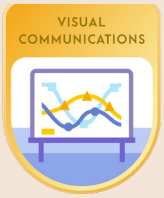
Engineered Bacteria for Ocean PET Degradation



Puloma Bishnu, Krishnaveni Dole, Yashvi Gosalia
BioBuilder Club, Andover High School, Andover, Massachusetts, USA

Reviewed on 11 May 2019; Accepted on 17 June 2019; Published on 28 October 2019

PET (polyethylene terephthalate), the plastic that water bottles are made from, is not naturally degraded and accumulates in oceans around the world, contributing to the problem of pollution and effectuating significant environmental damage. Our project utilizes a strain of gram-positive, spore-forming, genetically engineered halophilic bacteria, *Bacillus tianshenii*, that can break down PET in the presence of salt water. In their inactive spore form, the bacteria are placed between a bottle and its full-length bottle label, along with a dried medium of nutrients. When in salt water, the spores will germinate and consequently express the two genes needed for the decomposition of the bottle: PETase and MHETase, originating from the bacteria *Ideonella sakaiensis*. These bacteria express the two genes coding for the enzymes required for breaking PET down to its monomer, MHET (monoethylene terephthalate), and then further to environmentally benign compounds ethylene glycol and terephthalic acid. These two genes are ISF6_4831 (PETase) and ISF6_0224 (MHETase). Ethylene glycol and terephthalic acid can be utilized by the engineered bacteria, as well as other ocean-living bacteria, as a carbon source. The gene coding sequences are optimized for expression in *Bacillus* and for activity in salt water. They are driven by two constitutive *Bacillus subtilis* promoters (BBa_K143012 and BBa_K143013). Two strong ribosome binding sites (BBa_K780001 and BBa_K090505) and two terminators (BBa_K780000 and BBa_B0010) from *B. subtilis* are also used. A *Bacillus* bacteriophage phi29 is part of the design as a kill switch to control the population of the engineered bacteria in the environment if needed.

Keywords: (Poly)ethylene terephthalate, PET degradation, plastic, pollution, *Bacillus tianshenii*

Mentors: Lindsey L'Ecuyer, Patrick Holec
Direct correspondence to llecuyer@k12.andoverma.us

Watch a video introduction by the authors at http://bit.ly/31HWyOG

Background

Due to its configurational versatility and convenience in everyday living, polyethylene terephthalate (PET), a type of plastic that is commonly used, has taken a role in many aspects of our lives today in the form of plastic wrappers, bottles, and more. However, plastic takes an average of 1,000 years to decompose, which means that every piece of plastic ever created is still present on Earth (Gonzaga 2007). Considering its current rate of production, the use of plastic products is not sustainable. An estimated 5.25 trillion pieces of plastic are floating in oceans and make up 60 to 90% of all marine debris (Surfers Againts Sewage 2019). Furthermore, microplastics have become a significant health risk due to its increasing presence in our food sources, such as seafood. Synthetic plastic was created as a way to lessen the impact of human consumption on the natural world, but is now a major cause of ecological, economic, and human health problems.

Microplastics are everywhere. When ingested through food like the fish we eat, it's been proven to have adverse effects on humans. This is because when humans consume fish, they also consume these plastics (Borunda 2017). Though plastic ingestion has been found to cause inflammation and scar tissue, scientists are now more concerned that this consumption of plastic may be linked to cancer as well (Ciccarelli 2019). Additionally, some plastics break down into unsafe chemicals which can harm both humans and the environment. For instance, Bisphenol A, or BPA, can break down over time and let toxic chemicals, like plasticizers, leach out. BPA and other additives that break down in landfills hold the long-term risk of further contaminating soils and groundwater (Hopewell et al. 2009). When the groundwater is polluted, it can be swallowed by humans through their drinking water source, and is yet another way that microplastics can enter the body, resulting in severe side effects. BPA has been "recognized since the 1940s as an endocrine disrupting chemical that interferes with normal hormonal function" (Harth 2010).

