Engineering *C. reinhardtii* to Biodegrade the Pollutant Polyethylene Terephthalate

Abstract

In 2016, the bacterium *Ideonella sakaiensis* strain 201-F6 impressed researchers with its natural ability to biodegrade polyethylene terephthalate (PET) and assimilate its carbon. PET, used in the production of plastic bottles and more, is the most common form of plastic pollution. Genetic modification of the oligotrophic unicellular alga, *Chlamydomonas reinhardtii* strain CC-277, with the plastic degrading operons from *I. sakaiensis*, provides eco-friendly breakdown of PET. *C. reinhardtii* has been studied for decades and is known to be safe to humans, unlike recently discovered *I. sakaiensis*. Fed PET in specialized dark composters, modified *C. reinhardtii* will allow everyone to reduce plastic's environmental impact. A beneficial byproduct of *C. reinhardtii's* utilization of PET, is that the chemical energy produced can be harvested from its mitochondria, and transformed into clean electrical energy via inserted nanoelectrodes. This electrical energy can power the majority of the home or facility in which the composter is located.

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Project Description

Present Technology

The genetic modification of organisms is a recently developed, complex branch of biological research. Today, genetic engineering is utilized in commercial agriculture, as well as for experimental research aimed at various human processes. With new developments in genetic engineering, genes that contribute to specific traits can be used to replace genes in other organism's genomes to achieve desired changes in the organism's phenotype.

One such technology that contributes greatly to the genetic modification of organisms is the innovative concept involving the utilization of plasmids, the circular structures that all bacteria have in common. Plasmids have a unique ability, which allows them to replicate completely independently of their corresponding chromosomes. This allows them to promptly transfer their genetic material from one bacterium to another. Scientists can use restriction enzymes, or restriction endonucleases to cut plasmids' DNA. The endonucleases are programmed with a sequence of ~20 nucleotides that will cleave the DNA only upon its recognition of the correct sequence. When the endonucleases cut the plasmid, it creates sticky ends and the gene of desired attributes is placed in the plasmid utilizing a ligase enzyme as the binding component. The ligase combines the once separate plasmids together creating a recombinant piece of DNA which can be used to transfer altered genetic information from generation to generation, causing the desired effect in the organism.

A futuristic genetic engineering approach involves the development of synthetic chromosomes to either replace existing chromosomes in organisms, or insert entirely new chromosomes into the nuclei of unicellular organisms. Feasible methods of synthesizing DNA

molecules and assembling them into larger DNA molecules have been developed. These methods include the insertion of artificial minichromosomes and synthetic chromosomes into the cells' natural collection of chromosomes. Scientists have already successfully demonstrated synthesis of the entire genome of the bacterium *Mycoplasma mycoides*, as well as the creation of a synthetic yeast chromosome. These techniques to genetically modify organisms is useful for modifying and inserting entire organelles, like the chloroplast, into an organism. Scientists have developed two primary approaches for applying these techniques to various organisms. The most common approach is termed the "Bottom-Up" approach. This method involves the complete replication of a chromosome and all its key components, that is then inserted into the nucleus of the organism being modified. The second approach is termed the "Top-Down" approach. This approach uses existing chromosomes to produce a template from which a new chromosome is constructed with the desired attributes and traits.

Similar to the utilization of synthetic chromosomes, another newly developed genetic engineering method is CRISPR/Cas9 technology, which uses artificially programmed RNA molecules to cleave specific genes from the organism's' genome. The usage of CRIPSR/Cas9 has created a wave of excitement in the scientific community, for now it is possible to accurately modify an organism's genome down to the nucleotide easily and effectively. CRISPR/Cas9 was adapted from naturally existing genome editing system in certain bacteria like *Streptococcus pyogenes*. These bacteria contain CRISPR (clustered regularly interspaced short palindromic repeats) as a defense mechanism against plasmids and viruses that use RNA-Guided-Nucleases that target the cleavage of foreign DNA sequences. Foreign DNA sequences are imprinted into repeating sequences at the CRISPR locus. Then, these sequences are transcribed into an RNA molecule known as crRNA. The crRNA then hybridizes with a secondary RNA, known as the

tracrRNA. The Cas9 protein and tracrRNA interact with each other to form a complex. The complex then binds to the Cas9 nuclease, then crRNA guide the complex to the target DNA that is then cleaved by the Cas9 nuclease. Scientists can program small sequences of ~20 nucleotides in length into these RNA molecules to target specific parts of an organism's genome, thus allowing scientists to effectively insert desired genes into an organism's genome. The CRISPR/Cas9 genetic engineering method could be utilized in the genetic modification of *C. reinhardtii.*

History

Humans have been genetically modifying organisms for tens of thousands of years through the process of artificial selection, more commonly known as selective breeding. More than 100 species of dog have resulted from early artificial selection. Evidence from archaeological sites in Southwest Asia suggests that humans genetically modified plants, such as wheat and corn as early as 7800 BCE.

