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Using Polymerase Chain Reaction to Detect nifH and vnfDGK in a Novel Acidophilic Microbial Community

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Using Polymerase Chain Reaction to Detect *nifH* and *vnfDGK* in a Novel Acidophilic Microbial Community

by

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ABSTRACT

Acidophiles are widely known for their ability to exploit harsh environments, typically consisting of high temperatures, dissolved metal concentrations, and acidic conditions. Consequently, different acidophilic species are employed industrially in applications such as biomining and bioleaching. However, their full potential in bioremediation, and in industrial practices, has yet to be fully explored because many of these acidophiles are difficult to culture in laboratory conditions or have yet to be properly identified. This investigation focuses on a novel community sampled from the Richmond Mine site in Iron Mountain, California, consisting of a filamentous fungus and prokaryotes. This consortium can be cultured in laboratory conditions without the presence of organic nitrogen sources. Previous data has also revealed that the community has a nutritional requirement for vanadium ions. Therefore, I predicted that this bacterium is able to fix atmospheric nitrogen via the nitrogenase enzyme, and might do so using a rare variant that requires vanadium as a cofactor. My investigation will employ PCR amplification, ligation, and transformation as well as bioinformatics tools in attempts to isolate conserved *nifH* and *vnfDGK* gene fragments, that are found in standard nitrogenases and vanadium-dependent nitrogenases, respectively. Results from this investigation can be used not only to further understand the dynamics of the community found in these mine waters, but also to shed light on the process of nitrogen fixation in such a harsh environment.

INTRODUCTION

Site Description

Richmond Mine, located in the Shasta Valley region of Northern California, was once mined for its rich deposits of copper, gold, silver, and pyrite beginning in the late 1800's to the early 1960's, after which the mine was abandoned (Nordstrom and Alpers, 1999). The exposed ore at the abandoned mine creates runoff that is highly acidic and laden with dissolved metals, which leads to pollution of the local rivers and a decline in populations of aquatic species. Investigations into the mine began in the early 1980's with its addition to the National Regulation List under CERCLA regulations. The mine was also listed as a federal Superfund site by the Environmental Protection Agency in 1986. Upon investigation into the Richmond Mine complex, researchers found mine waters in some areas reaching an acidity of pH = -3.6 (the most acidic waters found anywhere in the environment), with sulfate concentrations as high as 760 g/L and specific metal concentrations being some of the highest ever reported (Nordstrom and Alpers 1999; Nordstrom et al. 2000). Despite these seemingly harsh conditions, a myriad of bacteria, archaea, and other eukaryotes were found within these waters, and have become the focus of many studies attempting to identify these organisms as well as their metabolic roles within this community (Baker et al. 2003; Edwards et al. 1999; Tyson et al. 2004).

Microbial roles in acid mine drainage

Acid mine drainage is largely controlled by specific microbes (Dopson et al. 2003; Singer and Stumm 1970). Iron sulfate (FeS_2), also known as pyrite, is easily

oxidized into sulfuric acid when it is exposed to environmental conditions via the following reaction:

$$FeS_2 + 14\ Fe^{3+} + 8\ H_2O \rightarrow 15\ Fe^{2+} + 2\ SO_4^{2-} + 16\ H^+$$

One would think this reaction would eventually come to equilibrium; however, many acidophilic microbes are able to accelerate the rate of pyrite dissolution by obtaining energy via the oxidation of Fe^{2+} to Fe^{3+}, thus driving the reaction forward (Edwards et al. 2000). The biogeochemical process mediated by microbial communities such as the one isolated for this study is believed to be the primary cause for the continual efflux of highly contaminating water from the mine site. Attempts to remediate the polluting effects of this site could, therefore, target the microbial communities.

