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HONORS THESIS

Microbial Communities of the Providence River

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ABSTRACT

The Providence River has been industrialized for over one-hundred years. Industries such as oil storage and metal recycling facilities have left high levels of pollutant metals, including lead (Pb), in the soil and water. The elevated Pb concentrations in these environments influence the selection of Pb resistance mechanisms in the bacterial community[1]. One mechanism of heavy metal resistance is the Pb efflux pump, consisting of proteins in the cell membrane that aid in the transport of Pb out of the cell[2]. In this study we investigated the co-occurrence of Pb efflux pumps and antibiotic efflux pumps in bacteria from Pb contaminated sediments. By using PCR techniques to identify Pb-resistant genes and sequencing bacteria to identify specific species with these genes, we are attempting to identify the types of bacterial species present and whether the same mechanism (encoded by similar genes) are responsible for both antibiotic and trace metal resistance. This helps us establish a genetic link between Pb resistance and antibiotic resistance.

INTRODUCTION

The Providence River has been industrialized for over a century. In the area there are metal recycling facilities, oil storage, and in the past railway yards, coal storage and manufactured gas plants. Activities at these sites has resulted in widespread pollution of the area including elevated concentration of heavy metals in the sediments and groundwater. The high levels of the toxic metals in the environment affects the micro-organisms. In order to survive, these organisms evolve mechanisms to remove the toxins preventing them from harming the cells. Some of these mechanisms are efflux pumps, which originated as protein channels imbedded in the cell membrane to allow for transport into and out of the cell. These channels can be modified in order to remove the metals from the inside of the cell before they can cause damage. Some of these modifications to the genes might also contribute to antibiotic resistance from sources such as the industrialization in the Providence River[1].

Bacteria are becoming resistant to antibiotics that are used in treatment[2]. This resistance is from the organism being able to attenuate the activity of the drug. This can be done in several ways such as modifying the drug so that it is less toxic to the organism, or mutation of the target of the drug so that it is ineffective[2]. Some studies notice a link between antibiotic resistances and trace metal resistance from areas with elevated metal levels[1]. These studies have looked at different marine environment with pollution and look for different types of antibiotics. These studies have been successful and do show that there is a link between pollution and antibiotic resistance. Genes that encode for antibiotic resistance have been identified in both and may correspond to the resistance in the bacteria[1, 3, 4.]

With similar mechanisms used to in both antibiotic resistance and methods used to survive in toxic environments we are studying the link between the two. The sites used in the study have pollution the areas along the shoreline with toxic metals such as chromium, arsenic, copper, silver, lead and zinc. In small concentrations the metals do not have many ill effects on biological organisms, however when they become concentrated they become toxic to the organism. At high concentrations the metals create molecules that the cell cannot process and thus disrupt the cell functions[3]. These functions include the production of proteins, and enzymatic activity that the cell needs to survive. In lower concentrations the metals do not

cause an effect, as the cell can remove or sequester the metal or the effect of the metal is sufficiently small that the cell can repair the damage[3]. Metals enter the cell by two different pathways, passive and active transport. Passive transport is when the metals enter the cell through the membrane by a protein channel that spans the length of the membrane. Active transport requires ATP or energy for the transport to work as it goes against the concentration gradient. The resistance genes we are looking for in this study may be more common in the areas that have bacteria that have the genes that encode for the channels, which can remove the metals from the interior of the cell allowing for the bacteria to survive in the metal rich environments[3].

METHODS

Sampling

Sediment samples were collected from three different contaminated sites along the Providence River. Sites were discovered to be contaminated with the trace metals of interest in a previous project[6]. The locations were at India Point Park (IPP), Oxford Street (OX), and Public way (PW). Sediment samples were taken from the surface of the intertidal zone as well as at depth, near the ground water this was between 4 and 12 centimeters. The sediments were stored in sterile packs and transported to the lab in a cooler with ice packs to preserve the samples. The sediment was then frozen to preserve the bacterial communities.

