An Integrative Plasmid Vector for Zinc Inducible Gene Expression in Cyanobacteria

Introduction

Genetic engineering works by introducing DNA with the genes of one organism into living cells of another organism. However, the expression of these new genes into proteins may be uncontrolled and unreliable in their new cells. The genes may be underexpressed or silent, which defeats the purpose of the experiment. Or they may be overexpressed and become toxic to the cell. The engineered genes are also cut off from the complex regulatory systems of their native cells which controls when their expression is turned on or off. Without such regulation, new genes are expressed constitutively (constantly) and are of limited use if even the simplest control is required of their expression.

Genes are introduced into bacterial cells on circular loops of DNA called plasmid vectors. Along with the genes of interest, these vectors may be packaged with simple regulation systems from a model organism such as E. coli. These systems often fail when introduced to evolutionary distant bacteria with wholly different metabolisms. For example, E. coli based regulators may involve a type of sugar to induce expression but cyanobacteria, being photosynthetic, do not uptake sugars from the environment. Instead, metal ions may be a better method for controlling the new genes because they enter the cells readily through membrane proteins and are not consumed by the host metabolism.

Gomez-Santos, et al[[1]](#endnote-1) created a set of plasmid vectors for controlled gene expression in *Myxococcos xanthus* using such a method. Existing methods for this species were either constitutive, hard to measure the effects of, or directly inhibited certain behaviors of the organism. The authors used the promoter region of a gene native to *M. xanthus* which was observed in an earlier study[[2]](#endnote-2) to be very responsive to the concentration of copper in the bacterium’s growth medium. The new plasmids were successful in that no expression was measured in the absence of copper and expression was observed to increase in direct proportion to the amount of copper to the growth medium without any inhibition of normal cell behavior.

SmtB[[3]](#endnote-3) [[4]](#endnote-4) [[5]](#endnote-5) is a repressor protein native to cyanobacteria genus *Synechococcus* which normally binds to the promoter of an operon of genes responsible for removing excess zinc from the cell. In the presence of zinc, the protein detaches from the DNA and RNA polymerase is able to begin transcribing the regulated operon as well as more copies of SmtB itself. This proposal aims to create a plasmid vector for *Synechococcus* cyanobacteria which uses SmtB to regulate the expression of genes of interest in response to elevated levels of zinc in the environment.

Experiment

The experiment will begin with constructing the plasmid *in vitro* by Gibson assembly with bacterial luciferase as a reporter gene. Next, the plasmids will be introduced to a naturally competent strain, Synechococcus sp. PCC 7002, for transformation and an antibiotic will be used to remove non-transformed cells. The transformed cells will then be tested with varying concentrations of zinc and expression of luciferase will be measured by a luminometer.

Gibson assembly[[6]](#endnote-6) [[7]](#endnote-7) is a simple and powerful way to build large DNA molecules without complicated mixes of restriction enzymes and secondary hosts. First, primers are designed in software so that they contain the sequences of biological interest (genes, promoters, etc.) and have complimentary regions at least 20 bp long on the ends which are to connect in the finished plasmid. That is, the 3’ end of one primer should be complimentary to the 3’ of the beginning of the next primer. The primers are ordered from a DNA supply company and amplified by PCR. The resulting DNA fragments are then combined simultaneously with a trio of enzymes: T5 exonuclease, Phusion DNA polyermase II, and *Taq* DNA ligase. The exonuclease chews off just the 5’ end of each DNA fragment. The untouched 3’ ends anneal together and the polymerase and ligase work together to fill in the missing nucleotides.

Synechococcus sp. PCC 7002 is a unique strain in that it is capable of natural transformation[[8]](#endnote-8) [[9]](#endnote-9), meaning that it can incorporate foreign DNA without electric shock, freezing, or chemical inducement. However, only linear DNA can enter the cell this way so our plasmid will need to contain a site for the restriction enzyme SphI or NsiI and be digested prior to transformation. Once transformed, the linear DNA reforms into a circular structure within the cell.

The process of natural transformation is relatively simple. Cultures of Synechococcus are added to bubbling tubes with the linearized plasmid and incubated overnight. At least one negative control culture is incubated without the plasmid. The cultures are then each centrifuged and spread over an agar plate containing an antibiotic for which resistance is provided in the plasmid. The plates are then incubated until colonies are visible. The colonies are restreaked several times on fresh plates of agar and the antibiotic and the purified strain is finally used to inoculate a liquid culture for the measurement procedure.

Bacterial luciferase[[10]](#endnote-10) [[11]](#endnote-11), gene luxAB, was chosen as a reporter instead of a more typical fluorescent protein because of possible interference from the cyanobacteria’s chlorophyll which fluoresces bright red under UV light. This would not be a problem for a quick qualitative measure (Is it glowing? Yes or no?) but would complicate a quantitative measure if the chlorophyll is glowing brighter than the reporter. Luciferase is an enzyme which catalyzes a reaction involving n-decanal and oxygen and therefore has some relevance to the synthetic biology pathways this experiment is intended to facilitate.

