyruvate, in media containing chloramphenicol and sodium acetate (7). They found that the cells were able to replenish their pools of acetyl CoA using sodium acetate, resulting in improved growth in chloramphenicol-containing media (7). A similar study also showed that the deprivation of acetyl CoA, as a result of the action of CAT, was a cause of strain CM2555 growth inhibition (6). In addition, the fact that the acetyl CoA levels in cells is directly correlated with growth rate (4), further shows support for the possible link between the action of CAT and the reproductive fitness of cells.

To confirm that the depletion of acetyl CoA is the main cause for growth inhibition of C584 in M9 minimal media with kanamycin and chloramphenicol, an exogenous source of acetyl CoA or its precursor, such as sodium acetate, should be added to the media and tested to see if this would resume growth. In addition, growth of C584 in M9 minimal media, with kanamycin only, can be examined. Without chloramphenicol, the intracellular pools of acetyl CoA should not be depleted and growth should occur normally in M9 minimal media.

In this experiment, we have observed that the removal of the CAT gene from pMOB3 reconstitutes growth of DH5α in M9 minimal media. This confirms that antibiotic resistance genes may play a role in inhibiting growth as observed by Lee *et al*. (3) and Valenzuela *et al*. (10). We speculate that the depletion of acetyl CoA is the main factor responsible for halting the growth of C584 in M9 minimal media, but further experiments must be performed to support this idea.

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