Functional analysis of the role of Toxin-antitoxin (TA) loci in bacterial persistence

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Running head: Analysis of TA loci in persistence

Summary

We have developed a method to analyze the functionality of putative TA loci by expressing them in *E. coli*. Here we describe the procedure for cloning recombinant TA genes into inducible plasmids and expressing these in *E. coli.* Following expression, toxicity, resuscitation of growth and changes in persister cell formation are assayed. This can confirm whether predicted TA loci are active in *E .coli* and whether expression can affect persister cell formation.

Keywords: Toxin-antitoxin; persistence; inducible expression; *E. coli;* antibiotic

1. Introduction

Toxin-antitoxin (TA) systems are found in many bacterial species and typically consist of a gene pair coding for a toxin and antitoxin gene. The toxins can typically bind and inhibit the function of a cellular target causing inhibition of bacterial growth [1]. The antitoxin gene codes for either a protein or an RNA molecule that inhibits the activity of the toxin under normal cellular conditions. One role for TA systems is in the formation of bacterial persister cells [2]. Bacterial persisters are a sub-population of cells that can tolerate and survive antibiotic or stress treatment, whereas the majority of the population is killed [3]. Expression of TA toxins can increase the population of tolerant persister cells [4,5]. An increase in persistence has been best demonstrated in *E. coli* following expression of a variety of toxins. One of the first examples of this was ampicillin treatment of *E. coli* cultures expressing the TA toxin HipA from an arabinose driven pBAD promoter [6]. Other toxins such as RelE, TisB and HicA have also been tested using similar methodology, expressing the toxin from an inducible promoter and then treating with antibiotic [7,5,4]. The method we have developed permits testing on the functionality of putative or predicted TA systems though expression in *E. coli.* Predicted TA systems native to the *E. coli* host or from other bacteria, such as *Burkholderia pseudomallei,* can be expressed in *E. coli* MG1655 [8,4]. The method requires cloning of putative toxin and antitoxin genes into separate compatible and inducible plasmids and firstly assaying for growth arrest/ reduction in culturability following expression of the toxin and resuscitation/restoration of growth following expression of the antitoxin. Following assessment of these phenotypes, the methodology can be implemented to assay for the effect of TA expression on the persistence of *E. coli* following treatment with various antibiotics. Failure of *E. coli* to express cloned recombinant genes and differences between toxin target(s) in the native host compared to target(s) in *E. coli* are potential limitations of this technique. However, in using *E. coli* as a host, a safe and high throughput method has been developed to screen a variety of predicted TA systems from a range of bacteria for

functionality. This method can be used to down select TA genes candidates for further phenotypic study in the native host or for structural studies.

2. Materials

1. *E. coli* MG1655 strain (F^-, $lambda^-$, rph-1)
2. Expression plasmids e.g. pBAD/his (Invitrogen) and pME6032
3. Antibiotics for plasmid selection, e.g. ampicillin (100 mg/ml) and tetracycline (15 mg/ml) dissolved in water
4. PCR reagents for gene amplification
5. Restriction enzymes for cloning
6. LB: Weigh 5 g Bacto-tryptone, 2.5 g yeast extract, 5 g NaCl and dissolve in 500 ml water. Autoclave for sterilisation
7. LB agar: Weigh 5 g Bacto-tryptone, 2.5 g yeast extract, 5 g NaCl, 7.5 g agar and dissolve in 500 ml water. Autoclave for sterilisation.
8. Arabinose 20% solution in water
9. Glucose 20% solution in water
10. Isopropyl β-D-1-thiogalactopyranoside (IPTG) 1 M solution in water
11. Universal tubes
12. 200 ml Conical flasks
13. 96-well plates
14. 24-well plates
15. Cuvettes: 1 cm diameter
16. 1.5 ml Eppendorf tubes
17. Spectrophotometer set for measurement at 590nm
18. Centrifuge(s), capable of speeds of 3000 *x g* for universal tubes and 13,000 *x g* for 1.5 ml Eppendorf tubes

3. Methods

3.1 Cloning into expression vectors

1. PCR amplify putative toxin or antitoxin genes using genomic DNA as a template and primers containing appropriate restriction sites
2. Clone toxin gene(s) into an inducible expression plasmid such as the pBAD/his vector (Invitrogen) via appropriate restriction sites **(fig 1a) (See Note 1).**
3. Clone antitoxin genes into a different inducible expression plasmid such as the pME6032 vector via appropriate restriction sites within the multiple cloning sites **(fig 1b), (See Note 2).**

3.2 Toxicity assay

The following protocols assume the use of the pBAD/his and pME6032 plasmids. If other plasmids are used, change the antibiotics for plasmid selection and sugar inducers/repressors as appropriate.