As a result of these problems, scientists are researching ways to significantly reduce plastic waste, such as biodegradable plastics. Without a sustainable solution, there will be damaging repercussions for life on Earth. We have developed a method of permanently removing plastic from oceans by degrading it into simple organic compounds using marine bacteria that are synthetically engineered to decompose PET. Our objective throughout this experiment was to create a tenable solution to plastic pollution by finding a way to fully disintegrate plastics. The plan we designed involves fixing our bacteria to the inside of a plastic water bottle label such that when it eventually reaches the ocean, it breaks down the bottle and label it was placed on. Plastic has become an integral part of daily life, thus it would be nearly impossible to universally stop its use. Our idea to genetically engineer bacteria to break PET down into two environmentally benign compounds can revolutionize the way we use plastics. The production of this bacteria can provide a sustainable way to retain the versatility and convenience of plastic while eradicating the debilitating effects of plastic pollution.

Systems Level

The basic components of our design (Figure 1) include a standard water bottle made of PET, a full-length bottle label also made of PET, and our synthetically engineered bacteria, *Bacillus tianshenii*. The bacteria will be placed in between the label and the bottle, along with dried nutrients, such as marine agar 2216, and an antibiotic reagent (American Type Culture Collection 2015). When this design interacts with ocean water, the bacteria will germinate, as the salt water concentration of the ocean is ideal for *B. tianshenii*. The bacteria will also utilize the nutrients provided to aid its growth. Once the bacteria have germinated and become fully active, they will express the inserted genes and synthesize enzymes PETase and MHETase to break down the bottle and its label.

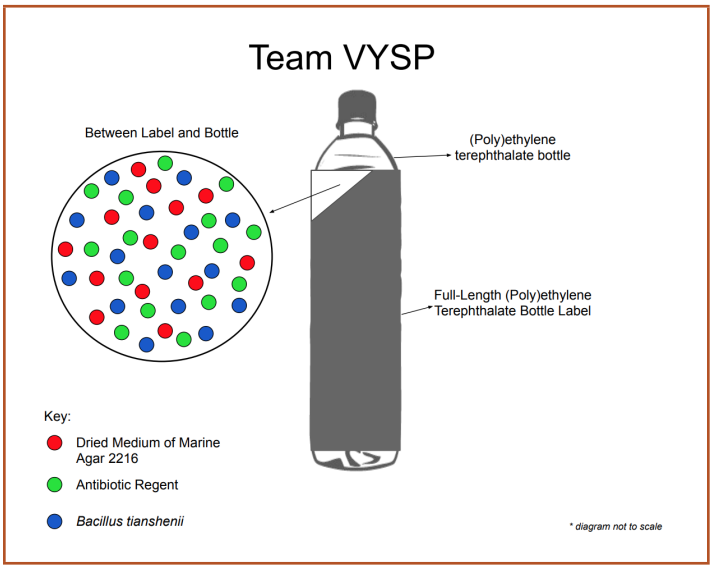


Figure 1. *Diagram of intended design*

Through the process of complete enzymatic hydrolysis, the enzyme PETase can degrade PET to its monomer, monoethylene terephthalate (MHET). Then, the enzyme MHETase will degrade MHET to ethylene glycol (EG) and terephthalic acid (TPA). These are two organic compounds that naturally occur in the ocean and can be

used as a carbon source for several marine organisms, including *B. tianshenii*. Our design has the capability to repurpose plastic pollution and yield a useful product for marine life.

Device Level

Upon beginning research, we delved into the chemical composition ofPET (Figure 2) as well as previous studies conducted on the nature of its decomposition. According to a recent study done, bacteria Ideonella sakaiensis 201-F6 have been found to hold the genes necessary for the production of PET-degrading enzymes (Yoshida et al. 2016).

