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Growth Inhibition of DH5α *Escherichia coli* on M9 Minimal Media is Linked to Specific Restriction Fragment(s) of pMOB3

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***Escherichia coli* strain DH5α is able to grow on M9 minimal media supplemented with 0.2% glycerol and 0.5 µg/ml thiamine. Following transformation with the plasmid pMOB3, DH5α loses the ability to grow on this media. Fragments of pMOB3 were ligated into the vector pBR322 and transformed into DH5α. The transformants were plated on LB-Amp to select for cells that had acquired a plasmid and screened for lack of growth on LB-Tet to confirm the presence of an insert. They were then evaluated for their ability to grow on supplemented M9 minimal media. Plasmids were isolated from colonies that did not exhibit growth on either LB-Tet or supplemented M9 minimal media, and inserted fragments were excised by digestion with restriction endonucleases. Our results demonstrate that a fragment of pMOB3 is responsible for the inhibition of growth of DH5α on supplemented M9 minimal media.**

Escherichia coli strain DH5α is able to grow on M9 minimal medium supplemented with 0.2% glycerol and thiamin (0.5 µg/ml). Kestell *et al.* (3) recently reported that following transformation with the plasmid pMOB3, this strain of *E. coli* can no longer proliferate on minimal media. We found this phenotype interesting and wanted to investigate it further.

While the inhibition of growth on minimal media has been shown to be caused by the presence of the plasmid pMOB3, the precise mechanism is unknown. Constructed by Schweizer *et al.* (7), pMOB3 is a 9.9 kb plasmid resistant to both kanamycin and chloramphenicol. Kestell *et al.* (3) suggested two possible reasons for the inhibition of growth on minimal media. First, it may be that M9 minimal medium simply does not provide adequate resources to allow the cell to maintain the plasmid while growing on such nutrient-limiting media. Second, pMOB3 may encode a gene product that inhibits a protein or metabolic pathway required for growth on M9 minimal medium.

In this study, we sought to identify the region of pMOB3 responsible for the observed inhibition of growth of *E. coli* on minimal media, and possibly narrow it down to a specific gene and/or gene product which would provide insight into the mechanism of growth inhibition. We demonstrated that a fragment of pMOB3 caused inhibition of growth on minimal media, as opposed to the entire plasmid, and we suggest a putative region of the plasmid responsible for this phenotype.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Three *E. coli* strains were used throughout this experiment: DH5α, strain DH5α/pBR322, and C584, all supplied by Dr. William Ramey (7). *E. coli* C584 is a DH5α-derived strain that was used in the isolation of the pMOB3 plasmid. pMOB3 confers resistance to both chloramphenicol (CAM) and kanamycin (Kan) which were used as selective markers in the isolation of the plasmids. *E. coli* DH5α/pBR322 refers to DH5α with the 4.4 kb plasmid pBR322; the tetracycline (Tet) and ampicillin (Amp) resistance genes on this plasmid allowed for its selection and use as the cloning vector in the subsequent protocols. *E. coli* DH5α was used in the preparation of competent cells for all transformations performed. All liquid cultures were incubated at 37°C with aeration for 24 hrs. Plated cultures were incubated aerobically in the dark at 37°C for 24 hrs, except for those on M9 minimal media supplemented with 0.2% glycerol and 0.5 µg/ml thiamine (M9) which were allowed 48 hrs for growth (5). All selective media contained 50 µg/ml of appropriate antibiotic.

Isolation of pBR322 and pMOB3 plasmids. Bacterial strains were grown in Luria Broth (LB) media supplemented with the appropriate selective antibiotic (Kan or Amp) to ensure retention of the plasmids. The cells were allowed to reach mid-exponential phase before being pelleted by centrifugation at 5000 rpm. The plasmid isolations were carried out using the methods described in the CONCERT High Purity Plasmid Maxiprep System (GibcoBRL). The pelleted DNA was dissolved in 500 µl of autoclaved distilled water and microcentrifuged at 12,000 x *g* at room temperature for 1 min to remove insoluble particles. The supernatant was then transferred to a new tube, diluted appropriately and the absorbance at 260nm and 280nm determined using an Ultrospec 3000 (Biochrom). Assuming 50 ng of DNA has an A_{260} of 1, the concentration of each plasmid suspension was determined. The values were 1.85 µg/µl for pBR322 and 4.21 µg/µl for pMOB3.

