Attempted construction of an arabinose-inducible pBAD28 recombinant expression vector containing acrD, an Escherichia coli aminoglycoside efflux porin

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The construction of pBAD28-acrD, an arabinose-inducible expression vector for AcrD, an aminoglycoside efflux pump, represents a considerable opportunity for investigation of the function of this membrane protein in an experimental setting. In this study, an attempt was made at the construction of this vector by PCR amplification of acrD from the genome of E. coli MG1655, isolation of the expression vector pBAD28 from E. coli DH5α, followed by restriction digestion using HindIII and XbaI, then T4 ligation of the digestion products. Following transformation of the ligation reactions into chemically competent E. coli® 5-alpha strain cells and characterization of putative transformants, it was determined that a successful pBAD28-acrD expression vector failed to be constructed, and the transforms were primarily empty pBAD28 vectors.

The efflux pump AcrD has been shown to confer resistance to various aminoglycosides (1). AcrD forms a tripartite complex with AcrA and ToLC (2), two other Escherichia coli membrane proteins. Mutation of the acrD gene has been shown to induce hypersusceptibility to many different types of aminoglycosides, including kanamycin, neomycin, and erythromycin (1). Previous research has also shown that loss-of-function acrD mutants are able to replicate and divide, but do not do so when the environmental temperature is increased (3). It has also been demonstrated that acrD has an effect on DNA synthesis and cell replication, as the mutants do not elongate after temperature increase (3). There has been a lack of research on the physiological effects of acrD manipulation alone on bacteria in terms of viability, population growth and antibiotic susceptibility.

Prior papers have detected acrD transcripts in putative ΔacrD strains (4). As AcrD is a transmembrane protein pump located on the outer membrane of a cell, the complete absence of this protein could disrupt the proper structural integrity of the cell membrane, which could be lethal to the cell. To prevent immediate cell death, the ΔacrD mutant would have to be complemented with a pBAD28-acrD expression vector.

The goal of this study was to construct a stable, transformable pBAD28-acrD plasmid in order to control expression of acrD and assess the effect of variable expression on bacterial cell physiology. Furthermore, we planned to use the pBAD28-acrD expression vector, part of a Lambda Red mediated recombination system, to produce AcrD in E. coli MG1655 cells with a complete knockout of the genomic acrD gene.

We planned to create the plasmid by ligating PCR-amplified acrD into pBAD28. The PCR amplification of acrD employed newly-designed primers, containing HindIII and XbaI restriction digestion sites, to allow the amplification of acrD with the appropriate restriction sites. The pBAD28 vector contains HindIII and XbaI restriction sites in a multiple cloning site downstream of its arabinose-inducible promoter, as well as ampicillin and chloramphenicol resistance selection markers (5, 6). The putative recombinant plasmid was transformed into E. coli DH5α, a high-efficiency transformation cloning strain (7). However, the transformation into DH5α strain was unsuccessful as we failed to identify any pBAD28-acrD positive colonies through plasmid isolation, restriction digestion, and gel electrophoresis analysis.

MATERIALS AND METHODS

Isolation and amplification of acrD. A 3171 bp fragment containing acrD was amplified from the E. coli K12 MG1655 genome using PCR. The primer RLSlacD-F that is complementary to the region upstream of acrD contains an XbaI restriction site. The primer RLSlacD-R that is complementary to the region downstream of acrD contains a HindIII restriction site. The use of these restriction sites allows the ligation of digested acrD into the digested and linearized arabinose-inducible expression vector pBAD28. PCR was carried out in a final reaction volume of 50 μl, and each reaction mix consisted of 1.25 U Taq polymerase (Invitrogen, 10342-020), 1X PCR Buffer (Invitrogen, 10342-020), 0.4 mM dNTP mixture, 1.5 mM MgCl2, 1 μM Primer RLSlacD-F, 1 μM Primer RLSlacD-R, and 2 μl washed MG1655 cells as described by Zhu (8). A Biometra TGradient 96 thermocycler was employed, and the protocol consisted of 15 minutes at 95°C, then 35 cycles of 1 minute at 95°C, 1 minute at 52°C and 3.5 minutes at 72°C, followed by 72°C for 10 minutes; reactions were kept at 4°C until further processing. The PCR products were cleaned up using a Fermentas GeneJET PCR Purification Kit (Thermo Scientific, K0701) and stored at -20°C.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’ – 3’)</th>
<th>Tm (°C)</th>
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<tbody>
<tr>
<td>RLSlacD-F</td>
<td>GGGCGCAAGCTTCATGTCGC</td>
<td>63.9 °C</td>
</tr>
<tr>
<td></td>
<td>CTTTTATTTCG</td>
<td></td>
</tr>
<tr>
<td>RLSlacD-R</td>
<td>TCTAGAAGAACACGAGGTC</td>
<td>55.4 °C</td>
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<td>CTCTTTTA</td>
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Isolation and preparation of pBAD28. Plasmid DNA was isolated from an overnight culture of E. coli K12 MG1655, grown
in 100 ml Luria Bertani broth (LB) containing 50 μg/ml ampicillin sodium salt (Sigma-Aldrich, 69-52-3) at 37°C in an incubator shaking at 200 rpm in a 500 ml Erlenmeyer flask. The plasmid was purified using alkaline lysis as described by Birnboim and Doly (9) with a 1:1 phenol chloroform extraction. The plasmid concentration was analyzed using a Thermo Scientific Nanodrop 2000 UV-Vis Spectrophotometer loaded with 1 μl of sample, set to read dsDNA, and used according to the manufacturer’s protocol.

