Bioengineering design for cyanide degradation

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Cyanide is a highly toxic product for humans and the environment and is present in wastewater generated from metal mining and other industries. Recent testing in my local community of Mead, Washington State, showed that cyanide, first detected in local wells in 1978, continues to persist in groundwater at levels almost ten times higher than allowable limits. A variety of methods for cyanide biodegradation exist, but they all have significant shortcomings. Although bioremediation methods are less expensive and more environmentally friendly than chemical treatments, they are also limited by the inability of the cyanotrophic microorganisms to survive at the higher cyanide concentrations present in landfills and other waste sites. I have designed a biological system capable of degrading cyanide that aims to overcome the existing limitations by having the ability to survive in high concentrations of cyanide and by providing a safe and efficient way to degrade cyanide. The system will use genes from Pseudomonas pseudoalcaligenes CECT5344, a cyanotrophic bacterium whose genome has been fully sequenced. This bacterium possesses an alternative respiration system, allowing it to be more resistant to cyanide than other bacteria. A plasmid containing the cyanide-degrading nitrilase genes, the cyanide-insensitive cioA and cioB genes, and two reporter genes will be inserted into the chassis, a safe and well-characterized strain of *Escherichia coli*. The two reporter genes, RFP and mTagBFP, will monitor both devices.

Keywords: Cyanide, degradation, *Pseudomonas pseudoalcaligenes*, bioremediation

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Watch a video introduction by the authors at https://youtu.be/2vjD8Yjf7nc

Background

Cyanides are chemical compounds that contain the cyano group, which has the chemical formula −C≡N. Hydrogen cyanide (HCN) and the alkali cyanide salts, sodium cyanide (NaCN) and potassium cyanide (KCN), are water-soluble cyanides that are powerful and rapid-acting poisons. HCN and free cyanide (CN-) are two of the most toxic chemicals for humans and the environment (Luque-Almagro, Cabello, Sáez, et al. 2018). In the cell, cyanide binds the iron atom in the enzyme cytochrome C oxidase due to its highly reactive nature. This disrupts the electron transport chain and causes cellular hypoxia, ATP depletion, and ultimately death in all living organisms. Because of the cyanide anion's high affinity for metals such as gold and silver, cyanide compounds have been used extensively in metal mining and other industries, which has resulted in cyanide-contaminated wastewater. Many large cyanide spills worldwide have ensued from mining-related accidents. One such example is the Baia Mare cyanide spill in 2000 caused by an Australian-owned gold-mining company. The cyanide eventually reached the Danube River and harmed aquatic species in three countries; for this reason, the event has been called Europe's worst environmental disaster since Chernobyl (Lenntech 2020).

Despite the dangers of using cyanide, global production has increased by forty percent in the last ten years and is expected to reach 1.5 million metric tons in 2021 (Merchant Research & Consulting 2014). Presently, cyanide is required in many industries for the manufacturing of plastics, adhesives, cosmetics, and drugs, and is widely used for extracting precious metals such as gold (Luque-Almagro, Moreno-Vivián and Roldán 2016). Cyanide in its different forms — free cyanide, HCN, NaCN, KCN, and CuCN — has been found in more than 460 of the 1660 U.S. superfund sites that existed in 2005 (Taylor 2010). Superfund sites have been identified by the Environmental Protection Agency (EPA) as the most serious hazardous waste sites in the nation and have been placed on the National Priorities List (NPL) for long-term federal cleanup activities. Cyanide accumulates in soil and groundwater over time, and it naturally degrades at a very slow rate.