Gregor Mendel, considered the father of genetics, conducted an experiment in the late 19th century to gather information on the genetic traits of organisms, specifically peas, and how their traits pass from one generation to another. All present research in genetics can be traced back towards Gregor Mendel's discovery of the laws governing inheritance traits.

The term genetics was coined in 1905 by the English biologist William Bateson. Subsequent research on the genetic modification of organisms led to what genetics is now. During the 1950s and 60s, key discoveries were made regarding the structure and function of DNA. In the year 1953, the double-helix structure of DNA was discovered by James Watson and Francis Crick. This discovery is considered the most significant in early genetics research. In

1967, scientists discovered DNA ligases, allowing for experimentation to be conducted into the genetic modification of organisms' genomes. Promptly afterwards in 1968, Werner Aber discovered restriction enzymes, which can cleave small sections of DNA from an organism. He proved the functionality of restriction enzymes during two experiments he conducted involving the removal of DNA segments from the bacterium *E. coli*.

In the 1970s, discoveries like type II restriction enzymes, and experimentation into gene splicing, ultimately paved the way for the creation of recombinant DNA (rDNA). In the 80s, 90s, and 2000s, innovations such as the first genetically modified animal, the development of polymerase chain reaction, and the discovery of CRISPR has allowed genetics to further advance, and allow for the development of genetic innovations like GMOs, and recombinant vaccines for Humans.

Future Technology

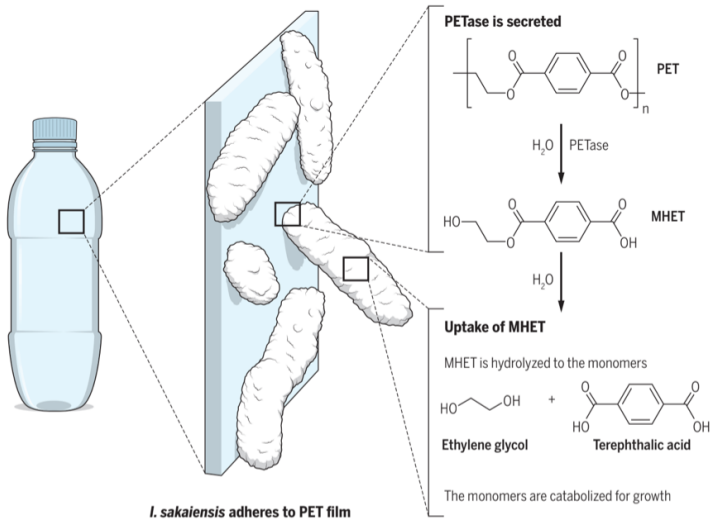
In the global battle against the ever-increasing plastic pollution entering our ecosystems, we have envisioned an innovation that will drive widespread recycling of polyethylene terephthalate (PET). PET is the world's most common form of plastic pollution, with ~65% of all plastic items made of PET. Our innovation genetically modified the bacterium *Chlamydomonas reinhardtii*, enabling it to unitize the carbon found in PET as its sole energy source.

Chlamydomonas reinhardtii is a unicellular oligotrophic alga that is easily grown. It is an autotroph, and can photosynthesize to produce its energy, or in the absence of light can use carbon as its source of energy. It has been extensively studied by scientists, and the full sequencing of its genome is available. It has also been successfully genetically engineered to

attack various human health issues. *Chlamydomonas reinhardtii* cannot naturally breakdown PET for carbon.

Researchers in Osaka, Japan recently discovered the bacterium *Ideonella sakaiensis*, which has the natural ability to breakdown PET, and use the carbon in PET as its only energy source. Taking operons in *I. sakaiensis* responsible for plastic degradation, and inserting them into *C. reinhardtii* will enable *C. reinhardtii* to degrade PET. Additionally, the chemical energy produced in *C. reinhardtii* can be harvested and transformed into clean electrical energy by inserting a series of small metal nanoelectrodes into the cells' mitochondria. *C. reinhardtii* recycling PET can be implemented easily into homes and offices, or facilities that recycle PET waste on a larger scale.

