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Expressing the β -glucosidase gene as a reporter in the hyperthermophilic archaeon *Pyrococcus furiosus*

by

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Introduction

One of the most common ways to track cellular activity in any organism is with a reporter gene, a sequence of DNA that can be inserted into a cell and provide information about gene expression within that cell. The organism I am studying, *Pyrococcus furiosus*, currently does not have any existing reporter genes developed for it. Due to the extreme temperatures and anoxic environment this organism lives in, it can be difficult to develop tools to study gene expression⁴. A promising reporter gene for *P. furiosus* is β -glucosidase, an enzyme that cleaves glucose monomers from polysaccharides and is naturally encoded by the organism¹. The goal of this study is to add the β -glucosidase gene to *P. furiosus* through a plasmid vector and determine its viability for use as a reporter gene by growth on media that takes advantage of the hydrolytic activity to enable tracking of gene expression levels. If glucosidase activity is high and consistent in *P. furiosus*, it is an indicator the β -glucosidase gene can be used as a reporter. Therefore, my research question is: Is β -glucosidase activity in *P. furiosus* high enough to be detected consistently? If so, can it be used as a reporter gene in *P. furiosus*? This tool would allow gene regulation mechanisms of *P. furiosus* to be studied more easily *in vivo* (live cells), and has the potential to be used to track levels of gene expression throughout the genome.

Background

P. furiosus was found and isolated from a very hot and anoxic environment at a marine hydrothermal vent. Its optimum growth temperature of around 95-100°C and lack of oxygen required for growth make it difficult to conduct genetic assays because most tools designed to carry out these types of experiments have been developed for bacteria and yeast, both of which are (largely) aerobic and grow at much lower temperatures⁴. The fact that β -glucosidase is a gene already encoded by *P. furiosus* naturally means that it has already evolved to be genetically

active at high temperatures and without oxygen. This combined with the fact that its hydrolytic activity can be tracked using specific media makes it a good candidate for use as a reporter gene. Several experiments demonstrate that β -glucosidase can be successfully used as a reporter gene in thermophilic archaea.

This notion is further supported by Santangelo et al., in which a reporter gene system was developed using a β -glucosidase gene from a thermophilic archaeon, *Thermococcus kodakarensis*, which is in the same phylogenetic order as *P. furiosus*⁸. They then tested this system on several ONP-based media to identify which gave the highest hydrolytic activity and found it was ortho-nitrophenyl- β -D-glucopyranoside⁷. Based on the results of this study ONP-gluco media will be used when testing β -glucosidase activity in *P. furiosus*. Another study conducted by Jonuscheit et al. successfully developed a β -galactosidase, which is very similar to β -glucosidase, reporter gene system for the thermophilic archaeon *Sulfolobus solfataricus*³. Though more distantly related to *P. furiosus* than *T. kodakarensis*, this study further demonstrates the viability of these kinds of hydrolytic enzymes for use as reporter genes in thermophilic archaea.

Relevance

Our lab studies the transcription machinery of *P. furiosus*. Transcription is the process by which a cell copies its DNA into RNA. The transcribed RNA may then be translated to make proteins, which serve essential cellular functions. A reporter gene for our organism is important because it will allow monitoring of transcriptional activity in a way that isn't possible without one. For example the reporter could be attached to promoter sequence, a short sequence of DNA where transcription starts, to see how actively the promoter is being transcribed.

Proteins and DNA sequences involved in transcription in the archaea are very similar to those used in eukaryotic transcription, so monitoring transcription function in archaea can also provide information about how our own transcription machinery operates⁹. Transcriptional systems of archaea also may be more similar to ancestral transcription systems than either bacteria or eukaryotes, so studying archaea could give further insight into how ancient cells operated⁹. Hyperthermophilic archaea are also of industrial interest because they have the potential to produce hydrogen gas to be used as a clean, renewable energy source¹⁰. The extremely hot and typically oxygen deficient environment of hyperthermophiles also make them good examples to study how life might survive in the extreme environments found on other planets. A specific, more realistic example comes from Groom, who describes the usefulness of a thermophilic β -glucosidase gene in assessing the viability for biofuel production in knockout mutants of several cellulose-degrading bacterial species¹¹.