Nitrogen Fixation

Nitrogen is an essential element for life and also the most abundant element in the atmosphere. It is a primary component of DNA, the building block of life, and is also present in all proteins. All eukaryotes and many prokaryotes are unable to use atmospheric nitrogen gas, however, and instead must use fixed forms of nitrogen such as ammonia and nitrate (Madigan et al. 2009). Certain bacteria and archaea, known as diazotrophs, have been able to accomplish this feat with help of an enzyme complex called nitrogenase. This complex is able to lower the activation energy required to break the dinitrogen triple bond into ammonium ions (NH_3), using 16 ATP per molecule of N_2, in a process known as nitrogen fixation (Canfield et al. 2010). The complex consists of two proteins: dinitrogenase reductase, which serves as the electron donor, and dinitrogenase, which reduces nitrogen into ammonia and one hydrogen atom at an active site containing an iron-metal core. The most common form of nitrogenase complex

operates using a dinitrogenase reductase enzyme encoded by *nifH* and a molybdenum-iron dinitrogenase protein (MoFe protein), encoded by a suite of genes collectively known as *nifDGK* (Hoffman et al. 2009; Oda et al. 2005). In some cases, particular diazotrophs can contain additional genes encoding alternative nitrogenases that can operate using iron (*anf)* or vanadium (*vnf)* in place of molybdenum when it is absent (Lee et al. 2009). The resulting iron-metal cofactor is the site of reduction from N_2 to NH_3 (Glazer and Kechris 2009; Rubio and Ludden 2005).

Description of study organisms

A group of unknown organisms composing of a filamentous fungus and some prokaryotes has been isolated from the waters of the Richmond Mine, and this group is the focus of my research. These acidophiles can be cultured in laboratory conditions without the addition of organic nitrogen sources. Unpublished work from Dr. Hung's lab has revealed that the growth of this community is enhanced by the presence of vanadium. This unclassified group, while adapted to pH ~2, is able to grow in pH 8 as well. From these observations, it was hypothesized that one or more of the members within this acidophilic community are capable of performing nitrogen fixation, and may further utilize the vanadium-dependent nitrogenase to do so. By using degenrate primers capable of identifying conserved regions of nitrogenase genes, PCR, cloning, and transformation were used in an attempt to amplify these genes.

METHODS

Samples of the symbiotic community were grown in a 0.1% (w/v) glucose solution (deionized water treated with reverse osmosis) adjusted to pH 2 with sulfuric

acid. Extraction of the DNA from the bacterium was performed using Zymo Research Soil Microbe DNA kit, according to supplied protocol. Using the Polymerase Chain Reaction (PCR), specified fragments within the DNA were selected and copied using primers, which flank each end of a region of interest. Universal degenerate primers described by Burgmann *et al.* (2004) were ordered and used to detect a conserved region 410 base pairs in size within the nitrogenase gene *nifH* (Burgmann et al. 2004). Samples for PCR were mixed in 25 ìl aliquots in the following way: 5 ìl 5x GoTaq Buffer, 14.3 ìl H_2O, 0.5 ìl Taq Polymerase, 2 ìl of 10 mM $MgCl_2$, 0.2 ìl of 25 mM dNTP, 1 ìl of 100 ìM forward primer, 1 ìl of 100 ìM reverse primer, and 1 ìl extracted DNA. The primers and enzymes were tested via PCR to determine optimal annealing temperatures. Expected results were obtained using *nifH*g1F and *nifH*g1R primers under the following cycle conditions: initial melting for 4 minutes at 95°C, followed by repeating 35 cycles of 20 seconds at 95°C, annealing time 15 seconds at 53°C, and extension at 72°C for 40 seconds, ending with a final extension time of 5 minutes at 72°C. Following amplification, I used agarose gel electrophoresis to determine the size of the amplified fragment. The gel consisted of 0.8% agarose in a 10x TBE buffer, and electrophoresis was performed at 85 volts for 90 minutes for optimal sizing of putative fragments.

