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# Investigating Homologs of the MarR transcription factor in *Methylobacterium extorquens*

## Introduction

The role of transcriptional regulators is often of interest due to the diversity of processes and signals they coordinate. One important class of transcriptional regulators is the MarR protein family. Proteins within the multiple antibiotic resistance regulator (MarR) category can be attributed to several gene activities within various kinds of microbes expressing this transcriptional regulator. In previously studied microbes, the regulation by this gene has been found to regulate stress responses, antibiotic resistance, and degradation of high-interest compounds, such as lignin-derived aromatics. In *Escherichia coli*, the bacteria in which MarR was initially studied, the protein was attributed to resistance of a variety of antibiotics.[2] Further studies have concluded that MarR can regulate stress responses, expression of virulence genes, and degradation of toxic aromatic compounds. Many common bacteria have been found to display the MarR protein, but the number of homologs associated within the genome can vary. While many of these proteins have been identified, the functions of these proteins often remain unknown.[2]

The strain of *Methylobacterium extorquens* that will be investigated in this study, PA1, has nine homologs of the MarR protein, with all but one having their function unknown. In prior studies conducted, the homolog Mext_0925 was found to play a role in mediating formaldehyde stress. This paper will outline the development of a high throughput phenotypic screen to begin determining the functions attached to the eight remaining homologs of *M. extorquens*. The elements that will be screened for are as follows: oxidative stress, antibiotic resistance, and phenolic compound stress. To investigate any phenotypic response, in-frame deletion strains of *M. extorquens* must first be created following an allelic exchange protocol, which requires assembling plasmids. From this point, mutant strains can be put under these stress conditions in the high throughput screen for analysis.

## Experimental

*Plasmid Development*

The first step in creating the deletion strains of *M. extorquens* is to assemble an integrative plasmid with homology regions that flank both sides of the target gene. For this procedure, the broad-host-range allelic exchange vector pCM433 will be used. This plasmid will be linearized using specific endonucleases at the *AatII* and *SacI* sites. Forward and reverse primers will be designed for each location (600 base pairs upstream and downstream from each gene targeted for deletion) and ordered from a laboratory supplier. A colony polymerase chain reaction (PCR) should be performed with the respective primer pairs to amplify the DNA, creating regions of homology. Gel electrophoresis with a 1% agarose gel should be performed on PCR products to check for correct size. To quantify the amount of DNA in these products, a NanoDrop spectrophotometer can be used by placing the sample in a cuvette and measuring at 600 nm.

After quantifying the DNA in the PCR products, a HiFi DNA Assembly Reaction can occur using PCR products, the linearized plasmid, and a HiFi DNA Assembly Master Mix like that supplied by New England BioLabs. After incubating this mixture at 50°C for 60 minutes for maximum efficiency, the products should be sealed DNA molecules containing the amplified regions of homology within the plasmid vector. These products can now be transformed into chemically-competent cells. Chemically-competent *E. coli* cells will be combined with the assembly product, let chill, and then heat-shocked at 42°C. From this point, Super Optimal broth media with Catabolite repression media will be added and then left incubated at 37°C for an hour with vigorous shaking. Cells will then be spread onto selection plates (LB + tetracycline). LB + Ampicillin plates will be used to plate a positive control sample of *E. coli*. Cells that are selected for will then be able to be used in an allelic exchange protocol as the donor strains, as these cells now have the amplified DNA regions that are required for conjugation.

*Bacterial Strains*

Triparental mating requires donor and helper *E. coli* strains to successfully complete this conjugation. The donor strain will contain the desired plasmid that will be transferred into the

wildtype *M. extorquens* strain. The helper strain contains a conjugative plasmid that helps mobilize the desired plasmid into the recipient. This donor strain should be grown in LB + tetracycline while the helper strain should be grown in LB + streptomycin plate to maintain its integrity. The PA1 wildtype strain should be grown in Hypho with 15 mM succinate and incubated at 4°C until good cultures have been acquired. All cultures will then be spun down and resuspended in their appropriate media without any antibiotics. To perform the triparental mating, all three strains will be mixed together on a nutrient agar plate and incubated overnight. Matings will then be added to Hypho liquid media and resuspended before being plated on selective Hypho plates + tetracycline. Transconjugants that arise should be restreaked on tetracycline plates twice for purification. A sucrose counterselection will be performed by streaking on a Hypho plate with 5% sucrose. After incubation, two sucrose resistant isolates should be restreaked for purification. Colony PCR should be performed on a colony from each of the first candidates and patched on a Hypho plate and a tetracycline plate. Good candidates will be sensitive to tetracycline and their PCR will align with expected results. These candidates should then be streaked on MPIPES media with 15 mM succinate. This strain should be saved, restreaked on MPIPES with 15 mM succinate, and colony PCR should be repeated on relevant colonies. This process should be repeated for each of the donor strains created to ultimately create eight mutant bacterial strains, each with a deletion of a MarR homolog.