In my local community of Mead, Washington State, cyanide has existed in groundwater for more than fifty years as a result of a smelter built in 1942 by Kaiser Aluminum. This facility used cyanide to extract aluminum needed for the construction of aircraft frames during World War II, and in 1978, cyanide contamination was discovered in private wells (Sowa 2013). Despite the closure of the Kaiser Aluminum plant in 2000, cyanide presence in groundwater remains a persistent environmental problem in my community (Hill 2019). The Kaiser superfund site has been on the EPA's NPL since 1982, and it is still considered active because the waste cleanup has not been completed. The 128,000 tons of waste at the site have been covered by an asphalt cap; however, this temporary solution has failed to prevent cyanide compounds from leaking into groundwater. In 2017, the results from testing wells in an area planned for urban development showed that cyanide was present in groundwater at a concentration level between 1.6 and 1.8 mg/L, which is almost ten times the allowable limit of 0.2 mg/L (Hill 2019, Washington Department of Ecology 2002). The Spokane Valley-Rathdrum Prairie Aquifer, which has been contaminated with cyanide, supplies water to the Spokane River. As a consequence, cyanide poses a serious threat to aquatic life and the environment.

In order to remove the cyanide in the Mead area, local officials have proposed using the natural oxidation method, which is based on the volatilization of cyanide under specific light and temperature conditions (Hill 2019). Alkali metal cyanides are very soluble in water, and when exposed to a low pH and in the presence of oxygen and light, they form HCN, which is then released into the atmosphere. This method is not efficient in cold or cloudy weather, as low temperatures and the presence of ice or snow interfere with the degradation of alkali cyanide salts (Taylor 2010).

A variety of physical and chemical methods for cyanide degradation also exist. Chemical oxidation is widely used for treating waste contaminated with cyanide and results in the conversion of cyanide to the less toxic cyanate. This method produces — but does not remove — free chlorine, chloramines, and iron cyanides, all of which are toxic to fish. Other remediation methods include electrolysis, photolysis, ozonation, and adsorption with activated carbon or resins (Taylor 2010). It should be noted that all these methods and other current technologies have significant shortcomings. The first drawback is that they are expensive; the planned cleanup in the Mead area is estimated to cost at least four million U.S. dollars for construction and one million per year for maintenance (Hill 2019). An additional concern is that they can create toxic sludge that will need to be remediated; the proposed system in Mead is projected to produce about one-thousand tons of sludge per year, which, depending on its toxicity, would need to be transported to either a landfill or a hazardous waste facility (Hill 2019). Lastly, physical-chemical treatments often require aerobic conditions and are usually ineffective for stable metal-cyanide complexes (Luque-Almagro, Cabello, Sáez, et al. 2018).

The biological methods for degrading cyanide by using microorganisms are less expensive than physical-chemical methods and are more efficient than natural oxidation (Table 1).

Table 1: *The different methods for cyanide degradation*

Degradation Method	Physical	Chemical	Natural Oxidation	Biological
What Is It?	Separate cyanide from water	Degradation of cyanide through oxidation reactions which destroy the cyanide nitrogen bond and transform the highly toxic cyanide in non-toxic or less toxic products.	When exposed to air under certain conditions (low PH, the presence of air and light), the free cyanide present in the water will form hydrogen cyanide which is released into the atmosphere.	Degradation of cyanide by different microorganisms.
Advantages			Inexpensive	• Less expensive than physical or chemical methods of cyanide degradation • Faster than natural oxidation • Besides removing cyanide, it enables the denitrification of ammonia which is produced during cyanide degradation • Inexpensive, effective and more environmentally friendly
Disadvantages	• Does not degrade cyanide • Can be very expensive • Creates toxic sludge which requires further remediation and recycling	• Expensive • The cyanate produced during degradation can, in large quantities, be toxic to fish	• Limited efficiency • Lower temperatures and the presence of ice/snow interferes with degradation	• Microorganisms are not able to survive at higher cyanide concentration similar to the one existing in hazardous waste sites and landfills • The majority of the microorganisms cannot survive in anaerobic conditions
Examples	• Electrodialysis • Reverse osmosis • Ion exchange	• Ozonation • Hydrogen peroxide • Alkaline chlorination		Microorganisms are able to degrade cyanide by different metabolic pathways involving four main reactions: Hydrolytic, Oxidative, Substitution, Reductive.

