The investigation of evolutionary relationships of exoelectrogenic bacteria via phylogenetic analysis of 16S rRNA gene sequences

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Plastic is a potential source of electrons that can be used to power microbial fuel cells. Given the right combination of enzymes and promoters controlling the genes coding them, a plan for using polyethylene terephthalate (PET) plastics as a source of electrons can be considered to drive such microbial fuel cells. *Ideonella sakaiensis* is a recently discovered bacterium with PETase and mono-2-hydroxyethyl terephthalate hydrolase (MHETase) enzymes, both of which are directly responsible for the breakdown of PET plastics to molecules that bacteria can use and metabolize to generate ATP. While there are problems with the use of *I. sakaiensis*, including its slow growth rate, synthetic biology solutions that enable the use of appropriate promoters coupled with appropriate open reading frames can be used to generate exoelectrogenic capacity in which electrons are transferred extracellularly. Very little is known about *I. sakaiensis*, including its evolutionary relationships with other bacteria. In this study, we explore the phylogenetic relationships of a broad group of bacterial species using 16S rRNA gene sequence comparison. Included in the group sample are species that have exoelectrogenic capacity and *I. sakaiensis*. We use this phylogenetic approach to propose bacterial species that may be studied further with the potential to release electrons and possibly have plastic-degrading activity. This exploration serves as the first step towards designing synthetic biology solutions by identifying potential species with usable synthetic biology parts. This may impact how different problems can be solved and can provide a different approach to solve these problems. For example, the problem of PET plastic waste may be solved through the use of bacteria, instead of only recycling.

Keywords: *Ideonella sakaiensis*, exoelectrogenic bacteria, microbial fuel cell, ribosomal RNA gene sequence, PET plastic

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Background

The ability to generate electricity by microorganisms is a fascinating idea to touch on and has also been a scientific interest for many years already (Sevda et al., 2018). Microorganisms that are able to generate electricity can be used in many different applications, for example wastewater treatments. Wastewater treatments require the use of bacteria to go through the process of respiration in order to produce electricity as a return. Wastewater treatments are basically huge microbial fuel cells that are capable of converting wastewater into usable electricity through the use of bacteria. These bacteria with the capabilities to generate power density and to be used to power microbial fuel cells are called exoelectrogenic bacteria. They generate electrical energy through the oxidation of organic matter (Jurtshuk, 1996).

Currently, plastic is a huge problem in countries all over the world. In 2016 alone, there were a total of 485 billion polyethylene terephthalate (PET) bottles produced, and it has been forecasted that a total of 583.3 billion of these will be produced in 2021 (Tiseo, 2021). Most of these PET plastics are being recycled. In fact, PET bottles are 100% recyclable and are actually deemed the most recycled plastic in the world (SpecialChem). In order for these plastics to be recycled, they go through a series of processes, including special washings or the use of chemicals to break down these plastics into their raw materials. The raw materials are then used to produce PET flakes. These PET flakes have several applications, including containers for food, beverages bottles, fiber for carpet, etc. This process of recycling seems to be good, without causing a lot of waste problems; however, there is still an issue regarding the method. Although PET plastic can be recycled, it takes a long time to degrade. According to the EPA, 29.1% of the recyclable PET bottles were actually recycled in 2018. Yet, in the U.S alone, 3.13 million tons of PET were discarded as of 2018 (LeBlanc, 2021). A huge percentage of the recyclable PET plastics were not put into use and are discarded into landfills instead. Humans can continue to recycle plastic, but this plastic would always be there. We can consider the burning of plastic waste, but that will only lead to greenhouse gas emissions.

Further research has shown that a bacterium called *Ideonella sakaiensis* has the capability to break down plastic through the secretion of PETase enzyme and MHETase enzyme (Tanasupawat, 2016). *I. sakaiensis* will secrete PETase and degrade PET to monomeric mono-2-hydroxyethyl terephthalate (MHET). MHET is then degraded by MHETase to produce ethylene glycol and terephthalic acid. The final product of ethylene glycol is used by the bacteria, by which it is metabolized and used as a carbon source. Terephthalic acid is oxidized into protocatechuate (PCA), which then goes through the TCA cycle, otherwise known as the citric acid cycle. This is a series of chemical reactions that produce adenosine triphosphate (ATP). The products produced from the degradation of PET by *I. sakaiensis* are therefore used as their energy source.

