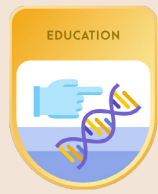
Using Synthetic Biology to Construct a PET Plastic Bio-tag to Improve Plastic Sorting at Recycling Facilities



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The plastic crisis is a well-known occurrence that is a major global issue today. It is negatively impacting many organisms on Earth, including humans. Plastic is used daily all around the world. However, many people do not realize how big of an effect it has on the environment and future prospects. By using synthetic biology to modify the bacteria *Bacillus subtilis* strain SCK6 to express a plastic "bio-tag", the process of sorting plastics into their seven number categories can be made easier. The main plastic focused on in this project is polyethylene terephthalate, or PET. This specific type of plastic is a food-safe plastic which is most commonly used around the world in the fast food industry. A methodology to transform *Bacillus subtilis* with a novel genetic construct is outlined, so that the bacterium expresses and secretes a series of fusion proteins used to bind to PET and fluoresce red. We hypothesized that the protein would specifically "tag" PET plastics, which would allow the sorting of plastics to be done more effectively, which in turn would help the plastic recycling industry become more efficient and productive. Overall, our team hopes that this will make a difference in the recycling world and help to solve the global issue of plastics in the environment.

Keywords: PET, recycling, synthetic biology, PET-hydrolase, hydrophobin, PETase, sorting plastic, bio-tagging

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Watch a video introduction by the authors at https://bit.ly/2OYg3ga

Background

There is more micro-plastic in the ocean than there are stars in the Milky Way galaxy (Smith, 2017). In recent years, plastic has become a significant issue that is greatly endangering the safety and well-being of organisms and ecosystems. Of all the plastic created on Earth, fifty percent is used only once, and only five percent is recycled (D'Alessandro, 2014). Our Lady of the Snows Catholic Academy (OLS) SynBio strives to find a solution to this global issue and to keep the environment a safe place for all future generations. The inefficient recycling of plastics is a well-known global issue. Plastic waste unnecessarily and frequently ends up in landfills and ecosystems (Cho, 2012). However, there is a common misconception; people think that this plastic dilemma is

caused by the actual recycling process, but what causes the majority of plastic waste to remain present in the world is the ineffectual sorting of plastics into their seven numbered categories (Duck, 2017, personal communication). Plastic that is poorly sorted cannot be properly recycled, and so ends up in landfill sites.

There are a multitude of substances that are classified as plastics. However, the recycling industry groups these materials into seven categories (Flor, 2017). The OLS SynBio team has chosen to target one: polyethylene terephthalate, also known as PET. This plastic is one of the most commonly used plastics in the world today, due to its food-safe properties. Synthetic biology can be used to help find a way to make a difference in the world of plastic sorting and recycling.

The OLS SynBio team's goal is to solve this prominent world-wide issue by using a simple chassis organism, the bacterium *Bacillus subtilis*, and to program it with a novel genetic circuit which will express protein "Bio-tags". The first protein expressed is a PET hydrolase (PET-ase) enzyme (originally identified in the bacteria *Ideonella sakaiensis*), which was identified for its potential in degrading PET plastic. In the construct, PET-ase is used to detect and selectively adhere to PET and is not utilized for its degradation activity. By fusing the PET-ase protein with a red fluorescent protein (RFP) called mCherry, this construct will allow both selective binding to PET plastic, and easy visualization through the RFP tag. The color mCherry was chosen specifically because it is the most vibrant, it is visible with the naked eye, and also appears brightly with a spectrophotometer. PET-ase is a valuable component to use because of its high degree of specificity, and its activity in the degradation of plastics, which is hypothesized to both aid in adherence and make the recycling process easier (Hampson, 2016).

The second protein construct is a bacterial hydrophobin called BslA. This hydrophobin, made naturally by *B. subtilis*, forms a water-repellent surface layer of the biofilm that can be used to stick itself to non-water molecules. Hydrophobins do not bond with water but they will bind to other substances, in this case, PET, to escape the water. These hydrophobins, when paired with PET-ase, are expected to aid in the binding of proteins to PET plastic. This will make the PET-ase mCherry fusion protein more efficient and effective as a selective bio-tag (Oberding, 2018, personal communication).

The genetic construct also includes a LipA secretion tag, that will help with sending the protein outside of the cell, and ensuring it will be secreted out of the bacteria. After the protein is secreted by the cell, the growth culture solution should be placed in a centrifuge to further separate the cells and protein. This process creates a supernatant at the top, which contains the separated proteins suspended in the liquid, whereas the bacteria will be forced to the bottom of the centrifuge tube. This supernatant will be used to tag the plastic, thereby minimizing the risk of bacterial contamination. In a real-world scenario, a protein bio-tag is a much safer tool to use than live bacterial cells.

The OLS SynBio team plans to perform a series of assays in order to ensure that these new protein constructs are selective in their binding to PET plastic, are visiblemas marker molecules, and have potential as real-world protein bio-tags. In order to ascertain the effectiveness of these proteins, modifying and experimenting with the expression and combinations of the proteins is important. By using the fusion proteins in both an RFP-marked and an RFP-unmarked form, in various combinations, we can increase the chances of success with this project (Figure 1).