1. Inoculate 5 ml of LB broth containing 100 µg/ml ampicillin with *E. coli* harbouring pBAD with cloned toxin gene and incubate at 37^{o}C, 200 rpm for 16 hours.
2. Dilute the culture 1:100 in 40 ml of fresh LB in a 200 ml conical flask supplemented with 100 µg/ml ampicillin. Grow cultures at 37^{o}C, 200 rpm until reaching an OD_{590}nm of ~0.1. (**See Note 3)**
3. Aliquot 2 x 10 ml of culture into universal tubes and supplement with either 0.2% (w/v) glucose or 0.2% (w/v) arabinose to repress or induce expression from the pBAD promoter respectively. Incubate cultures at 37^{o}C, 200 rpm.
4. At hourly intervals, remove 1 ml of culture (or 100 µl and mix with 900 µl LB for 1:10 dilution) and add to a cuvette to record the OD_{590}nm using a spectrophotometer
5. In parallel, remove 10 µl of culture and set up a serial dilution in a 96-well plate containing LB. Carry out a dilution range from 10^{-1}-10^{-6}. Spot plate the dilution range

onto LB plates containing 100 µg/ml ampicillin and incubate plates statically at 37^{o}C until colonies are visible for enumeration **(fig 2)**.

3.3 Co-expression assay

1. Inoculate 5 ml of LB broth containing 100 µg/ml ampicillin, 15 µg/ml tetracycline with *E. coli* harbouring pBAD/toxin gene and pME6032/antitoxin gene and incubate at 37^{o}C, 200 rpm for 16 hours.
2. Dilute the culture 1:100 in 50 ml of fresh LB in a 200 ml conical flask supplemented with 100 µg/ml ampicillin, 15 µg/ml tetracycline. Grow cultures at 37^{o}C, 200 rpm until reaching an OD_{590}nm of ~0.1.
3. Aliquot 4 x 10 ml of culture into universal tubes and supplement with either 0.2% (w/v) glucose or 0.2% (w/v) glucose, 25 mM IPTG or 0.2% (w/v) arabinose or 0.2% arabinose, 25mM IPTG to repress or induce expression from the pBAD and pME6032 promoters. Incubate cultures at 37^{o}C, 200 rpm.
4. At hourly intervals, remove 1 ml of culture (or 100 µl and mix with 900 µl LB for 1:10 dilution) and add to a cuvette to record the OD_{590}nm using a spectrophotometer
5. In parallel, remove 10 µl of culture and set up a serial dilution in a 96-well plate containing LB. Carry out a dilution range from 10^{-1}-10^{-6}. Spot plate the dilution range onto LB plates containing 100 µg/ml ampicillin, 15 µg/ml tetracycline and incubate plates statically at 37^{o}C until colonies are visible for enumeration. **(Fig 3).**

3.4 Resuscitation assay

1. Inoculate 5 ml of LB broth containing 100 µg/ml ampicillin, 15 µg/ml tetracycline with *E. coli* harbouring pBAD/toxin gene and pME6032/antitoxin gene and incubate at 37^{o}C, 200 rpm for 16 hours.