After repeated rounds of PCR to amplify the product, the fragment was sliced form the gel using a razor blade, and treated with Promega Wizard and SV Gel Cleanup System according to supplied protocol. Following, the fragment was cloned into a plasmid vector with the Promega pGEM-T Easy Vector Kit and protocol. The plasmid vector contained NotI restriction sites as well as ampicillin resistance for positive selection of *E.coli.* The protocol was adapted from the Promega kit, modified the

following way: 10 ìl 2x rapid ligation buffer, 1 ìl pGEM-T Easy plasmid vector (50ng), 8 ìl PCR product, 1 ìl T4 DNA ligase for a total volume of 20 ìl. Competent *E. coli* JM-109 bacterial cells were then transformed to take up the plasmid following Promega protocol with 20 ìl of plasmid used per 100 ìl JM-109 cells. The cells were then transferred to 900 ìl of Luria-Bertani (LB) broth and incubated with shaking at 37°C for 1.5 hours. Following this, a volume of 100 ìl transformed cells were plated onto 1.5% agar plates containing LB, Ampicillin, X-Gal, and IPTG for selection of *E. coli* with the plasmid insert containing the cloned fragment.

The cells were screened for uptake of the plasmid containing an insert. These white colonies were then treated with the Promega PureYield Plasmid Miniprep System to elute the inserted gene region from the plasmid adhering to the Promega protocol for larger culture volumes. A digestion with the NotI enzyme and PCR amplification of the DNA was then performed to determine if the correct gene region had been inserted. For the digestion, the following protocol was used: 8 ìl plasmid, 1 ìl 10x buffer, 1 ìl 10x BSA, 9.9 ìl H_2O, and 0.1 ìl NotI for a total volume of 20 ìl. If the fragment correctly matched the appropriate size outlined by Burgmann et al. 2004, this would indicate that the organism is capable of nitrogen fixation and will be sent off to the UIUC Biotechnology Center for genetic sequencing.

A similar method was executed using a different primer set used to detect a highly conserved region of 723 bp across three genes, *vnfDGK,* which codes for the vanadium-dependent nitrogenase protein (Betancourt et al. 2008; Loveless and Bishop 1999). The only PCR protocol changes made from the *nifH* protocol included the use of a high-fidelity polymerase for PCR in place of the Promega Taq Polymerase, which will ensure

accuracy in copying of the DNA fragment. Annealing temperatures were tested in the range of 48 - 58°C.

RESULTS

Using the primer set *nifH-g1,* I was able to amplify a fragment that appeared to be similar in size to the *nifH* gene (Figure 1). The bands were amplified to obtain a higher yield of fragments, before being cut from the gel. This excised fragment was then cleaned, before being cloned and transformed into a plasmid within a bacterial cell (Figure 2). Repeated experiments yielded a substantially low proportion of bacterial colonies that passed the IPTG/X-Gal screening and were able to uptake the plasmid containing the cloned fragment, with only 149 out of 1470 colonies passing the screening. Subsequent PCR of the products obtained from digestion of the fragment from the extracted plasmid showed the presence of the possible *nifH* gene region, but also a perplexing 1.5 kb band that was not inserted into the plasmid (Figure 3). Troubleshooting the cloning step via a blunt-end cloning kit and shrimp phosphotase yielded the same results. Despite this, the fragment at approximately 410 bp, although slightly larger than the expected size of *nifH* was sent off for sequencing at the UIUC Biotechnology Center.

Rigorous testing of the *vnfDGK* primers over a wide range of annealing temperatures yielded no amplifications (Figure 4). Therefore, I conclude that this organism does not contain the genes necessary for the vanadium-dependent nitrogenase.

DISCUSSION

Although the *nifH* primers amplified putative bands that fit the profile of the conserved *nifH* region, the identity of the 1.5 kb bands present after attempts at removing the fragment from the pGEM-T plasmid vector remains a mystery. In many trials, these bands also visually appeared to be at a greater density than the 410 bp bands of interest. Because the *nifH* bands were physically excised from the gel before cloning and transformation, I ruled out the possibility of sample contamination of PCR products. Using an alternative method of cloning the fragment into the vector also produced no changes in results. The digestion enzymes used for excision of the fragment from the plasmid, NotI and EcoRI, showed two cut sites on the pGEM-T plasmid map that flank the insertion region, and each was digested with the amplified fragment only to ensure there were no cut sites within the fragment (data not shown). Therefore, there is a low likelihood of fragments annealing together before insertion, or presence of an extra cut site within the plasmid that would cut the 3 kb plasmid into two 1.5 kb regions. The blue/white ratio of transformed cells was also low, with the success rate being <10%. Perhaps even more perplexing was when colonies that failed selection were digested as negative controls, I still observed the 410 bp fragment as well as the 1.5 kb band. Repeated transformations using varying concentrations of competent cells yielded similar low figures. I suspect that these two problematic steps may be interrelated, but only further troubleshooting methods will be able to make a definite conclusion. In addition, further confirmation of the negative results is needed to obtain confidence that the failure to amplify the relevant *vnfDGK* fragments indicate an absence of the DNA and not a result og technical errors.