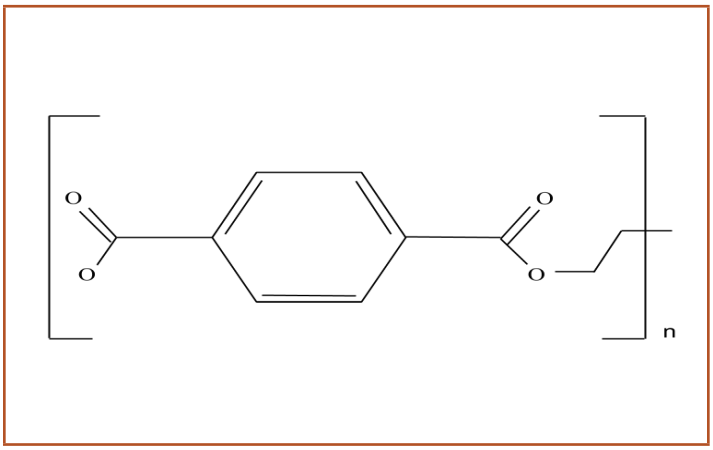


Figure 2. *Polymer structure of (poly)ethylene terephthalate, where n is the number of times this polymer structure is repeated*

I. sakaiensis 201-F6 has been found to hydrolyze a thin layer of PET film in an average of 6 weeks (Coghlan 2016). This analysis was done under a temperature of 30° C, and *Ideonella*'s maximum growth temperature for PET hydrolysis spans from 55 to 70°C (Universal Protein Resource 2016). However, noticeable degradation had been made after 6 weeks under its conditions, leading us to research other bacteria that may do this, but under the stipulation that they are halophilic and native to the ocean. With these set prerequisites in mind, we were able to distinguish the bacteria that could potentially produce optimal results for our desired purpose.

A study published in the International Journal of Recent Advances in Multidisciplinary Research revealed results of biodegradation of PET by a bacteria called Bacillus subtilis (Nakkabi et al. 2015). However, this bacteria is found to live in the gastrointestinal tract of ruminants and humans, as well as soil (Wickham Laboratories 2015). This would mean that B. subtilis is not well equipped to withstand marine environments. However, Bacillus's ability to become inactive by forming spores that protect it against extreme environments is ideal for our intended purpose. Furthermore, this genus has been thoroughly experimented on within the synthetic biology field. This means that many resources, such as parts optimized for Bacillus, are available to us for use. Therefore, rather than choosing an entirely different genus, we sought out an isolate of *B. subtilis*. Marine isolates of the strain can still grow starting from 15°C, and on average, the temperature of the ocean is reported to be 17°C (Windows to the Universe 2011). However, the average pH of the ocean is 8.1 and optimal growth was not found at this specified pH (Jiang et al. 2014). Taking this into consideration, we continued to search for marine isolates.

As a result, we were able to find a bacteria that grew optimally at 30°C, pH 7.0 and in the presence of 2–4% sodium chloride. This bacteria is *B. tianshenii*, isolated from a marine sample in the South China Sea. Though its optimal pH is 7.0, it can withstand a pH range of 6.0-9.0. Its temperature range is 10 to 50°C, and growth can occur in the presence of 0-7% NaCl. Additionally, it produces endospores in inhospitable environments, rendering itself inactive (Setlow 2014). This is a necessity if this bacteria will be placed where humans can potentially make contact. Additionally, the ability to form endospores ensures that the bacteria will survive while the bottle is in use or storage. From the information gathered, it can be inferred that *B. tianshenii* can survive in the ocean.