Restriction Enzyme (RE) Digests of pMOB3 and pBR322. *Complete Digests:* The restriction endonucleases used in this experiment included *BamH I*, *Eag I, EcoR V, Sal I,* and *Sph I* (New England Biolabs). All digests were performed using 5 µl of the appropriate plasmid (concentration above) unless otherwise stated. Ten units (U) of the desired enzyme was used along with 2 µl of 5X buffer, recommended by the manufacturer for optimal activity (New England Biolabs), in a 20 µl reaction. In addition, the following double digests were performed; *BamH I* and Sph I in React 6 buffer (Invitrogen), *EcoR V* and *Eag I* in *NE3* buffer 3 (New England Biolabs) and *EcoR V* and *Sph I* in React 6 buffer. All reactions were incubated at 37°C for 1 to 24 hrs prior to use.

Sau3AI *Partial Digests:* Partial digests were based on a protocol described by Sambrook *et al.* (1). Initially, 21.06 µg of pMOB3 was mixed with 10 µl of 10X NE buffer 4 (New England Biolabs) and 85 µl of autoclaved distilled water, mixed and 30 µl dispensed to a microfuge tube (tube 1). Twenty µl of the same mixture was added to tubes 2-4 and 10 µl in tube 5 and all tubes were put on ice. Twenty U of *Sau3A I* (New England Biolabs) was then added to the first tube mixed and 10 µl transferred to tube 2. Subsequent serial dilutions were performed by adding 10 µl of the reaction to tubes 3-5. All tubes were incubated at 37°C for 15 min before stopping the digest by incubation at 65°C for 20 min.

Restriction Map of pMOB3. Each of the complete restriction digests of pMOB3 previously described were run at 100 V for 1.5 hrs along with undigested pMOB3 and 10 µl of a 1 kb ladder (Invitrogen) on a 0.8% agarose gel containing ethidium bromide (1 mg/ml). The sizes of the fragments were determined based on distance migrated in the gel and comparison to the molecular weight standards of the ladder.

Construction of Recombinant Plasmids. The construction of recombinant plasmids required the ligation of both completely and partially digested pMOB3 fragments into a pBR322 vector. For each pMOB3 digest performed, pBR322 was digested with the same enzyme(s) to ensure compatible ligation sites. For all the *Sau3A I* fragments, pBR322 was digested with *BamH I* prior to ligation. Assuming a vector size of 4.4 kb and an average insert size of 3 kb (based on the restriction map of pMOB3), a 2:1 vector-to-insert ratio was determined for each ligation reaction. This corresponded to 2.2 µg of pBR322 and 0.76 µg of pMOB3 in a 20 µl reaction. One U of T4 DNA Ligase (Invitrogen) and 4 µl of 5X DNA Ligase Buffer (Invitrogen) were also added and the ligations were incubated at 24.5°C overnight.

Transformation of DH5a Competent Cells. *Preparation of Competent Cells:* In a 1 L Erlenmeyer Flask, DH5α *E. coli* was grown in 500 ml of LB to an OD_{600} of 0.602 and subsequently chilled on ice for 15 min. The culture was then transferred to two prechilled 250 ml centrifuge bottles and centrifuged at 5000 x *g*.. The supernatant was discarded and the pellet resuspended in 500 ml of ice-cold distilled water and centrifuged again for 20 min. After discarding the supernatant, the pellet was resuspended in the remaining liquid and transferred to two prechilled 50 ml centrifuge bottles and centrifuged at 5000 x *g*. The remaining pellets were each resuspended in 500 µl of cold distilled water and used immediately.