Because of the inability to successfully amplify a conserved *nifH* fragment and a conserved *vnfDGK* fragment, my current hypothesis that one of the members in this symbiotic community may be performing nitrogen fixation is not supported. It is possible the symbiotic community may not be able to perform nitrogen fixation. If this is indeed the case, I speculate that their ability to grow in the absence of added organic nitrogen sources could be due to a high capacity to scavenge trace levels of nitrogen in the reverse osmosis-treated deionized water. Alternatively, the consortium may be capable of performing nitrogen fixation, but the relevant genes cannot be amplified. Several factors may cause this result. Because the bacterium of interest is part of a symbiotic relationship with a fungus, DNA preparation of the sample may have resulted in a higher concentration of fungal DNA leading to a dilution of bacterial DNA and therefore a low representation of the genes of interest. The primer pairs used (*nifH* and *vnfDGK)* are very degenerate in order to be able to amplify nitrogenases from a wide spectrum of bacterial groups. This decreased specificity could have led to nonspecific bindings with another gene region similar in size to *nifH* , or they were just not specific enough to bind to the conserved region of the DNA. It is also possible, although unlikely, that the failure to amplify the relevant nitrogen fixation genes of this novel symbiotic bacteria is because they utilize a heretofore unknown set of genes.

Implications of research

The results of this research have a wide variety of implications. Insights into the nutrient flow throughout the community as well as the metabolism of the species found within the Richmond Mine could help further understanding of the roles and mechanisms of acidophiles, as well as eventually leading to remediation techniques of this Superfund

site. If this bacterial symbiont is confirmed to have the ability to fix nitrogen using vanadium co-factor, results could also shed light on one of the more uncommon forms of nitrogen fixation. Because the bacterial member of this symbiotic association has yet to be classified, this information could also be used to infer the relationship of this microbe to other organisms which share this suite of genes using bioinformatics tools such as BLAST.

FIGURES

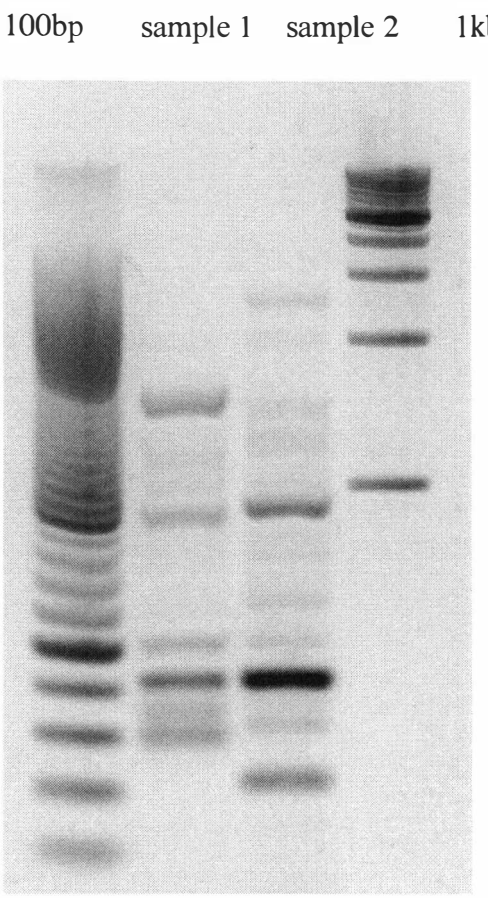

Figure 1. PCR results using extracted DNA and *nifH-g1* primer sets with annealing temperature at 53 °C. The lane containing sample 2 shows a dark band; sizing it with the 100 base pair ladder estimates the DNA fragment to be around 420 bp in length, approximately the same size as the expected fragment of *nifH*. Other bands within the lane are results of nonspecific bindings of the primer pair.