Extracting DNA

Total DNA was extracted from the soil using the Power Soil kit (Mo Bio Laboratories, Inc.) following the manufactures directions [8]. This technique extracts and purifies the DNA from contaminants that could affect or inhibit the PCR. The DNA is isolated from the sediment which contains living and dead cells. The cells are lysed and the DNA is exposed. Remaining cell debris and other contaminants are then removed. The solution is then filtered on a positively charged filter and the DNA, which is negative, will bind to the filter. The filter is

then washed to remove the DNA, which remains in the solution[8]. After the DNA is separated from the bacteria it is stored at negative twenty degrees Celsius for use in PCR.

Polymerase Chain Reaction (PCR)

PCR is a method that is used to amplify a specific gene of interest by cloning a specific region of the DNA. PCR creates the necessary conditions to copy DNA. To run the PCR first the DNA is mixed with the four bases of DNA, DNA polymerase, and the primers. Once combined, the mixture is heated to around 95ºC this separates the DNA allowing for it to be copied[10]. The next step is to allow the primers to attach to the regions that correspond on the DNA by cooling it to 55ºC. Then to synthesis the new DNA strand DNA polymerase an enzyme will attach to the primer and begin to copy the DNA in short fragments or genes[10]. This process is repeated several times (25-35 cycles) in order to amplify desired DNA fragments. Desired DNA fragments are chosen based on the primers and polymerase used. The cycles and temperatures of heating and cooling, vary in different methods; the one that is being used for this project was found to work over the summer in a similar PCR project[9].

The Primers used in this project was the 16srDNA primer. The gene which this primer targets is necessary for the production of proteins and without it the cell cannot survive. As this gene is important for life all bacteria have the gene. This is why the gene primer is commonly used to test if there is DNA at the site and if PCR was successful. This can also be used to amplify the gene for sequencing to test what organisms are at the site.

Gel Electrophoresis

To analyze the PCR reaction, the product is then run on an Agrose gel. The gel that was used was a one percent solution, it is porous and small strands of DNA will move through it faster than larger strands, separating the different lengths into bands based on sizes. The bands of DNA move on electrophoresis. DNA has a fixed negative charge to it and putting the gel into a solution and running a current through it will pull the DNA through the gel toward the positively charged terminal creating the bands[10]. The voltage that was used was 100 volts for 35 minutes. These bands are compared with a standard containing known sized of DNA at fixed concentrations. If the bands from the PCR match the length of a gene established from the literature then the PCR was successful and the gene was present[11]. If there are no bands on

the gel then the gene is most likely not there and the PCR will need to be run again for confirmation[11].

The primers that will target the gene, encoded with the Pb resistance efflux pump, to be copied was researched. Different genes that encode for the lead resistant efflux pump were found and analyzed by using Ugene as a software platform. This program allows for the access of many previously identified genes from to be compared and repeating patterns in the genes to be identified. Once the sequence for the primer is identified, from highly conserved regions of the genes, then it will be checked to see if there are any secondary structures that can result from the sequence with the program Oligonucleotides Properties Calculator. This is an open access program that will calculate the thermodynamic properties of the primer sequence to see if it will bind to itself and form secondary structures. The sequence cannot contain many of these as if it binds to itself then it would not work well as a primer.

The databases that was used to collect the different gene sequences is NCBI (http://www.ncbi.nlm.nih.gov/) and DOE-JGI (https://img.jgi.doe.gov/). These sites contain a collection of genes that can be accessed either full genomes of organisms or specific genes. NCBI is a medical research database that has several genomes that are involved in medicine. These genes can be analyzed for conserved regions in the software platform Ugene.

Cloning of 16srDNA Product

The 16srDNA product was taken and purified from the PCR to remove and purify the amplified DNA. The DNA was then made into a plasmid which also contained the gene for ampicillin resistance. This plasmid was then cloned into competent E. Coli cells. The bacteria cells had holes entered into their cell membrane to allow for the plasmid to enter the cell. The cells were then put on ice for 30 min to cool them then on the heat block set at 37^{o}C for 3 minutes to heat shock the cells. After they were put on the ice for another 15 minutes. The cells were given time to incorporate the plasmid into the cell and to start expressing the genetic data such as the ampicillin resistance gene in the incubator set at 37^{o}C. The cells were then grown on agar plates that contained ampicillin. This was to ensure that any cell that did not take up the plasmid and grow would not be able to survive. This process also amplifies the plasmid as each cell has one.