Construction of putative pBAD28-acrD. The prepared pBAD28 and acrD samples were digested overnight at 37°C, in a final reaction volume of 10 μl for pBAD28, and 45 μl for acrD. The pBAD28 digestion contained 30 μg pBAD28 sample, 1 μl NEB Buffer 2 (New England Biolabs), 1 μl 20,000 U/ml XbaI restriction enzyme (New England Biolabs), 1 μl 20,000 U/ml HindIII restriction enzyme (New England Biolabs) and 5 μl of sterile distilled water to a final volume of 10 μl. The acrD digestion contained 300 ng of acrD sample, 5 μl 20,000 U/ml HindIII restriction enzyme (New England Biolabs), 5 μl 20,000 U/ml XbaI restriction enzyme (New England Biolabs), 4.5 μl NEB Buffer 2 (New England Biolabs), and 0.5 μl sterile distilled water to a final volume of 45 μl. The digestion products were cleaned up using a GeneJET PCR Purification Kit (Thermo Scientific, K0701). 5 μl of the purified digestion reactions were run on a 1% agarose gel for 45 minutes at 120 volts, then incubated for 30 minutes in 0.5 μg/ml ethidium bromide and imaged with an Alpha Imager. Ligation of the digested pBAD28 and acrD was carried out in a final reaction volume of 20 μl consisting of approximately 18 ng acrD, approximately 1 ng pBAD28, 1X Ligase Reaction Buffer (Invitrogen, 15224-017), and 0.1 U T4 Ligase (Invitrogen, 15224-017). This reaction was incubated at room temperature for 1 hour, and then stored on ice until transformation.

Transformation protocols. Chemically competent E. coli®α strain was thawed on ice, then allowed to incubate with 5 μl ligation reaction for 5 minutes. The cells were heated to 42°C for 45 seconds, then recovered in 250 μl LB for 1 hour, shaking at 200 rpm at 37°C. Following the incubation, 100 μl of the recovered culture was plated onto non-selective LB agar and selective LB agar with 50 μg/ml ampicillin, and then incubated at 37°C for 18 hours.

Transformant screening protocols. 14 colonies were isolated from the selective LB-ampicillin (LB- Amp) plate that had been incubated overnight. These colonies were used to inoculate 14 5 ml tubes of LB broth containing 50 μg/ml ampicillin. The cultures were grown overnight at 37°C, shaking at 200 rpm. The following day, 50 μl of each of the cultures was examined visually on a slide using a light microscope, and observations were recorded, as there were concerns that some of the colonies might be contaminants. Plasmids were isolated from 1.5 ml of each culture using the RapidPURE Plasmid Mini Kit (MP Biomedicals, 112066200), and concentrations were measured using a Thermo Scientific Nanodrop 2000 UV-Vis Spectrophotometer. Each of the plasmid products was digested in a final volume of 50 μl, containing 395 ng (on average) of the plasmid mixture, 30 U HindIII restriction enzyme (New England Biolabs), 20 U XbaI restriction enzyme (New England Biolabs), and 1X NEB Buffer 2 (New England Biolabs). The digestions were incubated for 2 days at 37°C in 1.5 ml Eppendorf tubes, shaking at 200 rpm. 8 μl of these digestions were then run on a 1% agarose gel with 2 μl High DNA Mass Ladder (Invitrogen, 10496-016) for 45 minutes at 120 volts. The gel was then incubated in 0.5 μg/ml ethidium bromide for 30 minutes and imaged using an Alpha Imager.

Media and reagent information. LB was prepared by dissolving 10 g tryptone (Bacto), 5 g yeast extract and 10 g NaCl in 1 L distilled water, then autoclaving the mixture on liquid cycle. LB plates were prepared using the same recipe as above, but with 1.5% w/v agar. Antibiotic stock solutions were prepared at 2.5 mg/ml in 100% ethanol for chloramphenicol and 5 mg/ml in sterile dH2O for ampicillin. Working concentrations were 25 μg/ml and 50 μg/ml for chloramphenicol and ampicillin, respectively.