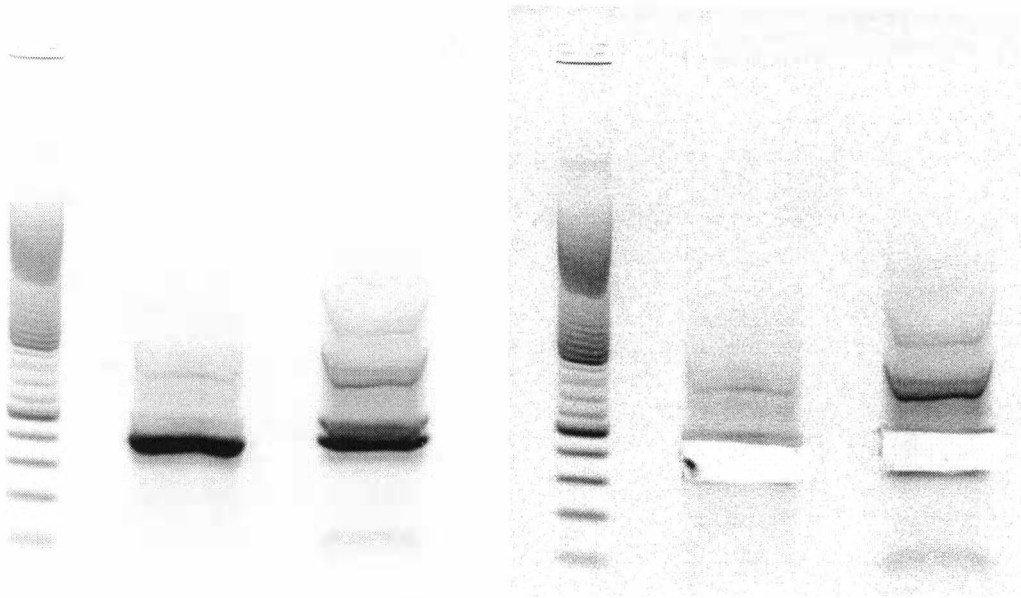

Figure 2. Excision of the suspected *nifH* fragment from the electrophoresis gel. These photos show the gel before (left) and after (right) the gel slice was removed using a razor blade for purification using the Promega Wizard and SV Gel Cleanup System.

1 – d1f + d5r
2 – d2f + d5r
3 – d3f + d5r
4 – d4f + d5r
+ - nifH-g1

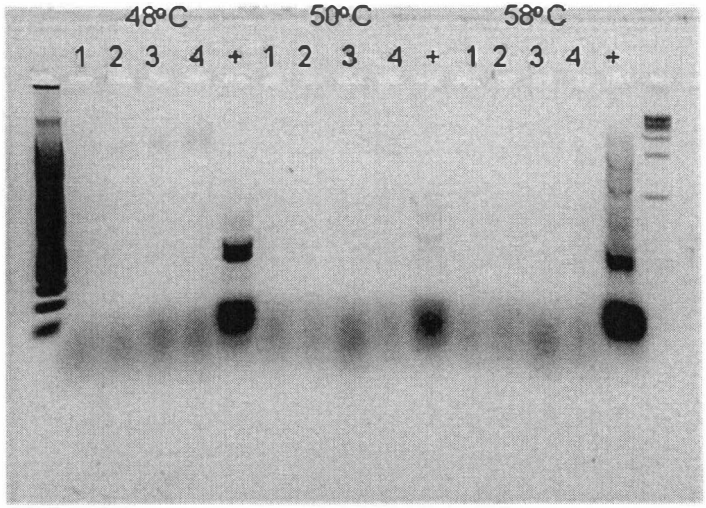

Figure 4. Results of PCR using *vnfDGK* primers. Here, 4 different *vnfDGK* primers were tested at 3 annealing temperatures. Fogginess near the bottom of the gel suggests that the reaction was functional, but there were no fragments amplified. Reaction indicated by a + represent PCR reactions using the nifH-g primer pairs as positive control to show that DNA and reagents are present.