*Oxidative Stress*

To measure the response of these homologs to oxidation, bacterial strains should be placed in a 96-well plate in a liquid medium of 3.5 mM succinate in MPIPES media at 30°C. Following a similar procedure, as used in *Corynebacterium glutamicum* to test for oxidative stress, mutants can be treated with a dilution of $H_2O_2$ as follows: 1 mM, 5mM, 10 mM, 50mM, 100mM.[4] Growth can be measured over 48 hours. While being incubated with $H_2O_2$ their optical density will be measured at 600 nm periodically to track growth. After being treated, these cultures can be visualized to detect any phenotypic change by plating a serial dilution. This serial dilution can be done by transferring 20 µL of undiluted sample and adding this to 180 µL of fresh liquid media. The serial dilution of 20 µL can be done seven times, reaching a dilution of

10-7. Using a small multichannel pipette, these dilutions can easily be plated onto MPIPES media with 15 mM succinate and incubated at 30°C to later have the colonies counted. The number of colony-forming units (CFUs) can be measured after a 36-hour incubation, along with information regarding cell stress and overall survival. These can be adjusted as needed based on the growth seen on initial assays with quantities described.

*Antibiotic Resistance*

To measure the response of the mutant bacterial strains to different classes of antibiotics, *M. extorquens* can be plated on MPIPES with 15 mM succinate and using antibiotic discs the diameter of clearing can be measured, similarly to how resistance to pine oil was tested on *E.coli*.[3] Antibiotics that target DNA synthesis, cell wall synthesis, and membrane-active compounds should be tested on each of the mutant strains. Antibiotics that will be tested are Ciprofloxacin and Nalidixic Acid which are members of the Fluoroquinolone class targeting DNA synthesis. Antibiotics that target cell wall synthesis belonging to the Penicillin class that will be used are Penicillin G, Penicillin V, and Piperacillin. Nigericin and Monesin will be used as well, as they belong to the Ionophore class of antibiotics which targets membrane-active compounds. Discs can be bought premade or made in the laboratory using a stock solution and untreated discs. A plate containing the wildtype PA1 strain with discs containing each antibiotic will be needed as the control for this growth assay. After placing these discs on plates with streaks of each mutant, a zone of inhibition should be recorded after a 36 hour incubation period at 30°C.

*Degradation of Phenolic Compounds*

To understand whether or not the MarR homologs are associated with the ability to degrade phenolic compounds, a procedure similar to that used in *Acinetobacter* will be used.[1] Mutants should be placed in a 96-well plate in a liquid medium of MPIPES containing 3.5 mM succinate at 30°C. Phenol in concentrations of 0.1, 0.25, 0.5, 0.75, and 1.0 mg/mL should be introduced to the strains using the PA1 wildtype as a control. Growth can be measured over a 48 hour incubation period by taking OD600 measurements. After treatment with phenol, cultures

can be plated on MIPIPES media containing 15 mM succinate after a serial dilution has been performed. This serial dilution will be the same as that for the oxidative stress assay, which is done by transferring 20 µL of undiluted sample and adding this to 180 µL of fresh liquid media. After repeating this seven times, the dilutions can be plated using a small multichannel pipette and incubated at 30°C for 36 hours before counting the CFUs.

**Discussion**

If this phenotypic screen were to be developed in a laboratory, it has the potential to guide further research into the identification of the function of additional MarR homologs in *M. extorquens* and other bacteria. For example, the oxidative stress assay would allow us to visualize any phenotypic changes to MarR deletion strains when placed under varying concentrations of hydrogen peroxide. If all mutants were able to grow unhindered, it would likely indicate that none of the homologs regulate function under oxidative stress. If mutant(s) were to show hindered or no growth when compared to a wild-type strain, it would be an indicator that the deletion of this homolog was related to this phenotypic change and hydrogen peroxide is then able to repress cell growth.

For the antibiotic resistance assay, the results from the control group would have to be considered when interpreting any results. It is currently unknown if the PA1 wildtype strain is resistant or susceptible to the antibiotics used in this experiment. If the wildtype is resistant to a particular antibiotic, any MarR deletion strains that were not able to grow in the presence of that antibiotics would be an indicator that that particular homolog is responsible for the action that was targeted by the class of antibiotic (e.g, DNA synthesis, cell wall synthesis, membrane-active compounds). Contrarily, if the wildtype strain is susceptible to an antibiotic, but the MarR deletion strain shows resistance, it would indicate that the protein is related to this susceptibility and may be involved in repressing the resistance of this bacteria to this class of antibiotics. From both perspectives, this assay is useful in beginning to understand the role of the MarR protein.

The degradation of phenolic compounds assay could similarly provide two different results. If the wildtype strain is resistant to the toxicity of phenol and a homolog is unable to grow in the same condition, it is an indicator that the homolog may play a role in the degradation

process. Alternatively, it can be hypothesized that the MarR protein acts as a repressor for this degradation process if the wildtype strain does not grow in the presence of these aromatic compounds, but a mutant deletion does grow. It can indicate the removal of this homolog causes this increased resistance. In each of these phenotypic screens, it can be assumed that if the mutant strains and the wildtype strain show similar results then the function of the homolog was not tested for and further screens are required. As the function of many MarR proteins is unknown, these growth assays can provide information to support further research in varying bacteria.

**References**

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