In order to degrade cyanide, microorganisms utilize one or more of the following enzymatic processes: hydrolytic, oxidative, reductive, and substitution reactions (Luque-Almagro, Cabello, Sáez, et al. 2018) (Table 2). More than thirty bacterial strains, including strains of *Klebsiella*, *Escherichia coli*, *Bacillus*, and *Pseudomonas*, have been identified as effective for cyanide degradation (Ibrahim, Syed, Shukor, et al. 2015). All of these strains are cyanotrophic, which means that they are able to metabolize cyanide and use it as a nitrogen source. Although bioremediation methods for cyanide are considered inexpensive, effective, and environmentally-friendly, they have two disadvantages. One limitation is that most microorganisms are not able to survive the higher cyanide concentrations usually found at hazardous waste sites and landfills (Taylor 2010). Another limitation is the risk of bacterial infections in immunosuppressed individuals (Hage, Schoch and Cunha 2013). In order to overcome these limitations, I have used synthetic biology tools to design a biological system involving bacterial strains that are safe for humans and are capable of surviving at high concentrations of cyanide.

Table 2: *Pathways for cyanide degradation in microorganisms*

Reaction Type	Enzyme	Reaction
Oxidation (Aerobic)	Cyanide dioxygenase	$HCN + O_2 + 2e^- + 2H^+ \rightarrow NH_3 + CO_2$
	Cyanide monooxygenase	$HCN + O_2 + 2e^- + 2H^+ \rightarrow OCN^- + H_2O$
Reduction (Aerobic)	Nitrogenase	$HCN + 6e^- + 6H^+ \rightarrow CH_4 + NH_3$
Hydrolysis (Aerobic/Anaerobic)	Cyanidase	$HCN + 2H_2O \rightarrow HCOOH + NH_3$
	Cyanide hydratase	$HCN + H_2O \rightarrow HCONH_2$
Hydrolytic Nitrile Degradation (Aerobic/Anaerobic)	Nitrilase	$R–CN + 2H_2O \rightarrow R–COOH + NH_3$
	Nitrile hydratase	$R–CN + H_2O \rightarrow R–CONH_2$
Substitution/transfer (Aerobic/Anaerobic)	3–Cyanoalanine synthase	$HCN + Cys \rightarrow 3–cyanoalanine + H_2S$
	Rhodanese	$HCN + S_2O_3^- \rightarrow SCN^- + SO_3^{2-}$

Systems level

In addition to the requirements for designing a system that is easy to monitor and safe for humans and the environment, my biological system aims to efficiently biodegrade cyanide and to withstand high concentrations

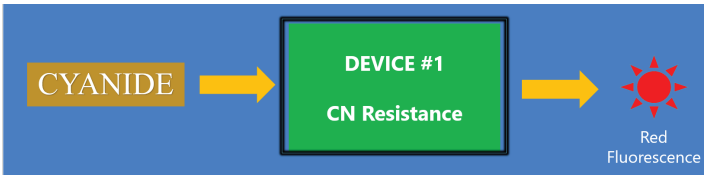

Figure 1: *The first device, designed for cyanide resistance, has cyanide as an input and red fluorescence as an output.*

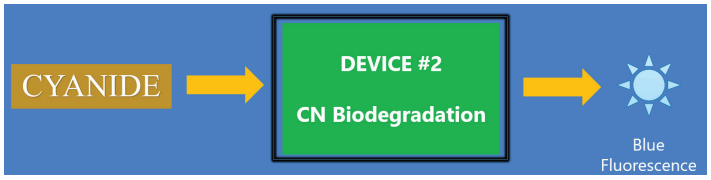

Figure 2: *The second device, designed for cyanide biodegradation, has cyanide as an input and blue fluorescence as an output.*

of cyanide. It consists of two devices, one for cyanide resistance and one for cyanide degradation (Figures 1 and 2). These two devices function independently of one another. My system requires the insertion of a plasmid containing genes for cyanide degradation and cyanide resistance into a safe chassis.