To measure luciferase expression, different amounts of zinc will be added to each of four different control groups of liquid cultures. At that time and every two hours afterwards for 48 hours, a sample of each cell culture is transferred to an empty vial. An open microcentrifuge tube of n-decanal is then carefully placed up right in the tube. The vial is sealed and placed under constant light for one hour. This just long enough for the decanal vapor to saturate the cell culture. A luminometer apparatus is then used to measure the bioluminescence of the vial and the figure recorded.

 Discussion

The results I expect to see are a steady increase in bioluminescence in response to an increase in zinc concentration. However, it is entirely possible that bioluminescence is unresponsive to higher zinc concentration or that the SmtB regulator is just as “leaky” as the lac repressor it is trying to replace. This leakiness would be shown as a relatively high amount of bioluminescence even if little or no zinc is added to the culture. One explanation for this would be the SmtB proteins of the host cells not binding to the foreign plasmids. This could be mitigated by including SmtB as part of the expressed operon because it binds to its own promoter both on the plasmid and on the genome. Assuming the newly synthesized SmtB is able to bind to smt and successfully block RNA polymerase, any initial burst of bioluminescence would almost immediately shut down until the arrival of zinc.

One possible negative result is poisoning the cells by an overabundance of zinc in the cell culture. The presence of zinc normally triggers SmtB to allow the expression of SmtA, a metallothionein which binds to zinc and renders it inert3. A preliminary experiment would have to be done first to find a maximum zinc threshold for cyanobacteria containing our plasmid, testing for survivability and not bioluminescence.

For follow up experiments, it would be interesting to explore plasmids regulated by other metalloregulators in the SmtB/ArsR protein family. These proteins are structurally similar to SmtB but vary in their metal binding domains and therefore which metals they are regulated by. An entire metabolic pathway could be placed on a single plasmid with several different regulators, each controlled the concentration of a different metal. This would allow very tuning for synthetic biology experiments trying to synthesize a complex compound with multiple enzymes or give systems biology a new tool to measure the network effects of changing the expression of many genes at once.

References

1. Gómez-santos N, Treuner-lange A, Moraleda-muñoz A, et al. Comprehensive set of integrative plasmid vectors for copper-inducible gene expression in Myxococcus xanthus. Appl Environ Microbiol. 2012;78(8):2515-21. [↑](#endnote-ref-1)
2. Sánchez-sutil MC, Gómez-santos N, Moraleda-muñoz A, Martins LO, Pérez J, Muñoz-dorado J. Differential expression of the three multicopper oxidases from Myxococcus xanthus. J Bacteriol. 2007;189(13):4887-98 [↑](#endnote-ref-2)
3. Vanzile ML, Chen X, Giedroc DP. Allosteric negative regulation of smt O/P binding of the zinc sensor, SmtB, by metal ions: a coupled equilibrium analysis. Biochemistry. 2002;41(31):9776-86. [↑](#endnote-ref-3)
4. Cook WJ, Kar SR, Taylor KB, Hall LM. Crystal structure of the cyanobacterial metallothionein repressor SmtB: a model for metalloregulatory proteins. J Mol Biol. 1998;275(2):337-46 [↑](#endnote-ref-4)
5. L. Botello-Morte et al., “Functional Genomics of Metalloregulators in Cyanobacteria”, Advances in Botanical Research, 65 (2013): 107-56 [↑](#endnote-ref-5)
6. Gibson DG. Enzymatic assembly of overlapping DNA fragments. Meth Enzymol. 2011;498:349-61. [↑](#endnote-ref-6)
7. Gibson DG, Young L, Chuang RY, Venter JC, Hutchison CA, Smith HO. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat Methods. 2009;6(5):343-5. [↑](#endnote-ref-7)
8. Xu Y, Alvey RM, Byrne PO, Graham JE, Shen G, Bryant DA. Expression of genes in cyanobacteria: adaptation of endogenous plasmids as platforms for high-level gene expression in Synechococcus sp. PCC 7002. Methods Mol Biol. 2011;684:273-93 [↑](#endnote-ref-8)
9. Frigaard NU, Sakuragi Y, Bryant DA. Gene inactivation in the cyanobacterium Synechococcus sp. PCC 7002 and the green sulfur bacterium Chlorobium tepidum using in vitro-made DNA constructs and natural transformation. Methods Mol Biol. 2004;274:325-40. [↑](#endnote-ref-9)
10. Liu Y, Golden SS, Kondo T, Ishiura M, Johnson CH. Bacterial luciferase as a reporter of circadian gene expression in cyanobacteria. J Bacteriol. 1995;177(8):2080-6. [↑](#endnote-ref-10)
11. Weiser J, Buriánková K, Kalachová L, Branny P, Pernodet JL. Construction and testing of a bacterial luciferase reporter gene system for in vivo measurement of nonsense suppression in Streptomyces. Folia Microbiol (Praha). 2006;51(1):62-4. [↑](#endnote-ref-11)