Research

The objective of this experiment is to determine the feasibility of using β -glucosidase as a reporter gene in the hyperthermophile *Pyrococcus furiosus*. The research necessary to attain this goal begins with identifying a way to express the β -glucosidase gene at high levels in *P. furiosus*. This was carried out using a plasmid containing the β -glucosidase gene. This plasmid would be added to *P. furiosus*, which is taken up naturally by our highly competent (takes up foreign DNA well) strain of *P. furiosus*, COM1⁵. These cells would then be grown on selective media to isolate transformants (cells that received the plasmid), which will be tested for expression of the gene.

A plasmid is a circular genetic element that can self-replicate independently of the rest of its host's genome. Genes can be inserted into a plasmid and can be replicated using the plasmid's

replication machinery. We are using a plasmid called pYS4 because it is specifically designed for genetic manipulation of *P. furiosus*². Even though β -glucosidase is a native gene, it is to be added to a strain of *P. furiosus* that has the β -glucosidase gene knocked out. The plasmid will introduce a form of the gene under control of a strong promoter (slpP) that will increase expression and therefore activity of the gene. The plasmid will also contain a gene known as a selectable marker, that allows only *P. furiosus* cells that acquired the plasmid to grow on specific media.

In order to select for the transformants, a selectable marker is included in the plasmid, allowing growth of only the transformants under certain conditions. Our selectable marker, *pyrF*, is involved in production of pyrimidines (nucleotides found in DNA/RNA). *pyrF* is essential for cell growth when the medium is not supplemented with the pyrimidine uracil^{5,6}. The host cells lack *pyrF* (it was intentionally deleted) so when grown in medium lacking the pyrimidine uracil, only the transformants that get the *pyrF* gene will grow. The plasmid was re-engineered to contain the *pyrF* gene, since the selectable marker it had previously (the gene HMG CoA reductase) was found to be unreliable^{2,4}.

The first step in plasmid engineering was to perform PCR (a DNA amplifying technique) to make the fragments of the plasmid, including the new selectable marker. The plasmid was then assembled through Gibson assembly; the process of annealing DNA fragments together and sealing with DNA polymerase and DNA ligase. Following assembly, the laboratory bacterium *E. coli* (strain XL-1 Blue) was transformed and grown on media containing ampicillin, an antibiotic, to identify successfully assembled plasmids because an ampicillin resistance gene is integrated into the plasmid². Two negative controls and one positive control were utilized. One negative control contained *E. coli* cells only and no plasmid DNA, so no selectable marker

therefore no growth, and the other contained *E. coli* cells with the backbone fragment only, which should not allow growth in the presence of ampicillin. The positive control contained *E. coli* cells and a pUC19 plasmid, which has a high copy number and should transform the cells very efficiently. The colonies that grow are potentially re-engineered to include the pyrF gene instead of the previous selectable marker, and were screened for correct assembly by PCR. The plasmid is to be sequenced by Sanger sequencing to verify proper construction.

Anoxic media would then be inoculated containing living *P. furiosus* with the re-engineered plasmids that contain the β -glucosidase gene. Only the *P. furiosus* cells that acquired the plasmids should grow because only they will have the pyrF gene. To check for successful expression of the β -glucosidase gene, the cells will be isolated, lysed and exposed to ONPG. ONPG is a substrate for β -glucosidase and changes color to yellow when glucosidase activity is present, and the level of this activity can be assessed by measuring the absorbance at 405 nm wavelength^{7,8}. Absorbance is to be measured using a spectrophotometer.

If β -glucosidase activity is observed, the next step would be to attempt using it as a reporter in *P. furiosus* by attaching it to both a promoter sequence and the same one with specific mutations to compare how actively genes are transcribed relative to each other. However, if hydrolytic activity is not observed for cells that contain the β -glucosidase expression plasmids, the next step would be to either attempt re-engineering of the plasmid to optimize it for expression, or move on to another reporter gene for *P. furiosus*.