Electroporation: A Bio-Rad Micropulser electroporation apparatus was used according to the manufacturer's instructions to perform all transformations. One µl of each ligation reaction was added to 40 µl of freshly prepared electrocompetent cells and pulsed on the setting "Ec1" (1.8 kV) in a 0.1 cm MicroPulser cuvette (Bio-Rad). Cells were immediately allowed to recover in SOC media (1) at 37°C with aeration for 1 hr. The samples were diluted in LB media and plated on LB-Amp at dilutions ranging to 10^{-2} for selection of successful transformants. Competent cells were also electroporated with pBR322 in the same manner and plated on selective media as a control. Finally, competent cell samples were transformed with both undigested pMOB3 and no DNA and plated on LB-Kan and LB, respectively, in dilutions of 10^{-4} to 10^{-6}.

Screening for Potential M-9 Growth Deficient Clones. Ten randomly selected colonies from each successful transformation reaction were transferred to LB-Amp plates with a grid imprinted on the agar. After incubation, the colonies were replica plated onto two LB plates containing Tet and Amp, as well as a plate of M9-Amp. The plates were incubated and then analyzed for the ability of each colony to grow on the media. Colonies that showed growth on LB-Amp but not LB-Tet or M9-Amp were streaked for isolation on M9-Amp and LB-Amp plates to confirm their phenotype. Lastly, colonies from the samples electroporated with pMOB3 and no DNA were plated in parallel on both M9 and LB media and analyzed for growth.

Analysis of Clones. Isolated colonies from the samples showing the M9 growth deficient phenotype were used to inoculate 5 ml LB-Amp cultures. The recombinant plasmids containing fragments from the partial digests with *Sau3A I* were isolated from the appropriate cultures using the CONCERT High Purity Plasmid Miniprep System (GibcoBRL) following the manufacturer's instructions. The remaining plasmids were isolated using the CONCERT Rapid Plasmid Miniprep System (GibcoBRL) again according to the manufacturer's instructions. The plasmids were subsequently digested using the method previously described with enzymes corresponding to sites used for the ligation. These reactions were then run on a 0.8% agarose gel containing 1 mg/ml ethidium bromide and electrophoresed for 1.5 h at 100 V.

RESULTS

pMOB3 is an 8 kb plasmid created by Schweizer *et al.* (Figure 1; 3). To supplement their map, we digested pMOB3 with *EcoR V, Eag I, BamH I,* and *Sph I*. pMOB3 was digested with these enzymes in single and double digests (see Table 1). The choice of restriction enzymes was derived from restriction sites located in the tetracycline resistance gene of pBR322. It is also of note that although many of the digests went to completion, some were only partially digested (data not shown).

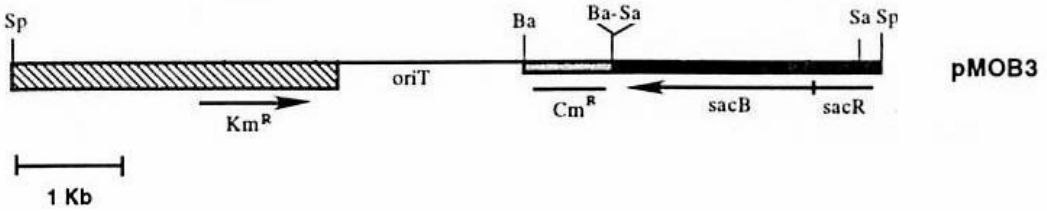


Figure 1. Physical and genetic map of the pMOB3 plasmid, modified from Schweizer *et al*. (4). The location and approximate extent of the functions on the plasmid are indicated by arrows (known orientation) or straight lines (orientation unknown). The origins of DNA are as follows: diagonal stripes, pHSS21 (6); solid line, pEMR2 (2); filled box, pMH1801 (4); shaded box, pBR322::Cm (4: M. Pato, University of Colorado). Restriction endonuclease sites are as follows: Ba, *BamH I*; Sa, *Sal I*; Sp, *Sph I*. Other abbreviations: Cm^R, chloramphenicol resistance; Km^R, kanamycin-resistance; *oriT*, origin of transfer from RP4; *sac*BR, *sac*BR loci from *Bacillus subtilis*.

Table 1. Single and double restriction enzyme digestions of pMOB3.