RESULTS

acrD was amplified from the genome of E. coli K12 MG1655. Using the information obtained by Zhu, a gradient PCR with annealing temperatures from 48°C to 56°C was tested with a template of washed cells (8). The product was visualized by agarose gel electrophoresis (Fig. 1). Bands were located between the 3kb and 3.5kb markers, falling in the range of the expected 3171 bp acrD fragment. All tested annealing temperatures gave a PCR product, with a 50°C annealing temperature giving a slightly more intense band on the gel (Figure 1). The acrD gene was successfully amplified with HindIII and XbaI restriction sites at the 5’ and 3’ ends respectively.

![FIG. 1. Colony gradient PCR amplification product, acrD](image)

Lane | Identity
--- | ---
1 | GeneRuler 1kb DNA Ladder (Thermo Scientific)
2 | acrD amplicon, 48°C annealing temperature
3 | acrD amplicon, 50°C annealing temperature
4 | acrD amplicon, 52°C annealing temperature
5 | acrD amplicon, 54°C annealing temperature
6 | acrD amplicon, 56°C annealing temperature
7 | acrD amplicon, 58°C annealing temperature

pBAD28 was isolated from the host strain E. coli DH5α. pBAD28 was isolated using alkaline lysis. The gel image containing undigested pBAD28 (Figure 2) shows several bands in Lane 2 at approximately 11000, 8000, 5700, 4500 bp when compared to the linear DNA mass ruler. Each of the pBAD28 bands visualized in Figure 2, Lane 2 potentially corresponds to various conformations of the plasmid: nicked, linear, supercoiled and single stranded circular (least migration to greatest, respectively). As each of these conformations move at different speeds during electrophoresis, only the linearized band (~5700 bp) corresponds to the size of pBAD28 (5777 bp). This is
confirmed by the linearized pBAD28 digest migrating the same amount (Fig. 2, Lane 3). The large diffuse band migrating below the smallest fragment from the mass ruler (250 bp) corresponds to where RNA contamination migrates.

**pBAD28 and acrD were restriction digested and subsequently ligated.** The isolated acrD and pBAD28 were double digested overnight with HindIII and XbaI restriction enzymes, and the digestion products were run on an agarose gel (Figures 2, 3). The presence of a band (~5700 bp) corresponds to the 5777 bp digested linear pBAD28 in Figure 2, Lane 3 and Figure 3, Lane 5. The presence of a band (~3200 bp) corresponds to the 3171 bp digested linear PCR-amplified acrD gene (Figure 2, Lane 4). The bands in lanes 3 and 4 (Figure 3) were individual digests that validate the functionality of restriction enzymes XbaI and HindIII, respectively.

**E. coli DH5α cells were successfully transformed with the ligation reaction.** Chemically competent E. coli DH5α cells were transformed with the ligation reaction, and recovered in rich media. The recovered cells were spread plated on selective and nonselective media. Colonies were isolated from both the selective and nonselective plates.

**Putative pBAD28-acrD transformants were determined to be empty pBAD28 transformants.** Following the overnight growth of the putative transformant E. coli DH5α cells, 14 colonies were isolated. Each of the 14 colonies was a potentially successful transformant, and as such, they were picked and inoculated into separate LB-Amp tubes and grown overnight. We were concerned, due to the low number of isolated colonies, that some or all of them may have been contaminants, so following their overnight growth, 50 μl of each of the cultures was examined on a slide under a light microscope, and the observations were recorded (Table 5). All of the samples were dense cultures of bacilli of a similar size. Plasmids were then isolated from these overnight cultures, and the concentrations were determined (Table 6). The purified plasmids were then digested using XbaI and HindIII. These digests were then run on a gel along with their undigested counterparts (Figure 4 A, B).

**DISCUSSION** The eventual failure to isolate successful pBAD28-acrD transformant cells was potentially due to several issues involved in the digestion and subsequent ligation of the acrD PCR product and in the isolation of pBAD28. The concentration of vector and insert used in the ligation was significantly less than what was indicated to be optimal by the manufacturer, and it is possible that because of this, an inadequate amount of successfully ligated pBAD28 was produced. The double-digested linear pBAD28 was designed to have incompatible sticky ends, preventing self-ligation, however, many of the final putative transformants were determined most likely to be pBAD28 according to their size (Figure...
This occurrence could be attributed to incomplete digestion of the pBAD28 fragment, leading to a cut site, which could easily reattach during ligation. It is much more likely, however, due to a complete lack of digestion, leading to intact plasmids going into the ligation step. Lastly, there is a small chance that it could be due to unsuccessful or inadequate cleanup of the linear digestion product, leaving the multiple cloning site fragment within the mixture free to ligate with successfully digested linear pBAD28 or the acrD amplicon. Issues are unlikely to stem from the T4 ligase or buffer employed, as they were new, and the manufacturer’s protocol was followed.