I. sakaiensis synthesizes the PET by using the enzymes PETase and MHETase apart of the α/β-hydrolase superfamily. *I. sakaiensis* secretes the enzyme PETase which converts PET to mono(2-hydroxyethel) terephthalic acid (MHET). The MHET is then collected by the bacterium where it is then hydrolyzed by the second enzyme, intracellular MHETase hydrolase. MHET is hydrolyzed into two separate monomers, terephthalic acid (TPA) and ethylene glycol (EG). These products are catabolized for *I. sakaiensis'* growth and development. Scientists have speculated that *I. sakaiensis* is utilizing carbohydrate-binding molecules (CBMs) to access the PET polymer fibers, although little research has been conducted on this particular aspect of the bacterium. The genomes of both *I. sakaiensis* and *C. reinhardtii* have been sequenced, making their genetic modification possible. The figure below shows the process of PET synthesis.



Delicious plastic. The *I. sakaiensis* bacterium discovered by Yoshida *et al.* (*5*) can attach to PET. It produces two hydrolytic enzymes (PETase and MHETase) that catalyze the degradation of the PET fibers to form the starting monomers. The monomers are then catabolized by the bacterium as its sole carbon source.

To gain deeper insight into the synthesis of PET, several researchers in the field of genetic engineering, as well as some of the scientists responsible for research on *I. sakaiensis* were contacted. We successfully communicated with two authors of the paper *"*Structure of the plastic-degrading *Ideonella sakaiensis* MHETase bound to a substrate". The authors Dr. Uwe Bornscheuer, Professor of Biotechnology and Enzyme Catalysis at the University of Greifswald, and Dr. Gert Weber, Professor of Macromolecular Crystallography affiliated with Helmholtz-Zentrum Berlin, replied to our inquiries and provided us with helpful information on the structure, function, and research done on *I. sakaiensis* showcased in the prior paragraphs.

Scientists at Stanford University have successfully inserted a metallic nanoelectrode into the chloroplast of *C. reinhardtii*, and produced a small electrical current. The scientists were able to capture mobile electrons moving between the chloroplast's thylakoids. This technology can be applied to *C. reinhardtii* upon its genetic modification, to allow for the production of clean electrical energy, helping to reduce fossil fuel energy usage, and in turn reducing the negative

effects of climate change. After the genetic modification of *C. reinhardtii*, based on results from later experimentation, the removal of *C. reinhardtii's* chloroplast could become reality.

To genetically modify *C. reinhardtii*, we plan on using the highly efficient CRISPR/Cas9 method. The operons in *I. sakaiensis* responsible for the enzyme creation, as well as PET syntheses, can be found between β-strand 7 and α-helix 15 of the α/β-hydrolase fold. The guide RNA segment in CRISPR/Cas9 will be programmed to target this specific branch of the bacterium. However, it remains uncertain where we would insert the snipped genetic information into *C. reinhardtii*.

This technology can be applied to function in homes, and other similar facilities across the globe. We envision the usage of "plastic composters" that utilize this technology to allow the everyday household to eliminate their impact of PET pollution. Users are able to place any PET waste used into the composter and the genetically modified *C. reinhardtii* will convert the PET into electrical energy collected by the previously installed clusters of joint metallic nanoelectrodes. *C. reinhardtii* is able to thrive in the environments it will be subjected to, as an oligotrophic algal species. *C. reinhardtii* will not be exposed to nutrient deprived environments due to the abundance of PET present, however, it is useful to have bacteria capable of sustaining their life for long periods of time without access to energy incase this becomes necessary.

Breakthroughs

To develop and implement this technology into homes globally, many technological breakthroughs are necessary. The genetic modification of *C. reinhardtii* by inserting PETase and MHETase operons is possible since both organism's genomes have been sequenced, however more research is crucial to *C. reinhardtii's* genetic modification to discover the exact place to insert the snipped operons from *I. sakaiensis*.

Also, technology involving the widespread commercial development of genetically altered bacteria needs to become reality. Technology involving the commercial insertion of nanoelectrodes into large number of cells also needs to be developed. This could be done in the case of *C. reinhardtii* by allowing large numbers of *C. reinhardtii's* cells to be punctured by the abrasive lining of a cluster of joint metallic nanoelectrodes, or similar.

The scientists who conducted the research involving the extraction of electrons from *C. reinhrdtii* successfully withdrew a small electrical current of ~250 nanowatts (nW) of electricity. Despite this small current, the scientists responsible for this research have stated that the research is still in its early stages, and there is plenty of room for necessary improvement.