Restriction Enzyme(s)	Fragment Size (kb)				
	1	**2**	**3**	**4**	**Total**
EcoR V	7.7	-	-	-	7.7
BamH I	6.6	0.7	-	-	7.4
Eag I	5.7	1.5	0.9	-	8.2
Eag I + EcoR V	3.4	2.5	1.0	0.8	7.8
BamH I + Sph I	3.1	2.5	2.3	0.9	8.8

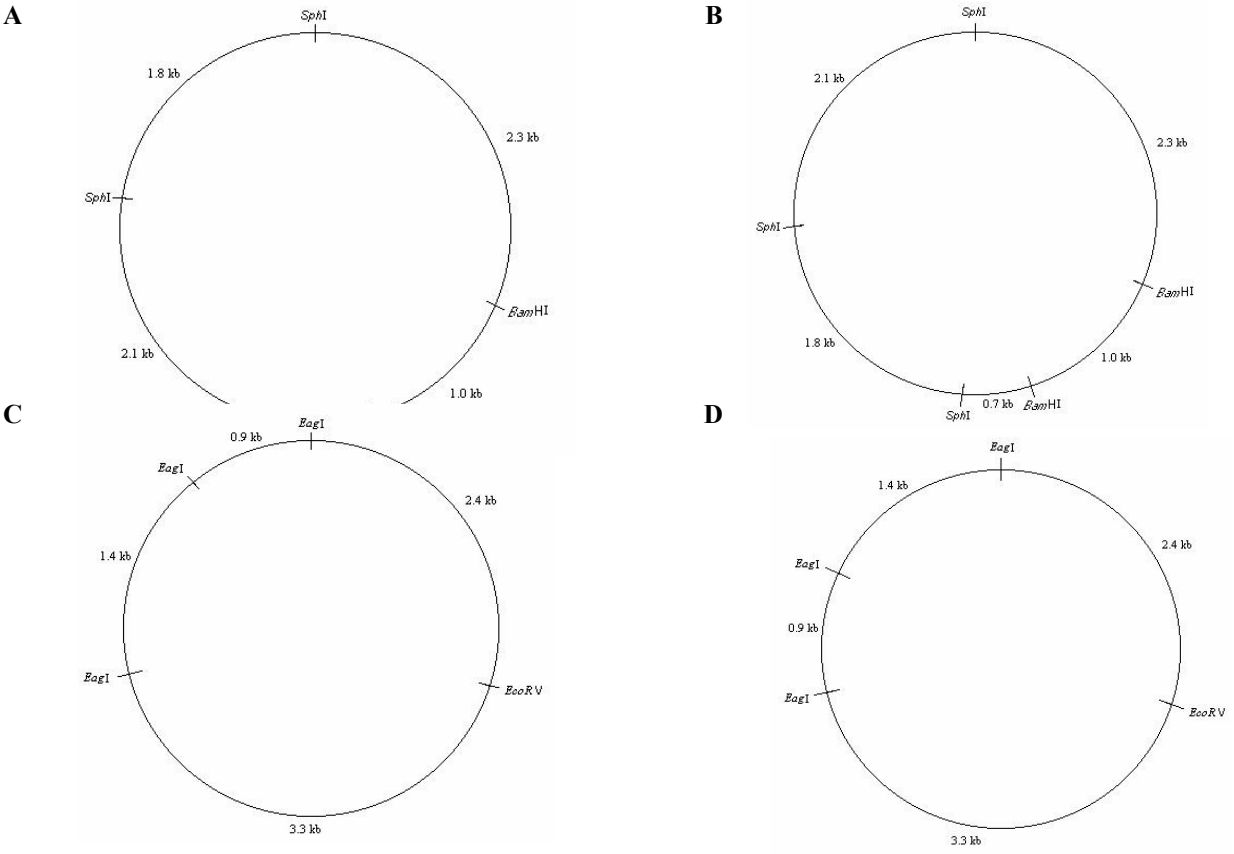


Figure 2. (A), (B): Putative restriction maps of pMOB3 indicating BamH I and Sph I cut sites and approximate fragment sizes. Two possibilities are shown. (C), (D): Putative restriction maps of pMOB3 indicating Eag I, and EcoR V cut sites and approximate fragment sizes. Again two possibilities are proposed.