Results

Two steps of the experiment were accomplished. The first is seen in figures 1 and 2 below; the fragments of the plasmid were amplified by PCR. Two fragments were utilized for maximum efficiency of assembly; the backbone of the plasmid containing the gene for ampicillin resistance, the *pyrF* selectable marker, the *slp* promoter, and other components required for replication in *P. furiosus* (see figure 3). The backbone fragment was expected to be 6465 base pairs long and the β -glucosidase gene was expected to be 1449 base pairs long. The agarose gels run for each of these PCRs confirmed the correct size of the fragments. The other step of the experiment that was accomplished was the Gibson assembly. The plates containing the β -glucosidase/backbone plasmid assembly grew less than the positive control (both too many to count), the cells-only negative control didn't grow at all, and the backbone-only negative control had about 10 colonies (likely due to ampicillin breakdown). These results are consistent with a successful Gibson assembly. The colonies have yet to be screened by PCR, and Sanger sequencing still needs to be performed before transformation of *P. furiosus*.

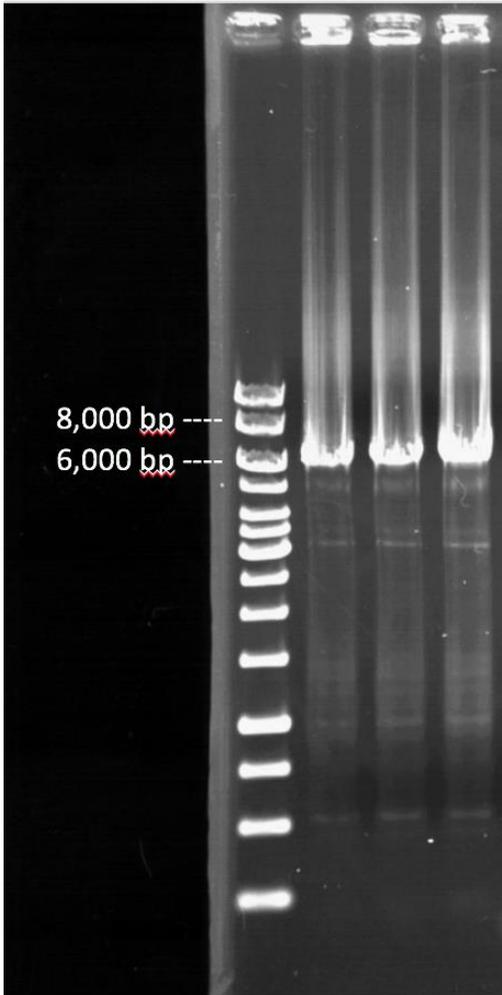


Figure 1: Agarose gel demonstrating size of backbone DNA fragment used in Gibson assembly of plasmid.