The plasmids were then collected using the Easy Vector Plasmid Kit. This allows for the removal of the plasmid by lysing open the cells and removing the cellular deprease. The plasmids were then sent to URI for sequencing. The genes were then looked at in PubMed to analyze what strain of bacteria was present.

Data Mining

The sequence that was received from URI was analyzed in the UGene computer program. The sequence then was searched for in PubMed by using a BLAST search. This type of search compares the sequence that we received from URI to a database of pre-sequenced organisms. The organisms with the highest match to the sequence that was found from the search was then shown.

RESULTS

The results of the PCR runs using the 16srDNA primer demonstrated that there was DNA in the sediment samples collected from the three sites. PCR data are included in appendix 1 figure 1. The two other primers, JK-OC-ZNTA and JK-Ch-ZNTA, designed to test for genes from two bacteria, *Oligotropha* and *Cytophaga*, and their respective cation efflux pump resistance mechanisms were tested in a sample from India Point Park. A complete list of primers used is in Table 2. Neither *Oligotropha* and *Cytophaga*, were found in this sample. This shows that the environments are different and the conditions that the bacteria needed to survive are not available in the Providence River.

Table 1: Primers that were used in the study

Primers Used	Gene Sequence	Target Gene
JK-OC-ZNTA-f	GCGGTGGTGTTTCTGTTCTT	*Oligotropha* Lead resistant Cation Efflux pump forward Primer
JK-OC-ZNTA-R	AATTTGGTCTGCGGTCAAAC	*Oligotropha* Lead resistant Cation Efflux pump reverse Primer
JK-Ch-ZNTA-F	CGTCCGGATGAGGTAAATGT	*Cytophaga* Lead resistant Cation Efflux pump forward primer
JK-Ch-ZNTA-R	CGTCCGGATGAGGTAAATGT	*Cytophaga* Lead resistant Cation Efflux pump reverse primer
16srDNA-F	AAGAGTTTGATCCTGGCTCAG	Ribosomal RNA used for making proteins forward primer
16rsDNA-R	CTACGGCTACCTTGTTACGA	Ribosomal RNA used for making proteins reverse primer

PCR product from the 16srRNA primer in the India point park sediment sample was sent to the University of Rhode Island for sequencing. This location had high levels of lead and had a higher chance to contain bacteria that had lead resistant strains. The sequence that we then received from URI was searched for in the PubMed data base to understand what the organisms could potentially be. Sequence Data is shown in Appendix B. The sequence was matched to different organisms that were in the database and the most probable were examined. The organisms that were identified include *Desulfabacillum alkenivorans* AK-1 and PF2803, common in areas with high metal[12]. Staniera *cyanosphaera*, *Desulfuromonas acetoxidans* strain DSM 684, *Geobacter uraniireducens* strains Rf4, *Geobacter sulfurreducens* strain PCA. Based on a literature review the organisms were found to be organisms that are commonly found in estuarine environments or environments that have high

concentrations of metal, commonly for the reduction in iron in the area, shown in Table 2[12,13,14,15,16].

Bacteria Identified and Area Located	
Bacteria Strain	**Typically found**
Desulfabacillum alkenivorans AK-1 and PF2803	Estuarine sediments Used for anaerobic Alkane biodegradation[12]
Staniera cyanosphaera	Estuarine sediments, Type of cyanobacteria [13]
Desulfuromonas acetoxidans strain DSM 684	Estuarine sediments used for suffer and iron reduction in the environment. [14]
Geobacter uraniireducens strains Rf4	Unspecified sediments, conserves energy from iron reduction. Can survive in areas with uranium. [15]
Geobacter sulfurreducens strain PCA	metal reducing bacteria found in areas with many different metals.[16]

Table 2: identified Organisms in India Point Park and functions.