These digestions indicate distances between the cut sites of the restriction enzymes. The data was not precise enough to construct an accurate map; however, it gave us a basis on which to compare the map provided by Schweizer *et al.* (7). Figures 2A and 2B illustrate the two possible alignments of the *BamH I* and *Sph I* recognition sites, and figures 2C and 2D illustrate the two possible combinations of *EcoR V* and *Eag I*.

Before doing any further research, we wanted to confirm the results reported by Kestell *et al*. (3). DH5α *E. coli* cells were transformed with pMOB3 via electroporation and plated on LB and M9 minimal media, and untransformed electroporated DH5α cells were plated on both media as a control. As expected, we saw that the untransformed cells grew on both LB and M9 minimal media. In agreement with the observation by Kestell *et al*. (5), DH5α cells transformed with pMOB3 formed colonies on LB, but did not appear to grow on minimal medium. The DH5α cells transformed with pMOB3 were also plated on LB-Kan to verify that they had acquired the plasmid.

After confirming that this phenotype does exist, we wanted to isolate the region of pMOB3 that was causing the cells to lose the ability to grow on M9 minimal medium supplemented with glycerol and thiamine. In order to do so, we ligated fragments of pMOB3 into a vector and transformed DH5α cells, then screened for colonies that were unable to grow on minimal medium. pMOB3 was digested into fragments using selected restriction enzymes, which were then ligated into the tetracycline resistance gene of pBR322, leaving the ampicillin resistance gene intact. In addition, pMOB3 was partially digested with different concentrations of the frequent-cutting restriction enzyme *Sau3A I*, yielding a number of random fragments of different sizes which were also ligated into pBR322. Competent DH5α cells were transformed with the ligation reaction products and plated on LB-Amp to select for cells that had acquired pBR322. Controls included DH5α cells transformed with pMOB3, pBR322, and untransformed (but had still undergone the electroporation procedure).

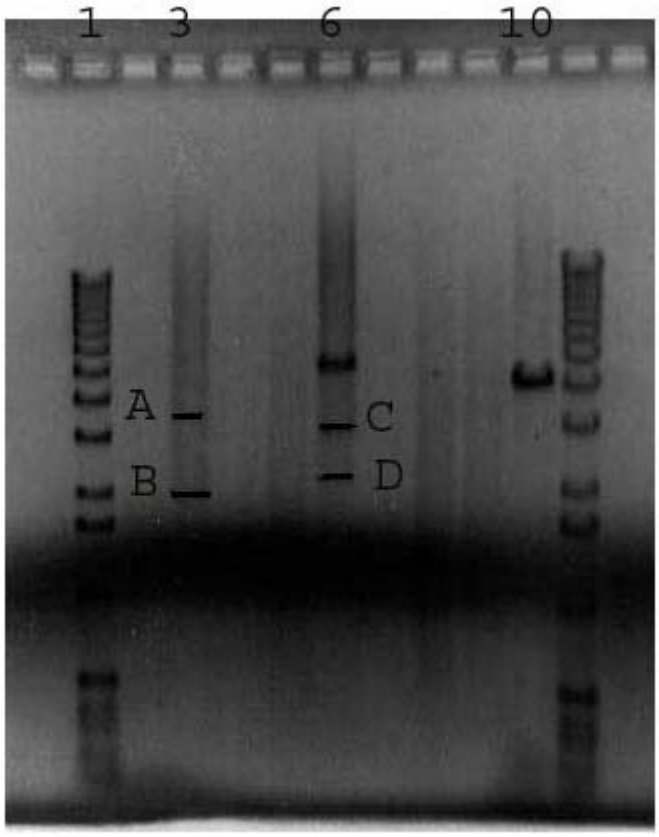
Table 2. Restriction endonucleases used to digest pMOB3 and the vector pBR322, which were used in the transformation reactions with the colonies that subsequently lost the ability to grow on M9 minimal medium.