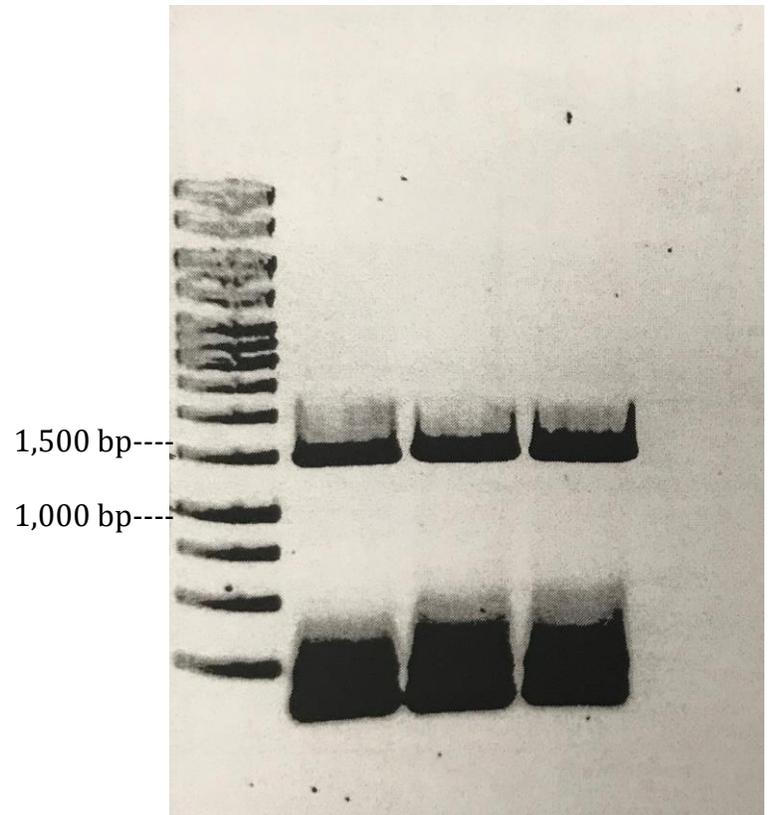


Figure 2: Agarose gel demonstrating size of β -glucosidase DNA fragment used in Gibson assembly of plasmid.

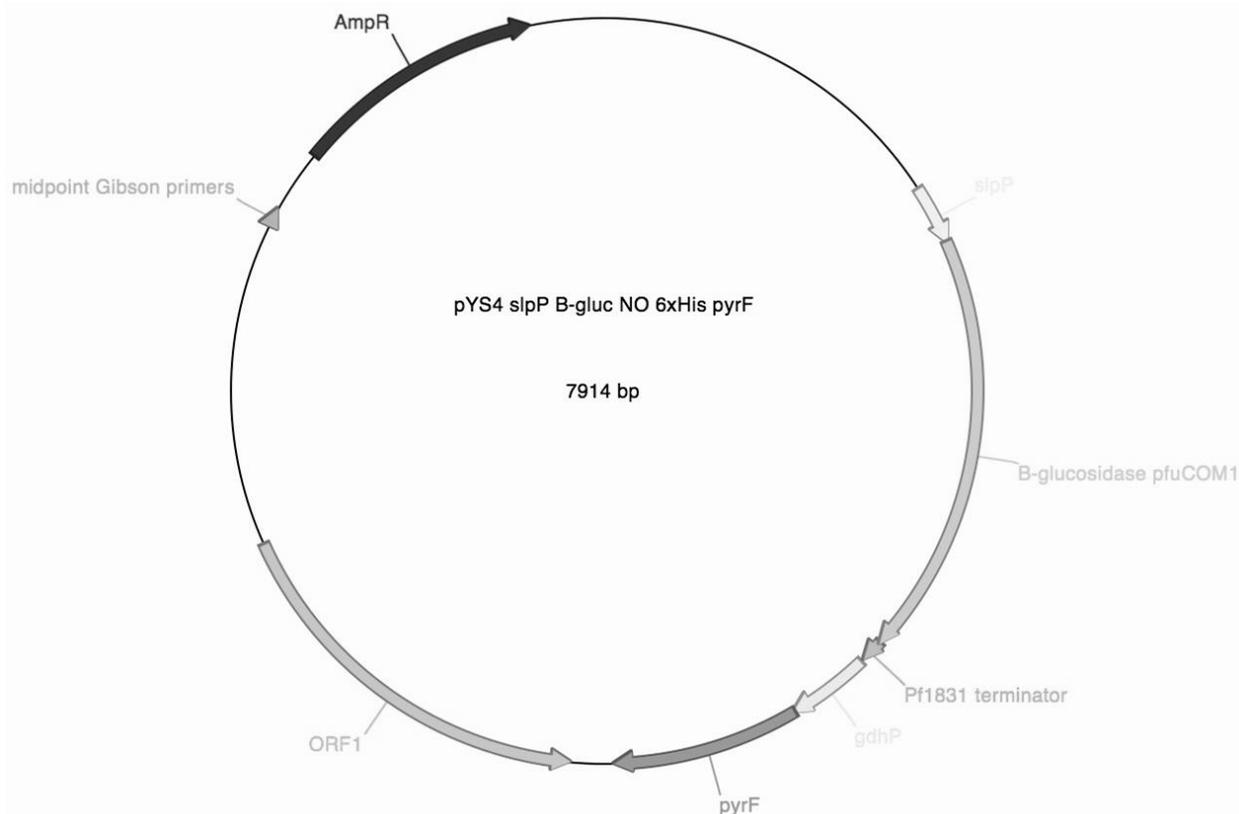


Figure 3: Graphical representation of constructed plasmid with labelled components