For the characterization of transformant colonies, it is possible that the 14 colonies were not enough to successfully screen for pBAD28-acrD ligations, as the ratio of successful ligations to self-ligated plasmids could be lower than 1:14. Only one LB-Amp (50 μg/ml) Petri plate was used to grow transformants and the transformation efficiency was not measured. This made it impossible to quantify the efficiency of the E. coli® strain transformation with pBAD28. When compared to manufacturer transformation efficiency, the measured efficiency could be an indicator of the quality of the input factors; poor measured efficiency indicating lack of quality in one or more of the following: divalent cations, conformations of DNA, or recovery media (10). Using more than one Petri dish would lead to a greater number of transformants to screen.

If the acrD insert had been present in one of the purified plasmids following the double digest, DNA fragments would be observed as bands corresponding to 5777 bp and 3171 bp (pBAD28 linearized vector and acrD insert, respectively) during gel electrophoresis. None of the digested samples shown in Figures 4A and 4B have more than one band, indicating a lack of insert within the plasmid. This was observed in transformants 1, 2, 4, 5, 6, 7, 8, 10, 11, 12 and 14, implying they were self-ligated pBAD28 or undigested pBAD28. An incomplete digest of pBAD28 would not be distinguishable from self-ligated pBAD28 on an agarose gel; hence the root of the empty pBAD28 vector cannot be identified. Undigested pBAD28 carried through to the ligation, and subsequently transformed into cells, would give rise to colonies, which have non-recombinant pBAD28. Double digests of the plasmids purified from transformations 7 and 13 did not result in any bands on the agarose gel (Figure 4B). The lack of these bands can be attributed to either spontaneous mutations or to unsuccessful plasmid preparations, yielding little to no successfully ligated pBAD28-acrD plasmid (7). The number of generations required to induce spontaneous ampicillin resistance in prior experiments is orders of magnitude greater than occurred in this study (11), and hence the spontaneous mutation hypothesis may not be feasible. Plasmid preparation issues where certain plasmid extractions from cultures were unsuccessful had been encountered in the past when samples of comparable cell densities were treated with identical protocols, and this may be the reason for the lack of bands. In conclusion, a successful pBAD28-acrD expression plasmid failed to be detected.

### FIG. 4. Analysis of digested plasmids from the putative pBAD28-acrD transformants. (A) Lane 1 was the high mass ladder. Lanes 2 - 14 contain double digested plasmids isolated from transformed E. cloni® strain colonies. (B) Lane 1 was the high mass ladder. Lanes 2 - 7 contain double digested plasmids isolated from transformed E. cloni® strain colonies. Lanes 8 - 14 contain undigested plasmids isolated from transformed E. cloni® strain colonies. The gradual change in distance migrated for bands of similar weight can be attributed to uneven gel thickness (right side of the gel was thicker for A while the left side was thicker for B).
FUTURE DIRECTIONS

In this study, a successful pBAD28-acrD expression vector failed to be constructed, and as such, future extensions of this project include the successful ligation of the acrD PCR product into a pBAD28 expression vector, and the use of this expression vector permit conditional survival of a genomic deletion of acrD in a lambda Red recombinase system. The most likely limitation was the amount of DNA in the ligation and transformation reactions, therefore, increasing the concentration to a verified 30 fmol of vector and 90 fmol of insert would determine if this was the cause. The occurrence of empty vectors might suggest that acrD is a harmful insert if the empty vector arises from re-ligation and therefore it would also be important to determine why the empty vectors arise. To determine whether the empty pBAD28 vectors in the putative pBAD28-acrD ligation transformants were due to snapbacks of the vector onto itself without insert, or undigested pBAD28 being carried through, a simple transformation of pBAD28 after its digestion without ligation would be valuable. Colonies arising from this transformation that grow on an ampicillin-selective plate would indicate that pBAD28 is not being cut by the restriction enzymes, and that there is the possibility that these molecules were carried over to ligations and transforming to provide false-positives in pBAD28-acrD screenings. If the false positives persist, then prolonging the digestion should decrease the number of detected false positives. In addition, a control reaction during the ligation (digested pBAD28 without acrD insert) could reveal whether pBAD28 is indeed becoming re-ligated, as the number of colonies from transformations with the control versus the acrD insert ligation products could be compared.

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REFERENCES