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

Mahmud Iqbal, Ke Li, David Reading, Kia Shahtaheri
Department Microbiology & Immunology, University of British Columbia

The construction of pBAD28-*acr*D, 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 *acr*D 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. cloni® 5-alpha strain cells and characterization of putative transformants, it was determined that a successful pBAD28-*acr*D expression vector failed to be constructed, and the transformants 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 *acr*D 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 *acr*D 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 *acr*D 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 *acr*D manipulation alone on bacteria in terms of viability, population growth and antibiotic susceptibility.

Prior papers have detected *acr*D transcripts in putative Δ*acr*D 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 Δ*acr*D mutant would have to be complemented with a pBAD28-*acr*D expression vector.

The goal of this study was to construct a stable, transformable pBAD28–*acr*D plasmid in order to control expression of *acr*D and assess the effect of variable expression on bacterial cell physiology. Furthermore, we planned to use the pBAD28-*acr*D 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 *acr*D gene.

We planned to create the plasmid by ligating PCR-amplified *acr*D into pBAD28. The PCR amplification of *acr*D employed newly-designed primers, containing HindIII and XbaI restriction digestion sites, to allow the amplification of *acr*D 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–*acr*D positive colonies through plasmid isolation, restriction digestion, and gel electrophoresis analysis.

MATERIALS AND METHODS

Isolation and amplification of *acr*D. A 3171 bp fragment containing *acr*D was amplified from the *E. coli* K12 MG1655 genome using PCR. The primer RLSIacrD-F that is complementary to the region upstream of *acr*D contains an XbaI restriction site. The primer RLSIacrD-R that is complementary to the region downstream of *acr*D contains a HindIII restriction site. The use of these restriction sites allows the ligation of digested *acr*D 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 RLSIacrD-F, 1 µM Primer RLSIacrD-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 1. Oligonucleotide primers used for the amplification of *acr*D. Primers were complementary to the regions upstream and downstream of *E. coli* MG1655 *acr*D. Restriction cut sites are indicated in bold.

Name	Sequence (5' – 3')	T_m (°C)
RLSIacrD-F	GGCGC**AAGCTT**CATGTCGC CTTTTTTATTGC	63.9 °C
RLSIacrD-R	**TCTAGA**AAACACGAGGTC CTCTTTTA	55.4 °C

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 30°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.

C**onstruction of putative pBAD28-*acr*D.** The prepared pBAD28 and *acr*D samples were digested overnight at 37°C, in a final reaction volume of 10 µl for pBAD28, and 45 µl for *acr*D. 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 *acr*D digestion contained 300 ng of *acr*D 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 *acr*D was carried out in a final reaction volume of 20 µl, consisting of approximately 18 ng *acr*D, 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.

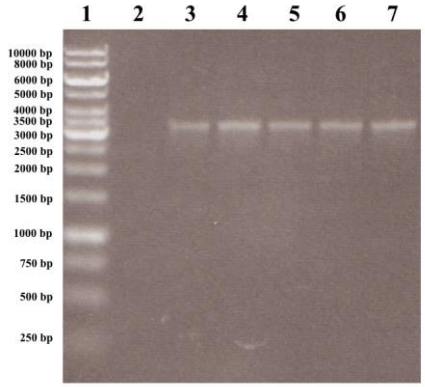
Transformation protocols. Chemically competent E. cloni® 5-alpha brand competent cells (Lucigen, 60602-1) were thawed on ice, then allowed to incubate with 5 µl ligation reaction for 5 minutes. The cells were heated to 42°C for 45 seconds, then recovered in 250 µl LB for 1 hour, shaking at 200 rpm at 37°C. Following the incubation, 100 µl of the recovered culture was spread 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 dH_2O for ampicillin. Working concentrations were 25 µg/ml and 50 µg/ml for chloramphenicol and ampicillin, respectively.

RESULTS

***acr*D 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 *acr*D 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 *acr*D gene was successfully amplified with HindIII and XbaI restriction sites at the 5' and 3' ends respectively.



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

FIG. 1. Colony gradient PCR amplification product, *acr*D. Lane 1 contains the GeneRuler 1kb DNA Ladder. Lane 2 shows an absence of PCR product. Lane 3 to 7 each contains PCR products amplified with different annealing temperatures.

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

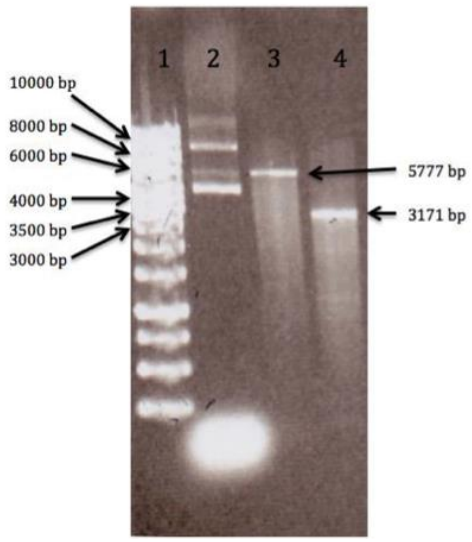
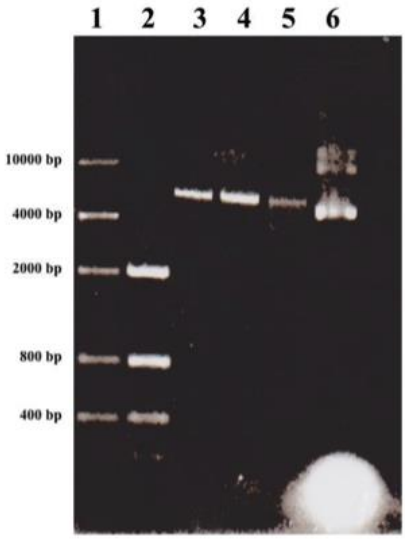