There are more than thirty microorganisms capable of cyanide degradation (Ibrahim, Syed, Shukor, et al. 2015). After a thorough review of the literature, I decided that the cyanide degradation genes from Pseudomonas pseudoalcaligenes CECT5344 would be the best choice for my system. P. pseudoalcaligenes is a versatile cyanotrophic bacterium capable of degrading cyanide, cyanate, and other cyanide compounds under both anaerobic and aerobic conditions. This bacterium can survive high cyanide concentrations up to 30 mM of free cyanide, which equates to 780.6 mg/L; this is much higher than the concentration of free cyanide in groundwater, which is between 1.6 and 1.8 mg/L. P. pseudoalcaligenes is the only cyanotrophic bacterium whose genome has been fully sequenced and its transcriptome analyzed through global transcriptome analysis (Cabello, Luque-Almagro, Olaya-Abril, et al. 2018).

The pathways for the assimilation and degradation of cyanide in P. pseudoalcaligenes are well-researched. In the nitrilase pathway, the cyanide reacts with oxaloacetate to form a nitrile, a step that requires the presence of malate:quinone oxidoreductase (Figure 3). This pathway is the only mechanism by which cyanide can be degraded hydrolytically in P. pseudoalcaligenes. A nitrile is an organic compound that contains a cyano functional group bound to an alkyl group. The general chemical formula of a nitrile is RCN, where R represents an organic group. The nitrile is then hydrolyzed to ammonium by the enzyme nitrilase. The ammonium is further incorporated into organic nitrogen (Luque-Almagro, Merchan, Blasco, et al. 2011).

In addition to having cyanide degradation capabilities, *P. pseudoalcaligenes* is more resistant to cyanide than other bacteria because it possesses an alternate respiration system that includes a cyanide-insensitive cytochrome.

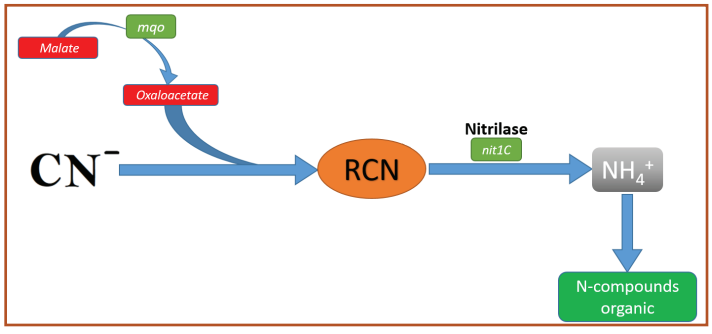

Figure 3: *The nitrilase pathway for cyanide degradation*

Device level

The designed plasmid will be inserted into the safe and well-characterized K12 strain of *E. coli* that will function as a chassis. *E. coli* is easy to grow in the lab, replicates quickly, and is well-characterized (Kuldell, Bernstein, Ingram, et al. 2015).

The system includes two separate devices in order to ensure that it is efficient and can be optimally monitored. It incorporates the nit1C gene cluster, the cioA and cioB genes, the RFP gene, and the mTagBFP gene.

The first device is responsible for the survival of the transformed *E. coli*, and it contains the cioA, cioB, and RFP genes (Figure 4). The cioA and cioB genes are clustered and encode a quinol oxidase insensitive to cyanide that ensures the survival of the system in the presence of high concentrations of cyanide (Quesada, Guijo, Merchan, et al. 2007). The RFP reporter gene encodes the red fluorescent protein, which emits red fluorescence to signal that the transformed bacteria is successfully surviving in the presence of cyanide. The red fluorescence in the bacterial cell culture would be visible with either the naked eye or with the help of an epifluorescence microscope.

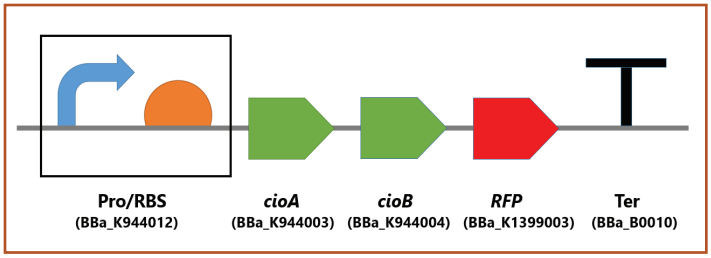

Figure 5. *The cyanide resistance device contains a promoter, an RBS, the genes for cyanide degradation, the reporter protein, and a terminator.*