CONCLUSIONS

In this project we attempted to find what the microbial population of the Providence River was in order to see if there was lead resistance in the organisms from the environment. No lead resistance were found using the primers from the literature as the organisms were not identified in the area. The genes that encode for the resistance against the different pollutions are not conserved among the different bacteria. Each one had its own way to make the

protein. By identifying what organisms are in each of the sites different primers can be made to test if there is resistance at the sites. Once the primers are made if lead resistance has been found then the sites can be checked for antibiotic resistance using known antibiotic resistance genes.

FUTURE DIRECTIONS

DNA will be sequenced from Oxford Street and Public Way locations. Those organisms will be searched to find those that are most probable to be in the area. The organisms identified will be researched to see if there are previously identified efflux pumps that are used for lead resistance. If any potential genes are found primers will be made for use in PCR. The sediment will then be tested for having the lead resistance and if any are found then tested for known antibiotic resistance.

APPENDICES

Appendix A – (PCR Results)

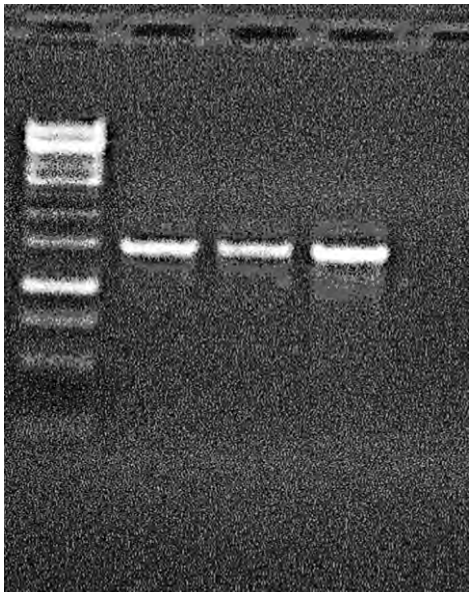

Figure 1: Lane:1 Gene ruler Lane 2:IPP Lane3: PW Lane4: OX
The gene size is 1500Kb

Appendix B – (IPP Sequence Data)