FIG. 2. HindIII and XbaI double digested products of pBAD28 and purified *acr*D PCR in 1% agarose. Lane 1 contains the GeneRuler 1 kb DNA Ladder. Lane 2 contains the undigested pBAD28, Lane 3 contains the digested, purified pBAD28 linear product (5777 bp), and Lane 4 contains the digested, purified *acr*D PCR product (3171 bp).



Lane	Identity
1	High molecular weight DNA Ruler (6 µl)
2	Low molecular weight DNA Ruler (6 µl)
3	pBAD28 Digest (2.6 g) with XbaI
4	pBAD28 Digest with HindIII (6 µl)
5	pBAD28 Digest with XbaI and HindIII (6 µl)
6	Undigested pBAD28 (6 µl)

FIG 3. Undigested pBAD28, bands corresponding to linearized fragment (further migration) and alternate conformation of the plasmid. DNA Rulers are in Lanes 1 and 2. Lanes 3 and 4 contain products confirming the functionality of individual restriction enzymes. Lane 5 shows desired double digested *acr*D product. The gradual increase in migration distance for bands of similar weight with increasing lane can be attributed to uneven gel thickness (the gel was thicker on the left side).

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 *acr*D were restriction digested and subsequently ligated. The isolated *acr*D 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 *acr*D 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* DH5a 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-*acr*D 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-*acr*D transformant cells was potentially due to several issues involved in the digestion and subsequent ligation of the *acr*D 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

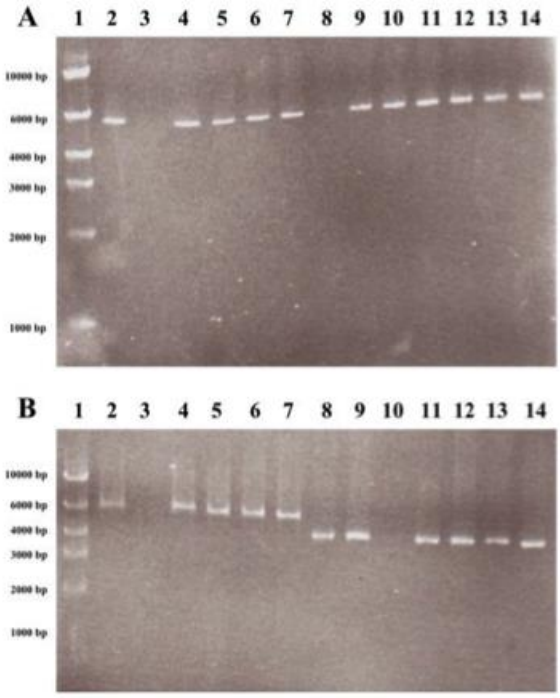


	Figure 4A		Figure 4B
Lane	**Identity**	**Lane**	**Identity**
1	High Mass Ladder (Invitrogen)	1	High Mass Ladder (Invitrogen)
2	Sample 14 (Digested)	2	Sample 8 (Digested)
3	Sample 13 (Digested)	3	Sample 7 (Digested)
4	Sample 12 (Digested)	4	Sample 6 (Digested)
5	Sample 11 (Digested)	5	Sample 5 (Digested)
6	Sample 10 (Digested)	6	Sample 4 (Digested)
7	Sample 8 (Digested)	7	Sample 3 (Digested)
8	Sample 7 (Digested)	8	Sample 10 (Undigested)
9	Sample 6 (Digested)	9	Sample 8 (Undigested)
10	Sample 5 (Digested)	10	Sample 7 (Undigested)
11	Sample 4 (Digested)	11	Sample 6 (Undigested)
12	Sample 3 (Digested)	12	Sample 5 (Undigested)
13	Sample 2 (Digested)	13	Sample 4 (Undigested)
14	Sample 1 (Digested)	14	Sample 3 (Undigested)

FIG. 4. Analysis of digested plasmids from the putative pBAD28-*acr*D 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).

4). This occurrence could be attributed to incomplete digestion of the pBAD28 fragment, leading to one 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 *acr*D 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-*acr*D 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. cloni® 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 *acr*D 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 *acr*D 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-*acr*D 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-*acr*D expression plasmid failed to be detected.

FUTURE DIRECTIONS

In this study, a successful pBAD28-*acr*D expression vector failed to be constructed, and as such, future extensions of this project include the successful ligation of the *acr*D PCR product into a pBAD28 expression vector, and the use of this expression vector to permit conditional survival of a genomic deletion of *acr*D 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 *acr*D 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-*acr*D 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-*acr*D 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 *acr*D insert) could reveal whether pBAD28 is indeed becoming re-ligated, as the number of colonies from transformations with the control versus the *acr*D insert ligation products could be compared.

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