2. Dilute the culture 1:100 in 60 ml of fresh LB in a 200 ml conical flask supplemented with 100 µg/ml ampicillin, 15 µg/ml tetracycline. Grow cultures at 37^{o}C, 200 rpm until reaching an OD_{590}nm of ~0.1.
3. Aliquot 2 x 25 ml of culture into 200 ml conical flasks and supplement with either 0.2% (w/v) glucose or 0.2% (w/v) arabinose to repress or induce expression from the pBAD promoter respectively. Incubate cultures at 37^{o}C, 200 rpm for 2 hours (T2).
4. Aliquot 2 x 10 ml of each culture into universal tubes and supplement one with 25 mM IPTG to induce antitoxin expression from the pME6032 promoter. Incubate cultures at 37^{o}C, 200 rpm for a further 2 hours (T4). **(See Note 4)**
5. At hours T0, T2 and T4 remove 1 ml of culture (or 100 µl and mix with 900 µl LB for 1:10 dilution) and add to a cuvette to record the OD_{590}nm using a spectrophotometer. In parallel, remove 10 µl of culture and set up a serial dilution in a 96-well plate containing LB. Carry out a dilution range from 10^{-1}-10^{-6}. Spot plate the dilution range onto LB plates containing 100 µg/ml ampicillin, 15 µg/ml tetracycline and incubate plates at statically at 37^{o}C until colonies are visible for enumeration **(fig 4).**

3.5 Persister assay

1. Inoculate 5 ml of LB broth containing 100 µg/ml ampicillin, 15 µg/ml tetracycline with *E. coli* harbouring pBAD/toxin gene and pME6032/antitoxin gene and incubate at 37^{o}C, 200 rpm for 16 hours.
2. Dilute the culture 1:100 in 30 ml of fresh LB in a 200 ml conical flask supplemented with 100 µg/ml ampicillin, 15 µg/ml tetracycline. Grow cultures at 37^{o}C, 200 rpm until reaching an OD_{590}nm of ~0.1.
3. Aliquot 2 x 10 ml of culture into universal tubes and supplement with either 0.2% (w/v) glucose or 0.2% (w/v) arabinose to repress or induce expression from the pBAD promoter respectively. Incubate cultures at 37^{o}C, 200 rpm for 3 hours.

4. Standardise cultures to 2 x 10^8 CFU/ml (OD_{590}nm 0.5) in a 5 ml volume and add 500 µl aliquots to a 24 well plate.
5. Add 500 µl of LB antibiotic stock at 200 x minimum inhibitory concentration (MIC) to each of the wells. (This gives 10^8 CFU/ml and 100 X MIC antibiotic per well) **(See Note 5)**.
6. Add 10 µl of standardised culture from step 4 to a 96-well plate containing LB for serial dilution. Carry out a dilution range from 10^{-1}-10^{-6}. Spot plate the dilution range onto LB plates containing 100 µg/ml ampicillin, 15 µg/ml tetracycle, 1 mM IPTG and incubate plates at statically at 37^{o}C until colonies are visible for enumeration. (**See Note 6)**
7. Incubate 24 well persister assay plate statically at 37^{o}C for 24 hours.
8. Remove cultures from 24 well plate and add to 1.5 ml Eppendorf tubes.
9. Centrifuge the tubes for 7 minutes at max speed in a microcentrifuge
10. Remove the supernatant and then re-suspend the pellet in fresh LB
11. Serial dilute the cultures in LB using a dilution range of 10^0-10^{-4} in a 96 well plate. Spot plate the dilution range onto LB plates containing 100 µg/ml ampicillin, 15 µg/ml tetracycle, 1 mM IPTG and incubate plates statically at 37^{o}C until colonies are visible for enumeration.
12. Persister frequency is determined as CFU post antibiotic treatment divided by CFU pre antibiotic treatment. **(See Note 7) (Fig 6).**

4. Notes

1. If using the pBAD/ his vector clone into the *Nco*I sites and *Eco*RI/*Hind*III sites to create a non-his tagged construct or *Sac*I and *Eco*RI/*Hind*III to create a his-tagged construct. The N-terminal his tag, although useful for protein purification and Western blotting, may interfere with the function of the toxin or shield the antitoxin binding site.

It is advisable to make both a his tagged and non his tagged version of the toxin for phenotypic analysis.