Figure 1: IPP-1 Sequence

ATGCAAGAAACTATTAAAAACGATGCTGAGGACCAAAACCAAAAAGCAGTCGATCAAAAACACGATCACGGTT
CTGGCAGTGAGAGTGACCATGATCACGATGCAGTAGATACAGAGGAAGAAAAAGCTGACGGATTTAAAAAAT
ATCTTCCCGCTATAATTTCCTTCGTACTACTAATCGGAGGAATTATTGCCGATAACCAGGTAACTAAATTTCAAA
GGCTGGAATTCTTCCAAGGATATGTCAGGTTAGCCTATTATCTTATACCCTATTTGCTCATTGGCTGGCCTGTGC
TGAAAAAAGCATACCGATCTATCTTGCGTCGTGACTTCTTTAATGAGTTCATGTTAATGAGCATAGCTACTCTTG
GAGCATTCTATATCAAGGAATATCCGGAAGGAGTTGCCGTAATGCTATTTTATCAGATTGGAGAATTATTCCAG
GATGCTGCGGTGCAACGGGCTAAAAGATCCATAAAAGCGCTGCTGGATATTCGTCCGGATGAGGTAAATGTA
CTTAGGAATGGCAAAGCGGAAAAAGTACATCCTACTAAAGTAGCTATAGGTGAAACCATTCAAATCAAGTCCG
GTGAAAAAATTGCATTAGATGGGGAGCTCCTTTCTGAAAAAGCATCCTTCAATACTGCTGCGCTAACTGGTGA
AAGCAAGCCGGATAATAAGTATAAAGGTGAGCCTGTATTGGCAGGCATGATTAATCTTGACACTGTATCTGAG
GTAAAAGTAACTGCTCTATTCAAAGACAGTAAATTGTCCAAGATACTGGAACTCGTACAGGATGCAACAGCAA
AAAAAGCAAAGACACAGCTCTTTATTTCACGATTTGCACGCAGGTATACACCTGTAATGGTGTTCCTTGCTATT
GCGATTACTGTTGTACCTTCTTTCTTTGTAGACAATTATGTTTTCAATGATTGGCTGTATAGAGCATTGGTATTTC
TTGTTATTTCCTGCCCTTGTGCATTAGTTGTATCTATTCCTTTAGGATATTTCGGGGGTATTGGATTAGCCTCACG
CAATGGTATCCTGTTTAAAGGCTCTAACTATTTAGATGTCATGACCGGTATTGATACTGTAGTAATGGACAAAA
CAGGAACGCTAACAAAAGGTGTTTTTAAAGTTCAGCAAGTAGAACCCAGCAATATTGAAAAAGATTCTCTTTTA
AAATATACAGCTGCATTAGAGACACATTCAACACATCCTATTGCAAAAGCAGTTGTTGCATATGTAGCTCTTAA
AGATTTACCGAATGCTGAAAATGTTGAGGAAATTGCAGGGTATGGATTAAAAGGTACTGTAGAAGGGAAACA
GATATTAGTAGGGAATCTAAAATTATTGAGCAAATTCAATATTGCCTTTGATGAACAAATTAAGAACATCACTG
ACACCATAGTTGTAACAGCCATAGACGGGAAGTATGCAGGCTATTTAACCATTGCAGATGAAATCAAGGAAGA
TGCCTTAGAAGCCATAAAGGCATTGCATGGACTAGGTATTAAGACTGTTATGCTTTCGGGCGATAAGCAGGCT
GTAGTAGATGCTGTCGCAAAGACATTAGGTATTGACGCTGCTTATGGCGATTTGCTTCCTGAAGGTAAAGTAG
AAAAAGTACAGGCCCTTAAAAATGAGGGGAAGAAGATAGCATTTGTAGGAGATGGTGTTAACGATGCACCAG
TTGTAGCTTTAGCAGATGCTGGAATTGCAATGGGTGGTTTGGGTTCGGATGCTACGATTGAAACTGCTGACAT
TGTTATTCAAAATGATCAGCCATACAAAATCTTCTCTGCTATTAAAATCGGCAAGCTTACTAAAAGAATTGTATG
GCAAAACATATCGCTAGCATTTGCTGTAAAAGTTATTGTATTGATTTTAGGTGCTGGAGGTTTGGCAACCTTAT
GGGAAGCTGTATTTGCCGATGTTGGTGTTGCTCTGTTGGCAATATTGAATGCTGTACGTATTCAAAAGATAAAA
TTATAG