To induce *B. tianshenii* into an inactive state, a dry environment will be provided to initiate the production of endospores, as this bacteria is halophilic and its spore form will deactivate in the presence of salt water. Spores are formed as a means of protection in unfavorable environments. Formed within the mother cell compartment of a sporulating cell, the spores are then released into the environment when the mother cell lyses, and become extremely resistant to most environmentally stressful conditions. Additionally, spores of the *Bacillus* genus have little to no metabolic activity and are considered inactive as a result, although just after their formation, a brief period may occur in which the produced spores display some metabolic activity. After this interval, however, spore metabolic activity has shown to be minimal and potentially nonexistent (Setlow 2014). Because of this, should anyone come into direct contact with these bacteria, it will theoretically have no harmful effect upon the subject, and an energy source will not be required to keep the bacteria alive for extended periods of time.

B. tianshenii has a positive gram stain, as is also preferred, due to the possibility of saline-lacking water or condensation appearing on the labels of water bottles. Gram-positive bacteria have been found to survive longer in PBS (phosphate-buffered saline) solutions compared to water (Liao and Shollenberger 2003). Therefore,

in addition to an unfavorable dry environment rendering it inactive, should any water infiltrate our design and reach our bacteria, it should not germinate, and in the event that it does, its survival rates will be low. For this reason, when choosing our bacteria, we kept in mind that gram-positive bacteria would be the ideal choice. Figure 3 depicts the difference in the cellular structure of gram-positive and gram-negative bacteria. Gram-positive bacteria typically have a cell wall consisting of a thick layer of peptidoglycan. Many bacteria in this group are also known to produce spores in response to starvation or harsh conditions (Gontang et al 2007). *B. tianshenii*, our chosen chassis, meets this condition in addition to its marine origin.

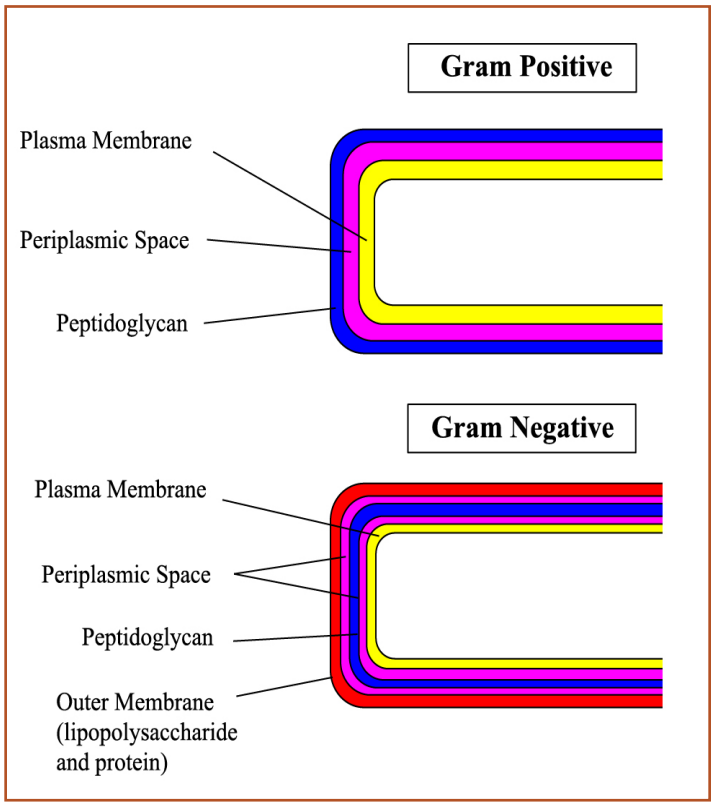


Figure 3. *A visual depiction comparing the cellular structures of gram positive and gram negative prokaryotic organisms*

Although *B. tianshenii* is aerobic, it will still survive near the water's surface, which is where the plastic bottle will be present. Thus, its preferred method of respiration will not pose an issue (Jiang et al. 2014).