I have decided to look into different applications of *I. sakaiensis*, such as the use of *I. sakaiensis* in microbial fuel cells, using plastic as its source of energy. In order to confirm that *I. sakaiensis* may have the potential to release electrons, I have chosen to use a phylogenetic analysis of the 16S rRNA gene sequences of different bacteria to determine whether they have the exoelectrogenic capabilities. The creation of a phylogenetic tree of all these bacteria will show their evolutionary relationships and hopefully find the possibility of *I. sakaiensis* being exoelectrogenic. Not only will this open a new idea to the possibility of breaking down plastic, but it will also do it in a manner that would reduce greenhouse gas emissions.

Materials and Methods

16S rRNA gene sequence analysis

The first step to this development was to find the FASTA file of the 16S ribosomal RNA gene sequence. The 16S rRNA gene is responsible to encode the small subunit of the ribosomal RNA in the ribosome. It is essential for many processes, including the conversion of genetic messages to the functional cell components through the translation of mRNA to proteins. The analysis of 16S rRNA sequence is able to reveal the conserved and variable regions To do this, I have used the National Center for Biotechnology Information to gather the information needed. Past articles/lab reports on electroactive microorganisms in the bioelectrochemical systems have provided a list of bacteria with their known power density. The 16S ribosomal RNA sequence of all of these bacteria have been compiled into a FASTA file, each bacterium with a name that is shorter and more understandable by the reader, for the purposes of analysis in future uses. Furthermore, 16S ribosomal RNA gene sequences of different *Ideonella* bacteria have been obtained to create a phylogenetic tree to determine which bacteria is genetically closest to it. A total of more than 50 16S rRNA sequences were combined in the file.

Not only was the NCBI search used in the process, NCBI BLAST was also used. The 16S ribosomal sequence of *I. sakaiensis* was pasted into the search bar, and BLAST-searched to obtain a total of 100 gene sequences that were genetically very similar to that of *I. sakaiensis*. 68 of these genes were downloaded into a text file, and each of the organism's scientific name was changed to be easily understandable by the reader (as seen below).

The compiled FASTA file was uploaded into Custal Omega for phylogenetic analysis. The information in the text file is compiled and is used to form a phylogenetic tree.

Laboratory Safety and Precaution

This investigation was solely done using computer software, so there was no need for any laboratory safety and precautions.

Results

rRNA gene sequence analysis of phylogenetic tree

Figure 1 shows an image of all the bacterial gene sequences that were obtained from NCBI. All of these

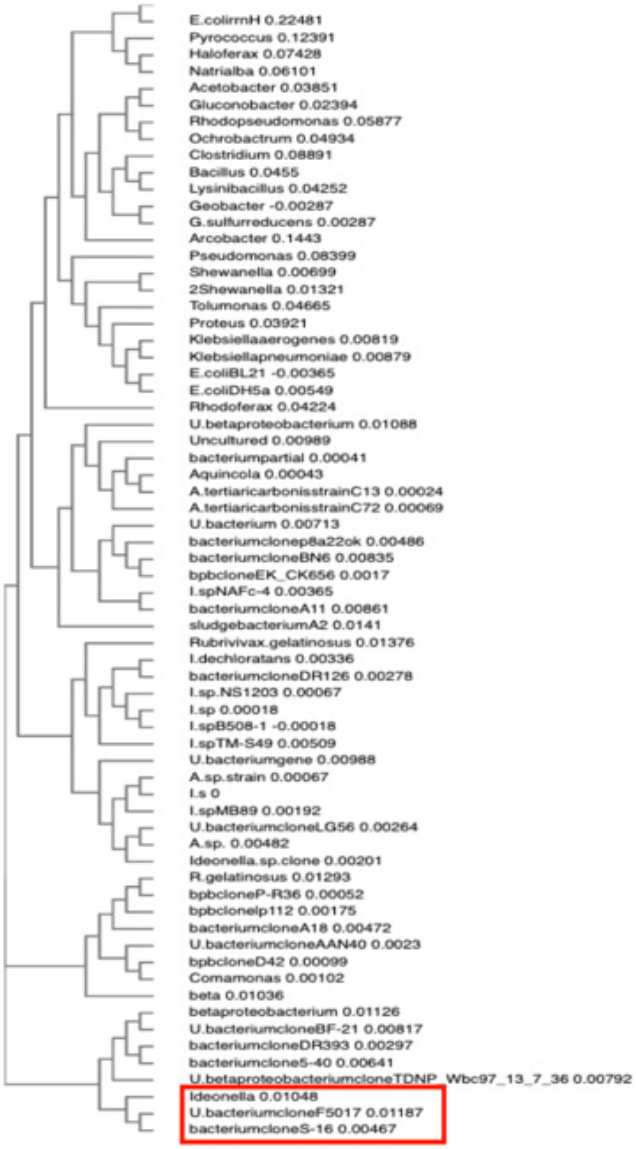

Figure 1. *Phylogenetic tree of 16S rRNA gene sequences similar to I. sakaiensis.*