2. Both inducible expression plasmids need to be in different incompatibility groups, as it is not possible to maintain two different plasmids in a single cell, which use the same mechanism for replication. The plasmids described in this manuscript fall into the pMB1 group (pBAD/his) and p15A group (pME6032). Both plasmids have a similar copy number per cell.
3. If cultures of *E. coli* harbouring pBAD with a cloned toxin are growing slower than a control *E. coli* harbouring empty pBAD, add 0.2% glucose to the media to prevent leaky expression from the pBAD promoter. Cultures should be harvested and re-suspended in fresh media prior to adding 0.2% arabinose if glucose is added during the initial growth step.
4. If addition of IPTG, to induce expression of the antitoxin from pME6032, fails to resuscitate growth of toxin induced cultures, a wash step should be added to the protocol. Prior to addition of IPTG, harvest cells by centrifugation at 3000 *x g* for 10 minutes. Remove the supernatant and re-suspend the pellet in the same volume of fresh LB. Add IPTG and fresh antibiotic.
5. Some antibiotic stocks, such as ceftazidime, need to be made fresh every time as they are susceptible to degradation and will not work properly after pro-longed storage.
6. 1 mM IPTG is included in the plates to induce antitoxin expression from the pME6032 promoter. This is to re-awaken/ resuscitate any non-culturable/ dormant cells induced by toxin expression. This is to achieve a more accurate measure of the total number of viable cells. If the total cell numbers are lower than expected the IPTG concentration should be increased.
7. Time 0 counts should be divided by 2, since only half of the standardised culture is added to the persister assay.

5. References

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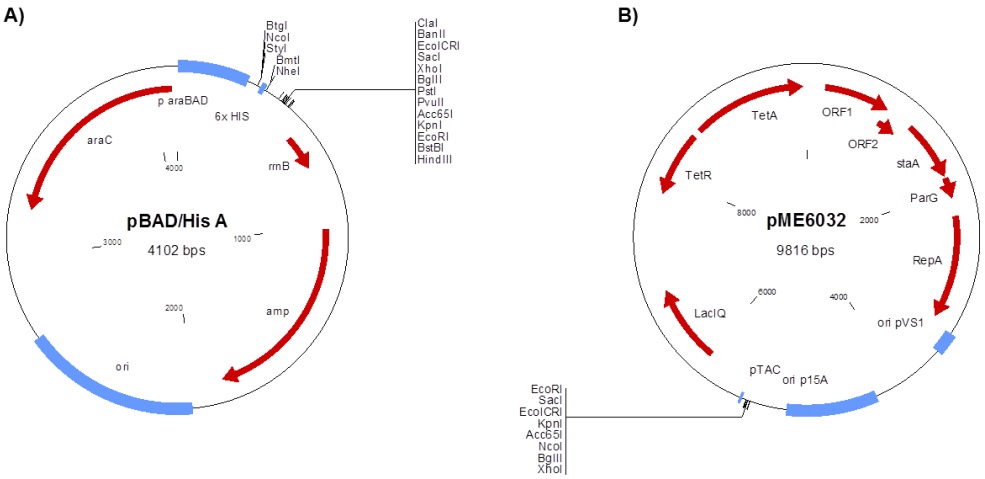

Figure 1. Expression plasmid maps. Key features such as antibiotic resistance genes., multiple cloning sites and origins of replication are shown A) pBAD/his. B) pME6032