When our modified strain of *B. tianshenii* makes contact with ocean water, it will germinate as a result of its ideal environment and a medium of nutrients, located under the label of the bottle alongside the bacteria. Once the spores have germinated, they will become fully functioning cells and will begin to transcribe the genes for PETase and MHETase, as our bacteria are designed to produce these enzymes. PETase breaks down PET into its monomer, MHET. Then, MHETase breaks down MHET to ethylene glycol and terephthalic acid. Ethylene glycol and terephthalic acid are organic, environmentally benign compounds. They can be used as carbon sources for several marine organisms, primarily bacteria, including *B. tianshenii*.

Parts Level

We have created a theoretical design to express the genes and their respective coding sequences needed to produce the enzymes that will break PET into its monomer, MHET, and then further into compounds ethylene glycol and terephthalic acid. These genes come from the aforementioned bacteria, *I. sakaiensis*, that produces enzymes PETase and MHETase to break down PET and MHET, respectively. The gene that codes for PETase is ISF6_4831, and the gene that codes for MHETase is ISF6_0224. The genes would be extracted from *I. sakaiensis* 201-F6 and put into our chassis, *B. tianshenii*. The gene coding sequences would be codon-optimized for expression in *Bacillus* and engineered for activity in salt water.

Our design utilizes two gene coding sequences; thus, two promoters, ribosome binding sites, and terminators are also required to express the integrated genes. The promoters that would be used are two constitutive promoters for *B. subtilis* (BBa_K143012 and BBa_K143013). Additionally, we have chosen two strong ribosome binding sites (BBa_K780001 and BBa_K090505). Lastly, we included two terminators (BBa_K780000 and BBa_B0010). The chosen parts are optimal for *B. subtilis* and can be codon optimized for use in our strain. A separate experiment regarding the duration of the bacteria's circuit and potential time mutations will be performed.

Safety

Using a selective, complex growth medium, such as a marine broth (ATCC medium formulation 2) with an antibiotic reagent, *B. tianshenii* can be germinated (American Type Culture Collection 2015). Upon the conclusion of its growth period (growth period dependent upon the number of bacteria needed to complete an analysis of *B. tianshenii*'s efficiency of PET degradation [will be obtained through bacterial titering]), the germinated bacteria will be placed on the label and dried over the course of 24 hours. Spores will be expected to form as a result of its adverse environment. Its spores are not known to produce any harmful effects on human life and our ecosystem. However, if necessary, the spore population can be eradicated with exposure to high pressure (15 psi) and high temperature (121°C).

In a lab environment, we will perform this process, place the bacteria on the label, and subsequently place the label on the bottle with a dried medium of nutrients underneath. The bottle will then be placed in a simulated ocean environment whose composition will consist of a 2.5% salt water solution. Over the course of 6 weeks, this simulated environment will be monitored and recorded. In an outside environment, should lab results show promise, the same procedure of the inducement of *B. tianshenii* spores will be performed. The environment in which the experiment is performed will be in a location with minimal to some occurrence of human activity, and in a medium-sized body of water with a tested salinity of at least 2.5%. A tracking tag will be put on the bottle.

As with all scientific designs, it is essential to consider its potential consequences. As mentioned above, our bacteria produces the enzymes PETase and MHETase to eventually break down PET into ethylene glycol and terephthalic acid, both of which are environmentally benign compounds that are common carbon sources for a variety of marine organisms. This means that, hypothetically, releasing our bacteria into the ocean or other salt water environments should not hold a directly negative impact on the ecosystem. However, it may affect population sizes, and could, therefore, impact the environment in various ways.

We recognize that the exact effects of our bacteria on the ecosystem cannot be predicted, and for this reason, our design includes a kill switch. Phi29, a bacteriophage that has been used for the targeted elimination of the *Bacillus* species, will be cultivated as a precaution if the engineered bacteria are released into the ocean (Meijer et al. 2001). Though this phage has been tested on *Bacillus* strains and has been proven to effectively eliminate colonies, a separate experiment regarding the effectiveness of phi29 and its ability to eliminate the engineered strain of *B. tianshenii* will be conducted.