Colony	Restriction Endonuclease(s) used to digest pMOB3	Restriction Endonuclease(s) used to digest pBR322
I – 1	*Sau3A I*	*BamH I*
I – 7	*Sau3A I*	*BamH I*
I – 14	*Sau3A I*	*BamH I*
I – 30	*Sau3A I*	*BamH I*
I – 33	*Sau3A I*	*BamH I*
II – 12	*Sau3A I*	*BamH I*
II – 31	*Sal I*	*Sal I*
III – 30	*BamH I + Sph I*	*BamH I + Sph I*
IV – 30	*Eag I + EcoR V*	*Eag I*
IV – 33	*Eag I + EcoR V*	*Eag I*
V – 3	*Eag I + EcoR V*	*Eag I + EcoR V*
V – 10	*EcoR V + Sph I*	*Sph I*
V – 14	*EcoR V + Sph I*	*EcoR V + Sph I*
V – 15	*EcoR V + Sph I*	*EcoR V + Sph I*

Of the 18 total transformations with the ligation reactions, all but one produced transformants that were able to grow on media containing ampicillin. Yields of approximately 50-200 colonies per plate were obtained, indicating the transformation reactions were efficient. 10 colonies from each plate were randomly selected and transferred to new LB-Amp plates. These colonies were subsequently replica plated onto LB-Tet and M9 minimal media to screen for transformants that contained inserts and had the desired phenotype, respectively. Fifty-two percent were unable to grow on LB-Tet, which demonstrated that most ligation reactions had been successful. 14 colonies did not exhibit any detectable growth on M9 minimal medium, and all of these colonies were resistant to ampicillin, yet susceptible to tetracycline, indicating the cells contained the vector with an inserted fragment. The colonies with the desired phenotype, and the corresponding enzymes with which pMOB3 and pBR322 were digested prior to

transformation, are listed in Table 2. Both fragments of known sizes digested with specific restriction endonucleases, as well as the random fragments produced by the partial digests, produced colonies with the desired phenotype.

Plasmids were isolated from eight of the colonies with the desired phenotype, and the insert was removed by digestion of the vector with the appropriate restriction enzymes. The inserts were then visualized by agarose gel electrophoresis, as seen in figure 3.



Lane	Contents*
1	1 kb ladder
2	II – 31
3	III – 30
4	IV – 30
5	IV – 33
6	V – 3
7	V – 10
8	V – 14
9	V – 15
10	Linearized pBR322
11	1 kb ladder

*Contents refer to the colony from which the plasmid was isolated. See Table 2 for corresponding restriction endonucleases.

Figure 4. Agarose gel electrophoresis of inserts of pMOB3 from colonies that were unable to grow on M9 minimal media. Fragments were excised from the vector pBR322 with specific restriction endonucleases listed in Table 2. Note: bands A-D are drawn in to overlay bands that appeared clearly in the photograph, but appear very faint in the print.

Of the plasmids producing the desired phenotype in DH5α cells, promising results were obtained from an insert isolated from a double digest of pMOB3. The *BamH I/Sph I* double digest of pBR322 containing pMOB3 fragment insert isolated from colony III-30 showed two very faint bands, seen in lane 3 of figure 3. This digest proved to be complete, as there were only two bands visible. The inserted pMOB3 fragment is represented by the lower molecular weight band that was found to be approximately 1.9 kb, and corresponds to the 1.8 kb fragment of figures 2A and 2B. The larger band of 4.1 kb represents pBR322 that has lost its 0.2 kb fragment between the *BamH I and Sph I* cut sites within the tetracycline resistance gene. Both of the bands are very faint indicating that there was very little plasmid used in the restriction digests.

Another clone with the desired phenotype, colony V-3, contained a reconstructed pBR322 with a inserted fragment of pMOB3 cut with *EcoR V* and *Eag I*. Lane 6 of figure 3 shows these results, with three visible bands. One band had an approximate molecular weight of 3.5 kb which would be consistent with reconstructed pBR322 (~4.4 kb) that has lost a 0.75 kb portion between *EcoR V* and *Eag I* cut site. The second band, with a molecular weight of approximately 2.4 kb, corresponds to the 2.5 kb region of the putative pMOB3 restriction map (Fig. 2c, d). The third and brightest band had a weight of 5.1 kb. The inefficiency of the restriction digest is demonstrated by the

discrepancy in the intensity of the lower weight bands, which are very faint, compared to the much brighter band at a larger molecular weight.