The role of the second device is to degrade cyanide (Figure 5). This device contains the nit1C gene cluster, the mqo gene, and the mTagBFP reporter gene. The nit1C gene cluster codes for the different nitrilase enzymes — NitC through NitF — all of which are cotranscribed (Estepa, Luque-Almagro, Manzo, et al. 2012). The mqo gene encodes malate:quinone oxidoreductase, the enzyme important for the reaction of cyanide and oxaloacetate to form a nitrile in the first step of the nitrilase pathway. The mTagBFP reporter gene encodes the blue fluorescent protein; the presence of blue fluorescence indicates that the cyanide degradation device is functioning properly.

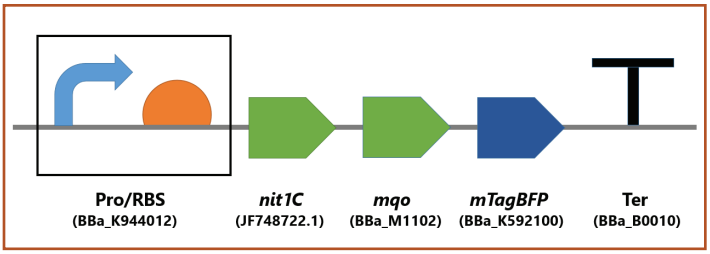

Figure 4. *The cyanide resistance device contains a promoter, an RBS, the genes for cyanide resistance, the reporter protein, and a terminator.*

Parts level

In the iGEM Registry of Standard Biological Parts, I was able to find BioBricks that match the requirements of my system. BioBricks are standardized, interchangeable DNA parts that can be used to help design plasmids. Both devices in my system contain a promoter, a ribosome binding site (RBS), an open reading frame (ORF), and a forward terminator (Part:BBa_B0010); these parts will be prepared in a linearized plasmid backbone (Figure 6).

Figure 6: The high copy number plasmid pSB1C3, which contains the BioBricks for both devices, will be inserted into the chassis, the safe K12 strain of E. coli.

The BioBrick Part:BBa_K944012 consists of a T7 constitutive promoter, an RBS, and a cyanide-dependent RBS transcriptional regulator and is included in both devices. The T7 promoter binds RNA polymerase, promoting transcription of the mRNA, a process which can either be enhanced or inhibited by the transcriptional regulator. The T7 promoter leads to the overexpression of the system's genes, contributing to the rapid, efficient response when cyanide is present in the environment.

The ORF is different for each device. The first device (cyanide resistance) contains BioBricks for the cioA (Part:BBa_K944003), cioB (Part:BBa_K944004), and RFP (Part:BBa_K1399003) genes. The cioA and cioB genes are part of the cioAB operon, which codes for the cyanide-insensitive terminal oxidase that is related to the cytochrome bd quinol oxidase.

The second device (cyanide degradation) contains the nit1C gene cluster sequence, which encodes the nitrilase enzyme. The sequence for the nit1C gene cluster from the P. pseudoalcaligenes strain CECT5344 can be found on GenBank (JF748722.1). The ORF for the second device also includes the mqo gene (Part:BBa_M1102) for malate-quinone oxidoreductase and the mTagBFP gene (Part:BBa_K592100).

The tables below show the parts for the two devices (Tables 3 and 4).

Table 3: *Parts for the cyanide resistance device*

Part	Description
BBa_K944012	T7 constitutive promoter, RBS, and RBS transcriptional regulator
BBa_K944003	ORF for *cioA* gene
BBa_K944004	ORF for *cioB* gene
BBa_K1399003	ORF for *RFP* gene
BBa_B0010	Forward terminator

Table 4: *Parts for the cyanide degradation device*

Part	Description
BBa_K944012	T7 constitutive promoter, RBS, and RBS transcriptional regulator
JF748722.1	Cyanide degradation genes: nit1C gene cluster (https://www.ncbi.nlm.nih.gov/nuccore/JF748722.1)
BBa_M1102	*mqo* gene for malate-quinone oxidoreductase
BBa_K592100	ORF for *mTagBFP* gene
BBa_B0010	Forward terminator

Safety

My design uses the safe, non-pathogenic K12 strain of *E. coli* which does not present any risks to human health. When I test my design, I would work in a biosafety level two (BSL–2) lab and would follow standard safety precautions and guidelines of BSL–2 labs: wearing protective clothing, chemical splash goggles, and safety gloves. The area would be well ventilated with a fume extraction system, and sterile techniques would be used in order to avoid any contamination.