Figure 2: IPP-2 Sequence Data

ATGACGCAGTCTGATGCATTCACGATCCGCATGCGGGTGGAAGGAATGGATTGCGCGAGTTGCGCGGTCAAG
ATTGAGAACGCGCTTCGGCGTATACCCGGCGTTGCGGAGGTAAAAGTATCGGTTTCGGGCGGCACGGTTACC
GTTCGAAATGATCGATTGAATGCCGAGGCGATAAAGACTCAGATTGTCAGGCTCGGTTATACGGTAACGGGCT
CCCGGGAGTTCAAAGGCGAAGCGGACCAGAGAGATGCCGTTGAAGTATCGACCTCAACCGGAAAGAGCGCTC
ATTCTCATACACATGAGAGTGATGATGACGACAAGCCATGGTGGCGGACAAGAAAAAGCGCTTTGACCATCGC
TTCCGGGGCAGCTTTGGCCGTCGCCTTCGTGGTCGGAAAAGCCAGCCCGGCGATCGAGCAGTGGGCATTTCTG
CTCGCGATGATGGTCGGACTCATTCCGATCGCAAAGCGTGCAGTTGTTGCCGCGATTGCCGGAACGCCGTTCT
CGATCGAAATGCTGATGACGATTGCTGCGGTTGGAGCAATCGTCATCGGTGCGACCGAAGAGGCGGCAGCGG
TGGTGTTTCTGTTCTTGATCGGAGAATTGTTGGAGGGGGTCGCTGCCAGCCGCGCCCGCGCAAGCATCCGGAA
TCTCGGTGATCTCGTTCCCAAAACAGCTCTCGTCGAAGACAATGGCCGACTCCGAGAGGTGCAGGCCGAGAGC
CTTGAGGTCGGAGCAACAATCCATGTCAGGCCGGGTGACCGAATCCCGGCCGATGGAGCGATCCTGTCAGGG
GATAGCTCGATCGACGAAGCCCCCGTTACGGGCGAAAGTACGCCGGTTCGGAAAGGTCCTGATGAGGTTGTT
TTCGCAGGAACGGTAAATGGCGATGGCCTTTTACGCATTCGGGTAACCGCCGCCGCAACAGACAACACCATCG
CGCGAATTGTCCGTCTCGTCGAGGAAGCCCAAGAATCAAAGGCACCCACGGAACGGTTCATCGATCGCTTTTC
CCGCTATTACACCCCCGGCGTGGTGGTGGTCGCGGCACTGGTCGCGATCATTCCTCCGTGGTGGTTCGGCGCA
GATTGGGGCGGTTGGATCTACAAGGGGTTGGCAATCCTCCTGATAGGCTGTCCTTGCGCCCTCGTTATATCGA
CGCCAGCAGCAATCGCCGCGAGCCTCGCCGCCGGTGCCCGCAGCGGTCTCCTCATGAAGGGAGGTGCCGTTCT
TGAACGCATTGGCAGAATTACGGTTGCTTGCTTTGACAAGACGGGCACGCTGACCGCCGGCAAACCCCAAGTG
ACGGATGTCATCGGCTTCGTTCGTCCGGAGACGGACGTGTTGCAAGTCGCAGCCGCGCTGGAATCCGGATCAA
GCCATCCACTCGCCATCGCAATCCTGGCATTGGCGTCCGAAAAGGGCATTCACGTTTCCTCCGCCGAGGATTCT
AAAGCGATCGGAGGGAAGGGCGTTCAGGCAAACGTCGAAGGAAAGAAAGTTTTCCTCGGATCGCCCGCAGCC
GTCGGGGAAATATCGGGTTTGACCGCAGACCAAATTGCCCAGATCGAAGCACTTAACGATGAAGGCAAAACC
GTTTCCGTGCTTTTGATCGGAGATGAGATCGCCGGCGCCATTGCCATGCGTGATGAGCCGCGTTCGGACGCGA
AGGACGGTCTTAAGCAACTGACAGCGGCAGGTATCCGCACTTTGATGCTGACCGGCGACAATCGACGTACCGC
AACAGCAATCGGCAACAGGTTAGGCATTGAGGTCAAAGCGGAACTGCTGCCGCATGACAAGCAGCGCCTGGT
AACCGACCTACAAAGGAGCGGACAGTCTGTTGCAAAGGTCGGAGATGGCATTAACGACGCGCCCGCCTTGGC
GGCGGCAGATGTTGGAATTGCTATGGGAGGAGGTACGGATGTCGCTTTGGAAACGGCCGATGCCGCGATTCT
GAACGGAAGAGTTGGCGACGTCGCGGCAATGGTCGCTCTATCCAAGCGGACTATGACGAACATTCACCAAAA
CATTGCCGTTGCTCTCGGGTTAAAGGCGGCATTCCTAGTCACGACCATTGCCGGTGTGACAGGCCTTTGGCCTG