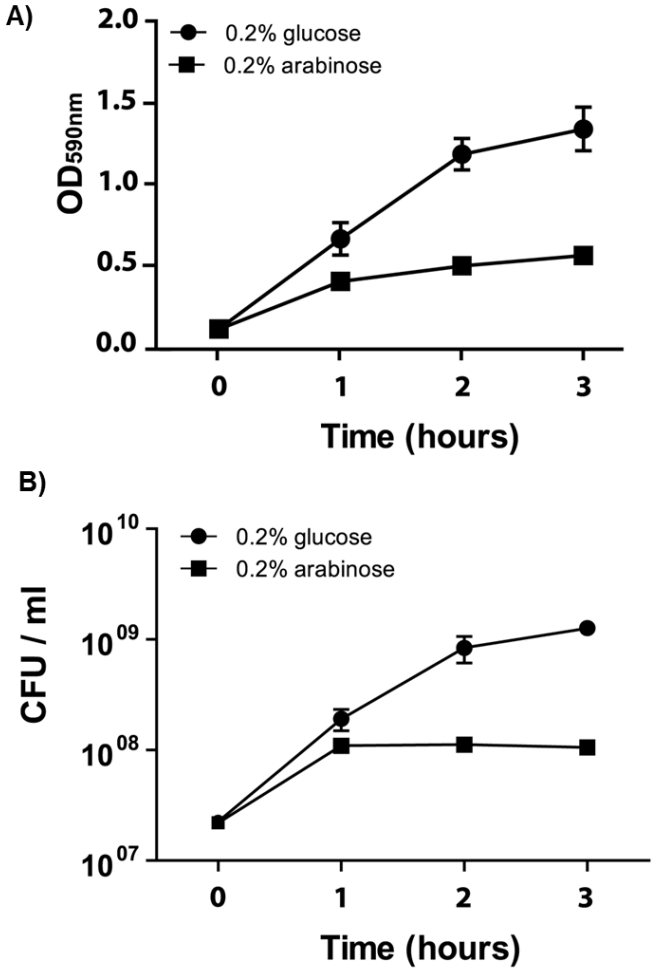

Figure 2. Effect of toxin expression from the pBAD/his vector on *E. coli* growth. 0.2% glucose or arabinose was used to repress or induce expression of the toxin respectively. A) Growth measured by optical density. B) Growth measured by CFU counts. Reproduced from [8] with permission.

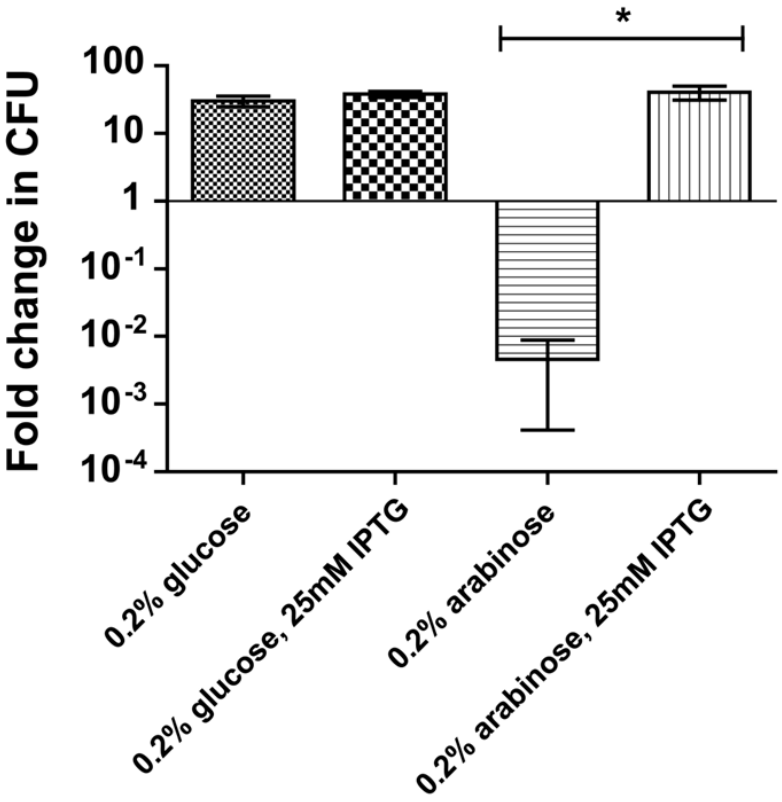

Figure 3 Co-expression of toxin and antitoxin from different inducible promoters in *E. coli* and the change in the number of culturable cells. 0.2% glucose or 0.2% arabinose was used to repress or induce expression of the plasmid cloned toxin respectively. 25 mM IPTG was used to induce expression of the plasmid cloned antitoxin. Reproduced from [8] with permission.