Several other colonies were isolated that gave the desired phenotype and therefore must have contained reconstructed pBR322 with pMOB3 insert (Fig. 3 and Table 2). However, when these colonies were grown overnight and plasmid isolation was carried out, very little if any plasmid was obtained as can be seen in lanes 2, 4, 7, 8, and 9 where no DNA is visible at all. Lane 10 of Fig. 3 shows the relative location of digested with *Sal I* (single cut) pBR322 at a size of 4.3 kb.

DISCUSSION

We confirmed that transformation of *E. coli* strain DH5α with the plasmid pMOB3 causes inhibition of growth on M9 minimal media supplemented with glycerol and thiamin. Following this observation, pMOB3 was digested with various combinations of restriction enzymes, producing fragments that were subsequently ligated into the tetracycline resistance gene of the vector pBR322. Of these, both the *BamH I/Sph I* and *EcoR V/Eag I* double digests of pMOB3 produced fragments, 2.1 kb and 2.4 kb respectively, that were successfully ligated into pBR322 and isolated from the transformed DH5α colonies III-30 and V-3, which were unable to grow on M9 minimal media. To better understand the location of these fragments within pMOB3, we ran digests in combinations to orient the enzyme cut sites. The results we obtained were not sufficient to allow us to create a complete restriction map. Nevertheless, we did determine that there is an additional *Sph I* recognition site in pMOB3 that is not shown on the map created by Schweizer *et al*. (7).

Further analysis of the possible locations of this putative *Sph I* cut site indicated that it may fall either in the kanamycin resistance gene, or closer to the *oriT*. However, given the data, we were able to eliminate the possibility of it being within the *sacB* portion of the plasmid. To support this conclusion, it has been shown that the *sacB* gene of the *Bacillis subtilis* from which portion pMOB3 was created does not contain a *Sph I* cut site (*sacB* gene accession #AY281049 from EMBL-EBI). Thus, we suggest that the 2.1 kb portion of pMOB3 corresponds to the 1.9 kb fragment is responsible for limiting the growth of DH5α on M9 minimal media (with thiamin and glycerol) (Fig. 4) may correspond to the *sacB* region of pMOB3.

The *sacB* gene has been included in plasmid constructs, such as pNPTS138, as a marker used for selection purposes. The gene encodes levansucrase of *Bacillus subtilis*, and when cloned into *E. coli, sacB* renders the strain sensitive to sucrose in media (3). Although sucrose was not included in the M9 minimal media, it is possible that components in the media caused similar catabolic intermediates in DH5α when transformed with *sacB* and grown on M9 minimal media supplemented with thiamin and glucose. This possibility opens another avenue of research in understanding the inhibitory effects of pMOB3 in DH5α when grown on M9 minimal media.

Analysis of the colony V-3 revealed a plasmid with 2.4 kb fragment derived from digestion of pMOB3 with *Eag I* and *EcoR V*. Because the Schweizer *et al*. restriction map did not contain cut sites for either *EcoR V* or *Eag I* enzymes, we could not draw conclusions based on their findings. Although we created a putative *EcoR V/Eag I* map that has the 2.3 kb and 3.3 kb sections adjacent to each other, we were unable to determine whether the 1.4 or the 0.9 kb fragments flanked the 2.4 kb fragment. We conducted several additional restriction digests in an attempt to further specify the relationship between the 2.1 kb *BamH I/Sph I* fragment and the 2.4 kb *EcoR V/Eag I* fragment. The data we obtained was ambiguous; however, some combinations we saw suggested that the 2.1 kb *BamH I/Sph* I and 2.4 kb *EcoR V/Eag I* fragments may overlap (data not shown).

Further restriction mapping of pMOB3 may elucidate the relationship between the inserts isolated from colonies III-30 and V-3. The most likely reason that we were not able to determine this relationship from our experiments was due to the combination of restriction enzymes used and the use of their respective buffers. Buffers were used that appeared close to the recommended salt concentrations for the digests, but they were not exact. Non-optimal conditions can induce star activity of restriction endonucleases, affecting their specificity and activity. We suspect that star activity was a contributing factor that impaired our ability to interpret the map data. The use of specified buffer concentrations during restriction digests may cut down on the undecipherable number of bands we observed in the gel (data not shown).