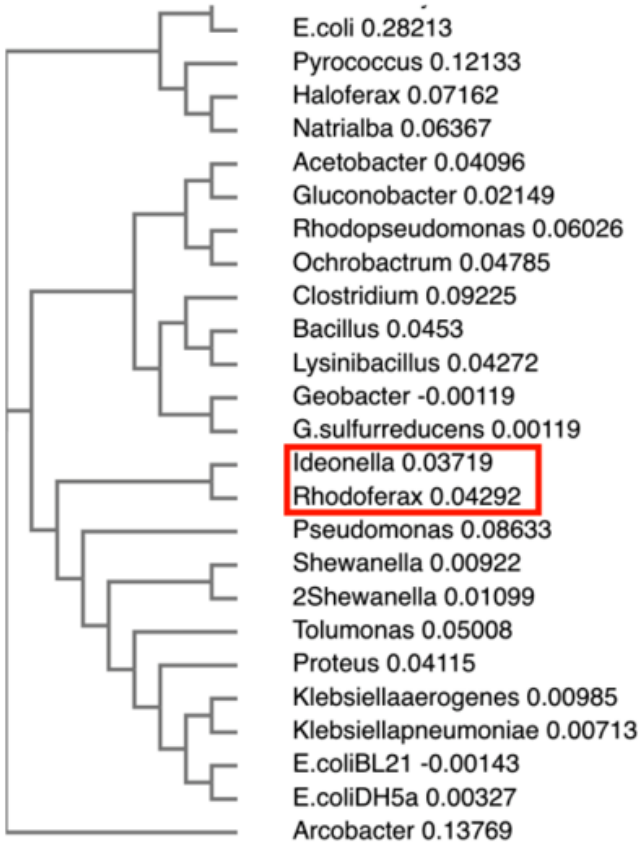

Figure 2. *Phylogenetic tree of 16S rRNA gene sequences from bacteria with known power densities*

sequences were combined to form a phylogenetic tree that displays the evolutionary connections between the bacteria. *I. sakaiensis* is at the bottom, along with a few other Betaproteobacteria clones. This shows that *I. sakaiensis* is genetically closest to these nearby bacteria.

Figure 2 shows a list of bacteria with known power density in comparison to that of *I. sakaiensis*. From this, we can further and closely see which of the bacteria are more similar to *I. sakaiensis*. We can see that Rhodoferax ferrireducens was put in the same bracket as

```
#
#
#  Percent Identity  Matrix - created by Clustal2.1
#
#

      1: Ideonella    100.00    91.99
      2: Rhodoferax    91.99   100.00
```

Figure 3. *Clustal 2.1 identity matrix for the 16S rRNA gene sequences of Rhodoferax ferrireducens and I. sakaiensis*

I. sakaiensis, and so we can deduce that they are genetically the closest among all of the other bacteria that are presented here. Because Rhodoferax is closest to *I. sakaiensis*, we compared the two bacteria by inputting

their 16S ribosomal RNA sequence into Clustal Omega, as can be seen in Figure 3.

Figure 3 here shows the percent identity matrix of the comparison of Rhodoferax ferrireducens and *I. sakaiensis*. From these four numbers, we can tell that the Rhodoferax and *I. sakaiensis* are 91.99% identical. The 100% in the numbers shows the comparison of *I. sakaiensis* and *I. sakaiensis*, as well as R. ferrireducens with R. ferrireducens. By looking at these figures, we can say that both these bacteria are genetically quite similar in terms of the 16S rRNA genetic sequence, however we do not know how similar this 91.99% is and if this similarity suggests that *I. sakaiensis* is exoelectrogenic.

Discussion

The percent identity matrix can show the similarity in the different bacteria that are being analyzed; however, we may not know to what extent this similarity is. When looking at Figure 3, we can see that both R. ferrireducens and *I. sakaiensis* are genetically quite similar, and we may assume that a lot of their characteristics are similar as well. The benefit of using a phylogenetic tree to determine exoelectrogenic bacteria is that we may be able to assume that a bacterium is able to produce electricity by analyzing whether or not the bacterium's "cousins" are also able to generate electricity. From Figure 1, we are unable to really deduce whether or not *I. sakaiensis* is able to generate electricity, as we do not know the power density of the values of the bacteria that it was genetically close to. However, in Figure 2, we have a clearer picture of which bacteria are closest to each other in terms of bacteria that already have their known values of power density. Additionally, in Figure 3, we see a percentage identity matrix of just R. ferrireducens and *I. sakaiensis*, and we can see that they are 91.99% identical, which means that they may share a lot of common characteristics. Considering that both of these bacteria are Betaproteobacteria, we can deduce that both of these bacteria could have exoelectrogenic properties. R. ferrireducens has a power density of 33 mW m-2 (Logan et al., 2019), which means that if *I. sakaiensis* also has exoelectrogenic properties, it may also have similar power density, and it could be more or less similar to that of R. ferrireducens.