Discussions

It is important to note that studies conducted on bacteria or fungi concerning the degradation of PET are still in the primary stage, as the compact chemical structure of this polymer creates difficulties to the manner in which one can decompose it. For this reason, it is not expected for the decomposition of PET to be swift. The potential that this design holds to remove plastic permanently proves to be our greatest incentive, as no significant actions have yet been taken to permanently eradicate PET.

The initial stage of our lab work is to use an online Molecular Biology Suite platform called Benchling. Using this platform, we plan to design our plasmid and identify restriction enzymes that cut the DNA at specific sequences. We will insert our codon-optimized DNA sequences, in addition to selecting and testing replication origins, selection markers, promoters, and other regulatory elements (Tolmachov 2009). The plasmid design will also include antibiotic resistance, which is just one of many methods of selection to shut down cells that are not transformed. Via our designed plasmid, we will integrate our gene expression cassette into the chromosome of the *B. tianshenii* by homologous recombination, a type of genetic recombination in which nucleotide sequences are exchanged between two similar or identical molecules of DNA. It is most widely used by cells to accurately repair harmful breaks that occur on both strands of DNA, known as double-strand breaks. The advantage of using homologous recombination is that it results in stable expression of the genes and non-transfer to other bacteria (Alberts et al. 2002). Additionally, we are considering the use of cell-free protein expression as a way to create proteins and test its ability to break down PET, as well as cell transformation to produce the proteins needed. This will allow us to test the efficiency of PETase and MHETase prior to expressing their genes in our chassis.

We are scheduled to begin experimental work soon. We are affiliated with the BioBuilders Organization, located at LabCentral in Cambridge, MA. With the help of our teacher, Lindsey L'Ecuyer, mentor Patrick Holec, BioBuilder founder and executive director Dr. Natalie Kuldell, and the BioBuilders team at LabCentral, we hope to physically construct a prototype of our design.

Acknowledgements

Team VYSP would like to thank the BioBuilders Organization and Dr. Natalie Kuldell as well as our teacher and leader of the Andover High School BioBuilders Club, Lindsey L'Ecuyer. We would also like to thank our mentor Patrick Holec. The aid of our mentors is what allows us to create and develop our ideas, and we are excited to continue doing so with their support.

This project was accomplished through participation in the BioBuilderClub, an after-school program organized by BioBuilder Educational Foundation. BioBuilderClub engages high school teams around the world to combine engineering approaches and scientific know-how to design/build/test their own project ideas using synthetic biology.

References

Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. Molecular Biology of the Cell.[Internet]. 4th ed. New York: Garland Science; 2002. [cited 2019 May 3]. Available from: http://bit.ly/2KNFXCw https://www.ncbi.nlm.nih.gov/books/NBK21064/

American Type Culture Collection. ATCC Bacterial Culture Guide [Internet]. Manassas (VA): American Type Culture Collection; 2015 [cited 2019 May 3]. Available from: http://bit.ly/2N4CLoN

Borunda, A. Ocean acidification, explained. [Internet]. United States of America: National Geographic Partners, LLC; 2017 April 27 [updated 2019; cited 2019 May 3]. Available from: https://on.natgeo.com/2YR3p-bC

Ciccarelli R. Plastic pollution could be working its way up the food chain, scientists warn [Internet]. Sydney (Australia): 9News; 2019 Feb 1[cited 2019 May 3]. Available from: http://bit.ly/2YR3I6g

Coghlan A. Bacteria found to eat PET plastics could help do the recycling [Internet]. New Scientist; 2016 March 10 [cited 2019 May 3]. Available from: http://bit.ly/2OZJ27B

Gontang EA, Fenical W, Jensen PR. Phylogenetic Diversity of Gram-Positive Bacteria Cultured from Marine Sediments. Applied and Environmental Microbiology. [Internet]. 2007 March 30 [cited 2019 May 3]; 73(10): 3272–3282. Available from: http://bit.ly/2KBejcX