Figure 3 represents plasmid analysis of all of the isolated colonies that demonstrated the desired phenotypes. Surprisingly, of these eight isolates, we were only able to isolate plasmids from two of them, derived from the double digests of *EcoR V/Eag I* and *BamH I/Sph I*. The other colonies did not contain detectable plasmids. It is possible that this poor plasmid yield was due to shortcomings in the plasmid isolation process, but due to number of times this procedure was repeated and the use of two different kits, it seems unlikely that this could entirely account for this unexpected observation. It is possible that the pMOB3 fragment that prevents the cell from growing on M9 minimal media encodes a protein product that provides no selective advantage to the cell. If this was in fact the

case, *E. coli* DH5α would experience selective pressure to lose the plasmid, since the only selective pressure included in the media was the presence of ampicillin, which ensures that the strain retains the vector pBR322 (with or without an insert). Thus, the cell may only replicate the plasmid enough to ensure that it can express sufficient resistance to ampicillin to survive and not any more. Effectively, this could mean that pBR322 goes from being a high copy number plasmid to a relatively low copy number plasmid when it is reconstructed with the fragment within pMOB3 that yields the inability to grow on M9 minimal media phenotype. Finally, the last possibility is that transformed strains are simply losing their plasmid for an undetermined reason.

In addition to the colonies III-30 and V-3, from which plasmids containing inserts were isolated, there were six colonies with inserted fragments from the partial digests with *Sau3A I* that exhibited the desired phenotype. While these fragments could not be localized to a particular region of the plasmid, this is supporting evidence that a specific region of pMOB3 is responsible for the inhibition of growth on M9 minimal media. When transformed with pBR322 alone, DH5α cells retained the ability to grow on minimal media, but this ability was lost when transformed with pMOB3 in its entirety. However, we have shown that the presence of full-length pMOB3 is not essential to inhibit the growth of these cells on minimal media. Consequently, we can reject one of the hypotheses put forward by Kestell *et al*. (5), which suggested that either the presence or maintenance of pMOB3 required too many resources to allow for the healthy growth of DH5α on M9 minimal medium. We have demonstrated that a fragment as small as 2 kb may be responsible for the observed phenotype, and therefore propose that a gene product encoded by pMOB3 inhibits a protein or metabolic pathway required for growth of DH5α cells on minimal media.

FUTURE EXPERIMENT

Although we have been able to confirm that pMOB3 is not necessary in its entirety to inhibit the growth of DH5α on M9 minimal media, we have not been able to precisely define the portion of the plasmid responsible. Attempts should be made to create a detailed restriction map of the plasmid, incorporating all of the cut sites which we have shown to be present in pMOB3 and focusing on the locations of the *Eag I, EcoR V, BamH I,* and *Sph I* sites which we had difficulty defining due to inadequate digest conditions. This detailed map could then indicate whether or not the two fragments we isolated overlap each other. In addition, attempts should be made to isolate fragments of pMOB3 from colonies for which we observed inhibition of growth on M9, but could not isolate a plasmid.

We have also shown the *Sau3A I* partial digests can be used to obtain plasmid fragments that confer the desired phenotype. Therefore, future experiments should employ this type of digest to obtain inserts of the smallest possible length, which could subsequently be sequenced. This could be used to further verify the location of the insert by comparing cut sites within the sequence. The sequence could also provide insight into the mechanism of growth inhibition by finding sequence homology to a protein of known function. Based on the potential function of the gene product, appropriate components could be added to the minimal media until the ability to grow is restored. This would allow for further characterization of the cellular pathways involved.

A third approach would be to transform DH5α with the plasmids from which pMOB3 was originally made: pHSS21, pEMR2, and pMH1801. pBR322 was also used to construct pMOB3, however, we have shown that it does not inhibit growth of DH5α on minimal media. Although it has not been previously reported, it is likely that whichever fragment of pMOB3 confers this phenotype exists in its entirety on its parental plasmid. The parental plasmid itself may also then confer the same. A parental plasmid that also renders DH5α unable to grow on M9 media may lend support to the location of the fragment on pMOB3, and further investigation of that region could be done.

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