As cyanide products are highly toxic, I would need to use suitable safety equipment and follow additional precautions regarding storage, use, and disposal. Highly toxic HCN gas, which is an inhalation hazard, is formed when cyanide compounds react with water or acids (Stanford University n.d.). Although certain cyanide compounds are toxic through ingestion and skin absorption, the primary concern would be the inhalation of toxic HCN gas. When handling cyanides, any procedures should be carried out in a fume cupboard (or equivalent); if a recirculating fume cupboard is used, the filter should be suitable for cyanides and must be regularly checked to ensure lack of saturation (University of Glasgow 2019). I would be working under

supervision and would have an emergency plan in case of exposure.

I plan to use diluted NaCN and KCN solutions that would be similar in concentration and toxicity to groundwater samples. The levels of cyanide will be between 0.1 and 1 ppm, which is significantly lower than the permissible exposure limits set by the Occupational Safety and Health Administration (OHSA). The diluted NaCN and KCN solutions will have a pH above seven, which will prevent the formation of HCN gas. By not using an acid solution and keeping the pH above seven, I am limiting the risk of HCN gas volatilization.

The system will initially be tested in a contained environment, a bioreactor, before being introduced into the environment. If the system is successful in a contained environment, I would add a kill switch to help ensure that the bacteria do not remain in the environment after cyanide has been degraded. There are significant concerns about having bioengineered bacteria in the environment since they may compete with other strains and exchange genetic material with other strains. I attempt to address this concern by carefully monitoring the microbiome and the viability of the engineered *E. coli* in a bioreactor. Toxicological testing will also be performed in order to ensure that the system is safe to be released into the environment.

In addition to being neither pathogenic nor toxicogenic, K12 strains of *E. coli* are devoid of all known *E. coli* virulence genes and have never resulted in any reported cases of disease (Kuhnert, Nicolet and Frey 1995).

Millions of pounds of cyanide are released every year into the atmosphere, surface water, or are injected underground (Taylor 2010). Cyanide persists in groundwater for many years, as shown by the situation in my local community. Chemical and physical methods for cyanide degradation are expensive and often produce new toxic waste. When biological systems are employed, the microorganisms are usually unable to tolerate high concentrations of cyanide and often pose a risk for bacterial infection. The background section explained both of these shortcomings in more detail.

Synthetic biology provides opportunities for improving biological remediation methods in order to make them less expensive, safer, and more environmentally-friendly. This design is the first bioengineered system for cyanide bioremediation using genetic material from *P. pseudoalcaligenes*. The next step of my project would be to test the system in a lab. Using math modeling equations based on a combination of Michaelis-Menten kinetics, ordinary differential equations, and stoichiometric relations, it is possible to calculate how much cyanide the system is capable of degrading in a given amount of time and to determine much transformed *E. coli* is necessary for efficient degradation (iGEM Team Oxford 2014).

Once lab testing is successfully completed, the system can be used to treat water or soil samples from contaminated water or sludge. In order to check whether the plasmid was taken up properly, I will send the transformed *E. coli* to be sequenced. P. pseudoalcaligenes is capable of degrading a concentration of cyanide up to 780.6 mg/L, which is four-hundred times the concentration of cyanide in groundwater; however, the cyanide concentration of each sample will need to be quantified so that it can be determined how much cyanide was degraded, a measure of the efficiency of the system. Since the system was designed to be capable of working in both anaerobic and aerobic conditions, when used in an anaerobic type reactor for wastewater treatment, it would be a significant improvement over existing methods.

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