Gonzaga D. Every single piece of plastic ever made still exists. Here's the story [Internet]. San Francisco (CA): Greenpeace International; 2007 Jan 6 [cited 2019 May 3]. Available from: http://bit.ly/2TCM1Bw

Harth R. Perils of plastics: risks to human health and the environment [Internet]. Arizona (US): Arizona State University BioDesign Institute; 2010 March 18 [cited 2019 May 3]. Available from: http://bit.ly/2OZ2jGe

Hopewell J, Dvorak R, Kosior E. Plastics recycling: challenges and opportunities. [Internet] The Royal Society Publishing. 2009 July 27 [cited 2019 May 3]; 364(1526): 1471-2970. Available from: http://bit.ly/2Za2pPh

Jiang Z, Zhang D-F, Khieu T-N, Son CK, Zhang X-M. Bacillus tianshenii sp. nov., isolated from a marine sediment sample. International Journal of Systematic and Evolutionary Microbiology [Internet]. 2014 Jun 1; 64. Available from: http://bit.ly/2KSrBAA

Liao CH, Shollenberger LM. Survivability and long-term preservation of bacteria in water and in phosphate-buffered saline. Letters in applied microbiology. [Internet]. 2003 [cited 2019 May 3]; 37(1):45–50. Available from: http://bit.ly/31MwXnJ

Meijer WJJ, Horcajadas JA, Salas M. φ29 Family of Phages. Microbiology and Molecular Biology Reviews. [Internet]. 2001 Jun [cited 2019 May 3]; 65(2): 261–287. Available from: http://bit.ly/2z2ehEv

Nakkabi A, Elmoualij N, Sadiki M, Saad IK, Fahim MF. Biodegradation of Poly (Ethylene Terephthalate) by Bacillus Subtilis. International Journal of Recent Advances in Multidisciplinary Research [Internet]. 2015 Dec [cited 2019 May 3]; 02(12): 1060-1062. Available from: http://bit.ly/2Zb1E8A

Setlow P. Germination of Spores of Bacillus Species: What We Know and Do Not Know. Journal of Bacteriology [Internet]. 2014 Apr [cited 2019 May 3]; 196(7): 1297–1305. Available from: http://bit.ly/2KCVDK0

Surfers Against Sewage. Plastic Pollution - Facts and Figures [Internet]. Cornwall: Surfers Against Sewage; 2019 [cited 2019 May 3]. Available from: http://bit.ly/2TGrdsX

Tolmachov O. Designing plasmid vectors. Methods in molecular biology. [Internet]. New York City: Humana Press; 2009 Feb 24 [cited 2019 May 3]. Abstract. Available from: http://bit.ly/2Z5virF

Universal Protein Resource. UniProtKB - A0A0K8P6T7 (PETH_IDESA) [Internet]. Cambridge (UK): European Bioinformatics Institute (EMBL-EBI); 2016 May 11 [modified 2019 Jan 16; cited 2019 May 3]. Available from: http://bit.ly/2yRaSrH

Wickham Laboratories. Fact Sheet: Bacillus subtilis [Internet]. Gosport (UK): Wickham Laboratories; 2019 [cited 2019 May 3]. Available from: http://bit.ly/2Has-5Bc

Windows to the Universe. Temperature of Ocean Water [Internet]. Boulder (CO): National Earth Science Teachers Association; 2011 Feb 16 [cited 2019 May 3]. Available from: http://bit.ly/2z6AYHz

Yoshida S, Hiraga K, Takehana T, Taniguchi I, Yamaji H, Maeda Y, Toyohara K, Miyamoto K, Kimura Y, Oda K. A bacterium that degrades and assimilates poly(ethylene terephthalate). [Internet] New York Avenue (WA): American Association for the Advancement of Science. 2016 March 11 [cited 2019 May 3]; 351(6278): 1196-1199. Available from: http://bit.ly/2Nb4gwS