Furthermore, if we assume that *I. sakaiensis* does in fact show exoelectrogenic properties, they may be able to be used in a microbial fuel cell, by using plastic as its main source of energy. As explained in the background section, *I. sakaiensis* is able to break down plastic into necessary products used as energy sources, and with the hypothetically discovered electrogenic properties, we can say that we might be able to break down plastic in a much more sustainable manner. This can lead to many new innovations and new ideas of how we can approach both the energy problem, as well as the PET plastic problems we have right now.

However, this way of determining whether a bacterium has exoelectrogenic properties also has its limitations. We cannot fully confirm that *I. sakaiensis* has the capabilities to produce electricity. We have used a phylogenetic approach to this problem. However, without conducting any further experiments, we cannot fully say that *I. sakaiensis* really has exoelectrogenic properties. It is possible that even with the high percentage identity, the remaining 8% could include the differences in terms of having exoelectrogenic properties or not having those properties. There are many factors to take into consideration when looking at the gene sequence of two bacteria. This 8% difference occurs on the 16s rRNA gene. However, there is probably a much greater difference at the whole genome level. 8% can cause a huge difference too. We can take humans as an example, we humans are genetically 99.9% identical, however the remaining 0.1% difference is the reason why some of us get certain diseases while others do not. 0.1% may not sound a lot, but the amount that this 0.1% holds is a lot (National Human Genome Research Institute, 2018)

Another limitation is that we do not have the information of power densities for enough bacteria. The bacteria closely related to *I. sakaiensis* listed in Figure 2 may have exoelectrogenic properties, but due to the lack of data, we are unable to link these bacteria directly to *I. sakaiensis*. Due to this limitation, our data and our interpretation may not be as accurate as it can be. If we were able to find a bacterium with exoelectrogenic properties, as well as having the identity percent matrix of higher than 92%, then our interpretation of that data would be more accurate than what we have right now. Due to lack of data, we were unable to fully confirm the presence of exoelectrogenic properties in *I. sakaiensis*.

Next Steps

In the near future, I plan to perform this investigation experimentally. As of right now, it is hard to confirm whether or not *I. sakaiensis* really has exoelectrogenic properties. To determine this hypothesis and this claim, it is good to test it out, and take in results to conclude whether or not our claim was true. One way to test this claim out could be to use *I. sakaiensis* as the bacterium in a Microbial Fuel Cell. If voltage could be produced through this method, then it may mean that *I. sakaiensis* has exoelectrogenic properties.

Alternatively, we may also express these *I. sakaiensis* plastic-degrading proteins in an organism with more standardized genetic parts, like Shewenella, which, as seen from above, has been shown to have exoelectrogenic properties. This way we can accurately assume

that this new bacteria with the genes of *I. sakaiensis* and Shewanella has both plastic-degrading properties-, as well as exoelectrogenic properties. Thus, it makes it plausible that this bacterium can also function in a Microbial Fuel Cell.

We may also look into trying to find the presence of exoelectrogenic properties in bacteria that are very closely related to *I. sakaiensis* based on the blast search conducted on NCBI. Some of these bacteria found were genetically really close to *I. sakaiensis*, and if these bacteria were to be proven to have exoelectrogenic properties, there might be a higher chance that *I. sakaiensis* also does. If it is not possible to obtain *I. sakaiensis* bacteria, then we may resort to a different method, which is to take another bacterium available that is genetically similar to *I. sakaiensis*, and test it out.

Another experiment that can be done before jumping into the lab is to determine if there are any conserved genes or gene clusters that are responsible for these exoelectrogenic properties. It may be easier to compare the similarity between these genes with that of *I. sakaiensis* to determine if there are any analogous genes present. A simple blast/alignment can be used to determine the similarity index of these genes with that of *I. sakaiensis*.

It would definitely be helpful to confirm our claim through the use of laboratory experiments. To expand this project, the team goal would be to first, to test out the hypothesis, and second, to attempt to improve plastic degradation properties of *I. sakaiensis*. Because *I. sakaiensis* is known to be slow at plastic degradation (Andersen, 2019), it will be a good idea to engineer the bacterium and improve the plastic degradation properties. This improvement will be able to successfully improve the efficiency of the system.

Author Contributions

The entirety of this article was written by Yu Tung Lee, which includes the results and all the research that was done.

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