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Summary of Samantha Flower's Honor's Independent Study Experience

Samantha carried out a series of exercise using bioinformatics for her independent study. Using the material she learned from the BIO3960 course, Samantha performed the following tasks for the genome of the organism "*Ferroplasma acidarmanus*" strain fer1:

1. Annotation of new genes in the organism using BLAST, InterPro Scan, T-COFFEE, and other bioinformatics tools.
2. Examination of the histidine biosynthesis pathway in this organism, using KEGG pathway tools, BLAST, InterPro Scan, and other bioinformatics tools.

For her first task, Sam used tools to identify whether the gene in the organism fer1 matches other previously identified genes. Using bioinformatics analyses, she assayed the likelihood of the gene being identified before and offer a conclusion.

For her second task, Samantha used similar techniques, but this time, she applied it to 10 genes in a single biochemical pathway. Samantha examined the 10 genes in the pathway to determine whether these predictions are accurate or not. In her work, she identified one gene in the pathway that is known to be involved in histidine biosynthesis, but which is predicted to be absent in our organism.

Samantha successfully completed both tasks for her independent study.

Sincerely,

Narrative of Honor's Independent Study Experience

Samantha Flowers

My independent study took place in Fall 2010 with Dr. Kai Hung. This study consisted of using bioinformatics tools in order to study and compare genes, their pathways, and their functions. Bioinformatics is the use of computer software and algorithms to properly analyse, predict, and organise complex biological data. In a growing age of molecular biology and the enormous amount of information gathered so far on complex genes and proteins, the use of computer software to help organise and understand this data has become essential. Many bioinformatics tools and corresponding databases are open-source, which allows scientists from around the world to upload, edit, and view this information. This fast-growing field has shown great promise in allowing scientists to see the big picture within molecular data, and will be an incredibly helpful and ubiquitous tool to scientists within this field as time progresses.

For this study, I undertook a bioinformatical approach to study select genes within the acidophilic archaeon "*Ferroplasma acidarmanus*" strain fer1. To begin, I used a variety of bioinformatics tools (including BLAST, T-COFFEE, InterPro Scan, P-sort, ExPASy) to annotate unknown genes within the organism. Using the information gleaned from these tools, I gathered information about the gene and its corresponding protein, as well as its similarity to genes found in other organisms within an open-source database. I was then able to gather conclusions about the identity of these gene and predict its functions. Following this, I then analyzed the histidine biosynthesis pathway of this organism using the KEGG database, as well as the bioinformatics tools mentioned above to annotate the 10 genes that compose this pathway. From this, I was able to confirm the predictions put forth by different programs were accurate and in some cases update the information found in these databases. Interestingly, from this study I identified one gene that is involved in protein synthesis but was not predicted to be found within *Ferroplasma acidarmanus* or any other closely related species. This strange finding is currently being investigated by a graduate student of Dr. Hung's in the laboratory to see if the histidine pathway is functional in this organism and if so, it is utilizing the gene that was predicted to be absent. A third aspect of this independent study was using MAUVE software to compare three different strains of *Yersinia pestis,* a bacteria famously known for its involvement in various human plagues, and compare their genomes and the genes involved in conferring pathogenicity.

Through this study, I came to realize the importance of bioinformatics and how vital it is to understanding and organizing of molecular data. Through using various programs I was

able to do a wide variety of statistical analysis using gene sequences, from predicting protein structures to gene functions to comparing sequences against an entire database of organisms to analyzing genes responsible in entire biochemical pathways. Bioinformatics is a fast growing field whose importance and open-source nature is vital to understanding the big picture and organizing a complete database of genomes that are available for analysis by any scientist wishing to find information. By simply analyzing published data on the histidine pathway, I stumbled upon a question concerning one of the genes within the pathway that is able to be answered using laboratory experiments. I feel like through this work I was able to get a glimpse of this big picture, and in the process learned to how to use a variety of tools to help with my future endeavors as a molecular biologist.