Discussion

This experiment did not accomplish its main goal of assessing the viability of β -glucosidase as a reporter gene in *P. furiosus*. Many obstacles presented themselves along the course of the experiment. The first was a problem with the original selectable marker being used in the plasmid, simvastatin resistance. Simvastatin is an antibiotic, so a gene that encodes resistance to it was to be used as a selectable marker after transforming *P. furiosus*. The problem was that simvastatin is sensitive to the high temperatures required for growth. This did not allow for selection of cells that contained the β -glucosidase gene because lack of simvastatin likely allowed cells that hadn't received the selectable marker to grow. This required re-engineering of the plasmid to replace the simvastatin selectable marker with the gene pyrF.

Problems then presented themselves when trying to create PCR fragments of the plasmid. At first attempts were made to amplify the entire backbone and β -glucosidase gene, but after several attempts there was no success. It was then decided to use several smaller fragments in hopes of increasing the success of the PCR since longer fragments tend to be harder to amplify. This approach seemed to work but when the PCR product was purified it gave odd quantitation curves. However, these fragments were used in a Gibson assembly experiment to test if enough DNA was present to assemble properly. When screening the Gibson assembly, primers for the slp promoter (upstream junction of the β -glucosidase gene, figure 3) were used and showed that it was successful. After receiving the Sanger sequencing data, it showed two things were wrong: there were several single base pair mutations in the β -glucosidase gene and there was an issue with the downstream junction where β -glucosidase meets pyrF (see figure 3). The single base pair mutations were found to be due to the fact that the wild type *P. furiosus* β -glucosidase gene sequence was being used as a reference instead of the COM1 version. The issue with the junction was likely because the fragment containing the pyrF gene was not integrated in the assembly but the one containing the slp promoter was, likely due to low DNA concentration or contaminated DNA.

It was then decided to take a step back and optimize the PCR for more reliable DNA fragments to use in the assembly. Previously the PCR program used a 2°C touchdown annealing temperature from 56-52°C, so a gradient PCR was set up with differing annealing temperatures ranging from 48-56°C. It was determined that a constant 54°C was the optimum annealing temperature. This allowed us to amplify the full, long backbone fragment instead of breaking it into several pieces, which should introduce less variables and therefore prevent false

confirmation of assembly in colony PCR as happened previously. This fragment along with the β -glucosidase gene were used in the latest Gibson assembly.

Though this experiment is still in progress, there may be an even bigger issue with the plasmid that was recently discussed in our lab. It contains a pUC19 origin of replication, which is a high copy number plasmid. Our plasmid backbone is about 6500 base pairs, which is fairly large. This puts a lot of stress on *E. coli* cells that are forced to uptake this foreign DNA, and this stress is amplified by the high copy number it maintains due to the pUC19 replication origin. Replacing the replication origin with one from a low copy number plasmid may enable more successful transformation of *E. coli* with this particular plasmid.

Conclusion

While *P. furiosus* was not transformed with β -glucosidase and its viability as a reporter gene wasn't able to be tested, this project has demonstrated an important principle: the uncertainty of biology. While one may think they understand what is going on in a particular experiment and everything is laid out in a way that should theoretically work, it is not uncommon that the actual results differ from expectation, since there are so many variables that need to be controlled. Working in a biology lab, and any science lab I'm sure, takes patience and faith in the work you do, but also an open mind to recalibrate your thoughts when things don't go as expected so you can approach the issue from a different angle. This opportunity has taught me all of these things and even though I wasn't able to accomplish the initial goal of the experiment, I feel like I learned so much more than just the biological knowledge this project required and am thankful for that.

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