Specific research and experimentation are required to take place to determine *C. reinhardtii's* capability to express PETase and MHETase operons. The genetic modification of *C. reinhardtii* involves the complex modification of multiple genes. No organism has been genetically modified to this drastic standard, so experimentation would need to be conducted into multi-gene genetic modifications. To theoretically test this, a synthetic operon for both PETase and MHETase that are capable to synthesize PET will be produced. To achieve this, the modification of 12 β-PETase and 12 β-MHETase genes extracted from *I. sakaiensis* to express the construct in *C. reinhardtii*, which creates two synthetic operons. This is crucial to allow for the genes' expression in *C. reinhardtii*. The exact regions of these genes in *I. sakaiensis* remain unknown, however with more research and analysis of *I. sakaiensis'* genome, this could be discovered. The synthetic operons created involve the combination of the (estimated) 4 primary operons into two separate operons, resulting in the creation of the two operons responsible for encoding the bacterium with PET synthesizing properties. However, as mentioned previously,

nothing has been genetically modified over multiple genes, and no experimentation or research has been done on the topic.

After the operons responsible for PET degradation are inserted into *C. reinhardtii*, research should be conducted to determine the efficiency of this new species of bacterium. Currently, *I. sakaiensis* can eat through a plastic bottle in about a day. If the results with this new species are not optimal, then small changes may be made to the operons genes, in which specific genes and nucleotides thought to bring faster PET degradation could be added to the organism's MHETase, or PETase operons as necessary.

Design Process

Before reaching the current spot in our project's development, we went through various phases of planning and design revisions. These original features include the modification of an entirely different bacterium, *Aquincola tertiaricarbonis* strain MS86-16S, different approaches to extracting the chemical energy derived from PET, as well as different approaches for genetically modifying the cell.

To begin with, we originally planned to modify the bacterium *Aquincola tertiaricarbonis* strain MS86-16S with the PETase and MHETase operons extracted from *I. sakaiensis*. The bacterium has more than a 99% genetic similarity to *I. sakaiensis* which allows for potentially easier genetic modification. However, this particular strain is not able to thrive and survive as well as *C. reinhardtii* is able to in the various environments we would be subjecting these organisms into. Also, *A. tertiaricarbonis* is not as accessible or widely produced as *C. reinhardtii*. These factors convinced us to develop the technology necessary to modify *C. reinhardtii* opposed to *A. tertiaricarbonis*.

Additionally, we originally planned to use singular nanoelectrodes to be inserted into *C. reinhardtii's* chloroplast. Later research deemed the use of singular nanoelectrodes inefficient, and with more analysis of the processes behind *I. sakaiensis's* plastic synthesis process, determined the withdrawal of electrons from the cell's chloroplast useless, as the cell's energy will be produced elsewhere. The adaptation of the previously mentioned cluster of joint metallic nanoelectrodes was chosen to allow for maximum energy collection. It was also decided after more research and analysis, that opposed to collecting the cell's energy from the inefficient chloroplast, the nanoelectrode would be inserted into the cell's mitochondria to allow for the extraction of electrons.

In the earlier stages of the project, we planned on using the plasmid method to genetically modify *C. reinhardtii*. Based on the research we did, we automatically knew that the usage of either the plasmid or CRISPR/Cas9 method would be the most beneficial for commercial standards and efficiency. Upon more research, the application of the CRISPR/Cas9 system was adapted. This is mainly due to CRISPR's natural ability to precisely cleave the target DNA's genome at exact locations. This is useful in the situation of *I. sakaiensis* and *C. reinhardtii*, because multiple sections of DNA will need to be cleaved. The only plausible solution to this is the usage of CRISPR/Cas9 due to its well-known efficiency and speed at genetic modification.

Consequences

Any creation of scientific technology impacts the scientific community tremendously. Whether that impact is positive or negative, it is important to analyze the pros and cons, and face the challenges.

The use of this technology would positively impact marine ecosystems, reducing the millions of metric tons of PET entering their habitats annually. The average household uses ~75

kilograms of PET annually. With the elimination of this plastic debris, humanity could reduce its plastic pollution footprint, inspiring positive changes in countless communities and ecosystems across the globe.

This technology also brings negative consequences, such as the potential introduction of this species into the wild, which is strictly forbidden among the scientific community internationally. The probability of this happening is low, since the bacterium will synthesize PET in homes and similar facilities such as office buildings, etc., however accidental introduction of the bacterium could cause unpredictable environmental damage.

Another negative consequence of the usage of this technology could be the cost. While it is unknown, the upfront cost of the technology could prevent many households from implementing this innovation into their daily lives. However, with the increased development of genetic engineering technology, it is predicted that the cost will decline in the future.

With the use of the genetically modified *C. reinhardtii* to degrade plastic in homes and similar facilities globally, humanity can make a difference and unite to counteract the untold damage PET is causing in ecosystems.

With persistence, creativity, and technological advancements such as the innovation we've proposed, polyethylene terephthalate pollution will be no more.

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