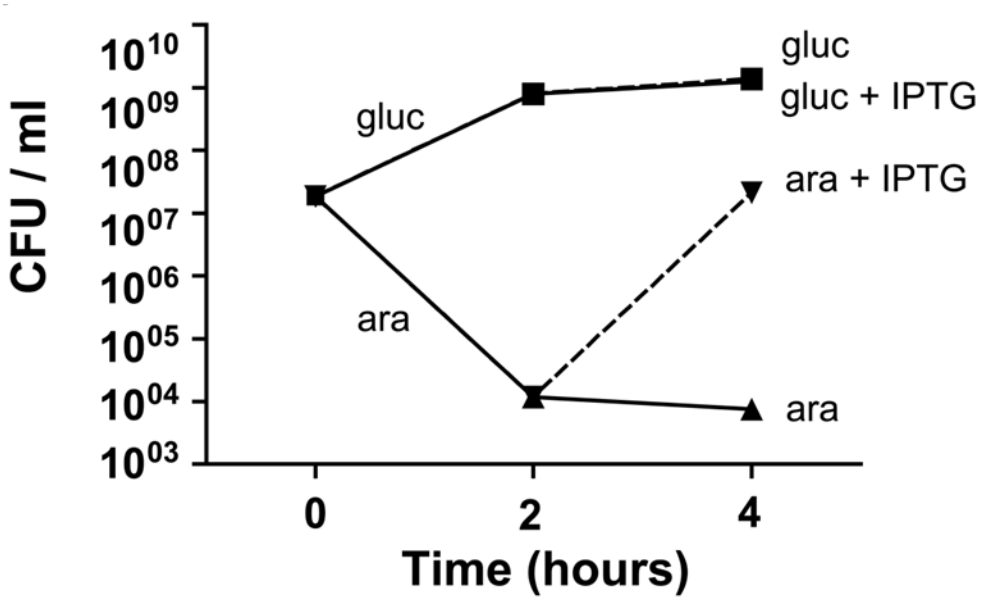

Figure 4. Resuscitation of growth by antitoxin expression. 0.2% glucose or 0.2% arabinose was used to repress or induce expression of the plasmid cloned toxin respectively. 2 hours later 25 mM IPTG was used to induce expression of the plasmid cloned antitoxin before plating for enumeration. Reproduced from [8] with permission.

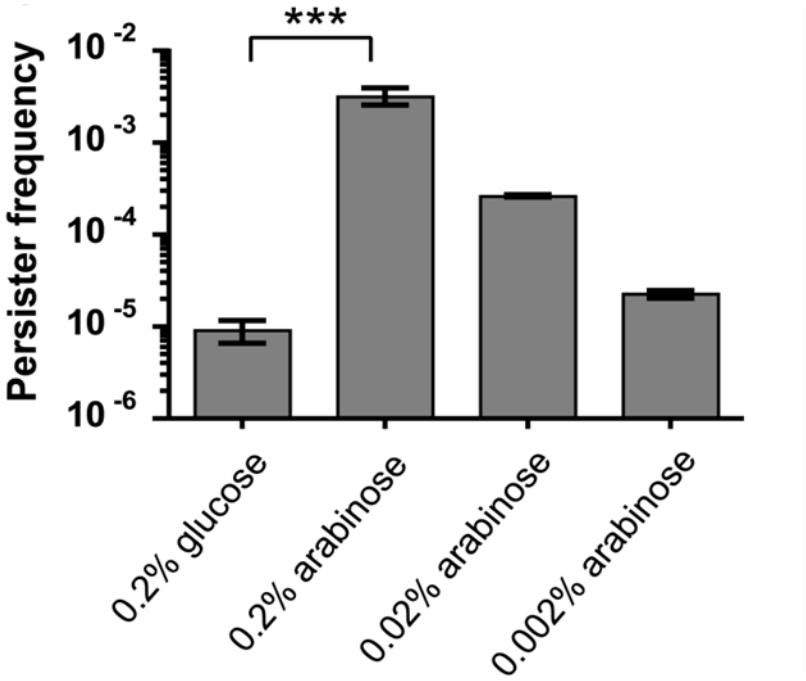

Figure 5. Persister cell frequency following 24 hour treatment of *E. coli* cultures with 100 x MIC ciprofloxacin. Toxin expression was repressed with 0.2% glucose or induced with a range of arabinose concentrations for 3 hours before ciprofloxacin treatment. Reproduced from [4] with permission.