CGATCCTTGCCGATACTGGAGCAACCGTCTTGGTGACTCTTAATGCGTTGCGCCTGCTTTCGCCAAAGGACCGA
AGTCGATCTTGA

Figure 3: IPP-3 Sequence

ATGAACGTCACCGCACGGGCTGCGAAAGTCCGCTTCCGCGTGGAGGGAATGGATTGCGCGTCGTGCGCCACC
AAGATCGAAACTGCTCTGCGACGGGTACCGGGTGTTACCGAGGTCGCCGTCTCCGTCACCGGAGGCACGGTG
ACCGTCAGTCGCGACAATGACCGCGTCGATGACGACAAGCTCCGCAATAGCATTTCAGATCTCGGCTATCGCG
TGGAGACGGCCCGCAACGGTCTCCATGACGACGATCAGGGTGTCTCGCCGCGGCAAGACGATCCGCACGCTC
ACAGTCACGCCTCGGTCGACGGATCTTGGTGGCGGACCCAAAAGGGCTTGCTCACCTTGGCGTCCGGCATTGC
ATTAGTCGTCGCGTTTGCGATCGGCAAAGTTGTCCCCGTCACCGAGCGTTGGGCGTTTCTGCTGGCGATGCTG
GTGGGTCTCGTTCCGATTGCGCGACGCGCACTCTCTGCCGCACGCTCCGGAACGCCGTTCTCGATCGAAATGTT
GATGTCGATCGCCGCCGTGGGGGCGGTTATCATCGGGGCGACGGAAGAGGCGGCGACCGTCGTATTCCTGTT
CCTGATCGGCGAACTCTTGGAAGGCGTCGCAGCCAGCCGCGCCCGCGCCAGCATCCGGGACCTCACCAAGCTC
GTTCCGACGACCGCGCGCCTCGAAGACGGCGGGCAGGTCCGGGAGGTGCAGGCTGACAGTCTTGAGGTGGG
AACGACGATCCAGGTCAGGCCGGGCGACCGCATTCCGGCGGATGGCGTCATTCTGTCCGGCGAAAGCGCGAT
CGATGAGGCCCCGGTGACGGGGGAAAGTACGCCGCTCCGAAAAGGGCCGGACGCACTCGTGTTCGCCGGCA
CCGTAAATGGCGATAGCCTGTTGCGGGTGCGAGTGACCGCCGCCGCGGCTGACAACACCATTGCGCGGGTCA
TTCGCCTGGTCGAGGAGGCGCAGGAGTCGAAAGCGCCGACGGAACGCTTCATCGACCGCTTCTCACGCTTCTA
CACGCCGGGCGTGGTGGCGGTTGCCGCCTTGGTTGCCATCGTTCCTCCTCTGTCGTTCGACGGGGATTGGAGC
GGTTGGATCTACAAGGGTCTGGCCATCCTGTTGATTGGTTGTCCTTGCGCGCTGGTCATCTCGACGCCCGCTGC
GATCGCCGCCAGCCTGGCCGCCGGGGCGCGCCGGGGTCTTCTCATGAAGGGCGGCGCCGTCCTGGAACAGGT
CGGCAAGATCACGGTGGCGTGCTTCGACAAGACCGGAACGTTGACGGCGGGGAAACCTCAGGTCACGAACGT
TATCGGCTTCGCGCGCAGCGAGGCGGATGTCCTGCGCCTCGCCGCCGCGTTAGAATCCGGCTCAAGCCACCCG
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TCGGAGGCAAGGGCGTCCAGGCGACGGTCGACGGGCAAAGCGTCTTTCTTGGGTCGCCTGCGGCCGTCGCGG
AATTGTCGCCTCCGACCTCCGAGCAGACCGCGCGGATCGCGGCGCTCAATGACGAAGGCAAGACCGTCTCGCT
GCTCTCGATCGGAAATGAACTCGCGGGCGCAATCGCGATGCGCGACGAGCCCCGTCCCGACGCAAAGGCAGG
GCTTAAGCGTCTGACGGACACGGGAATCCGGACGCTGATGCTGACCGGTGACAACCGCCGCACCGCGCACGC
GATCGGTCAAGACTTGGGAATCGAGGTCAGAGCGGAATTGCTGCCGCAGGATAAGCAGCGGATCGTCGGTG

AGTTGCGGAAAGGCGGTGGCTCGGTCGCCAAGATCGGCGACGGCATCAACGATGCGCCGGCGTTGGCGGCG
GCCGACATCGGCATCGCGATGGGAGGAGGTACCGACGTGGCGCTGGAAACCGCAGACGCCGCTGTGTTGCAC
GGCCGCGTCGCGGACGTCGCCGCGATGGTCGACCTGTCGAAGCGGACCATGGCCAACATCCGGCAGAATATC
GCGATCGCGCTGGGACTCAAGGCCGTCTTTCTGGTCACCACGATCGTCGGGCTGACCGGATTGTGGCCGGCGA
TTCTCGCCGACACCGGTGCGACCGTCCTCGTCACCATAAACGCGCTTCGCCTGCTCACGCAGGCGAAGGCCTG
A

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