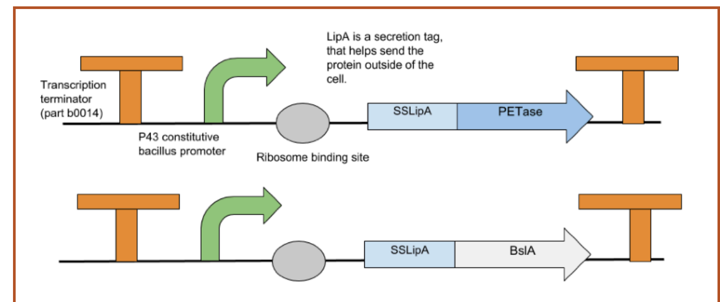

Figure 1. *Schematic diagrams of genetic circuits coding for protein bio-tag elements.*

Protocol 1: *Bacillus subtilis* SCK6 Competent Cells and Transformation

The following protocol outlines the process to make competent cells from the super-competent *Bacillus subtilis* SCK6 strain, in which competency can be induced using 1% xylose (Benchling.com, 2018a).

Requirements

- Luria Bertani (LB) medium with 1% xylose
- LB medium
- LB plates with 1 µg/mL erythromycin
- 1 mg/mL erythromycin
- *Bacillus subtilis* SCK6 strain
- Plasmid/Gibson to transform

Procedure

1. Two days prior to transformation, make a glycerol stock of SCK6, streak out a plate (1µg/mL final erythromycin) and grow at 37°C overnight.
2. To prepare the seed culture, a single colony from the plate produced in step 1 is inoculated into 25 mL of LB medium containing 1 µg/mL erythromycin in a 125-250 mL flask. After this is done incubate the cell culture at 37°C at 200 RPM in a rotary shaking incubator for 8-12 h (Benchling.com, 2018a).
3. To induce super-competence, the absorbency of the seed culture at 600 nm is measured and the culture is concentrated/diluted at 37°C to A600 = ~1.0 by adding freshly prepared LB medium containing erythromycin, 1 µg/mL. The culture can then be centrifuged, the supernatant poured off, and the cell pellet resuspended in fresh media to the same volume in fresh LB. Add D-xylose into the diluted

culture at the final concentration of 1% (w/v) OR resuspend in premixed LB 1% xylose. Incubate the diluted cell culture in a rotary shaking incubator for another 2 h, and then the cells are ready for direct transformation.

4. To store the competent cells, gently mix them with 50% glycerol until the final concentration of glycerol is 10-15% and then store the competent cells at -80°C for future transformations
5. Mix 1–2 µL of the plasmid/polymerase chain reaction (PCR) product with 100 µL of competent cells in a cell tube. Incubate cells in a rotary shaking incubator at 37°C at 200 RPM for 1.5 h to complete the transformation.
6. Spread the transformed competent cells on an LB plate with the appropriate antibiotic. Incubate the plates at 37°C for 8-12 h to select transformants.
7. Gently mix the competent cells with 50% glycerol to make a final concentration of glycerol to be 10-15%, and then store the competent cells at -80°C for future transformations.

Notes

1. IMPORTANT: Grow cultures in a large, sterile flask as this will ensure proper aeration and growth to an optical density $OD_{600} > 1$.
2. To avoid drastic changes in cell culture conditions, the freshly added LB medium should be prewarmed to 37°C before diluting the seed culture.
3. DO NOT add too much PCR product into competent cells for transformation since the PCR solution decreases the transformation efficiency.
4. *Bacillus subtilis* prefers to be transformed with multimeric plasmids vs traditional cloning methods. To get good quality plasmids, extract from freshly grown overnight culture, not more than 12 h old (otherwise DNA can be severely contaminated with genomic DNA and multimers).

Protocol 2: Plasmid Purification

This protocol is to purify the *Bacillus subtilis* SCK6 strain, after conducting the experiment. This process separates the proteins from the *B.subtilis*, so it can be used as a tag (Benchling.com, 2018b).

Requirements

- LB Medium (for overnight culture)
- P1 buffer (cold), P2 buffer , P3 buffer
- Isopropanol
- Cold 70% ethanol / reagent alcohol
- Water (Molecular-grade)
- TRIS buffer
- TE buffer
- Bacterial cultures
- Culture tubes
- 2.0 mL microcentrifuge tubes
- 1.5 mL microcentrifuge tubes

Procedure

1. Grow 2.5 mL culture overnight and use 2 mL for purification, keeping 0.5 mL subculture/glycerol stock.
2. Pellet cells in a 2 mL microfuge tube by centrifuging at maximum (14000 RPM) for 2 min.
3. Pour-off the supernatant; Repeat with more culture if necessary.
4. Vortex the pellet in 300 µL P1 (keep on ice if RNAse is added). Perform the next step quickly and gently (~1 min max).
5. For *B. subtilis* ONLY! Add 20 µL of 20 mg/ml lysozyme in 10 mM TRIS buffer, incubate at 37°C for 10 minutes.
6. Add 300 µL P2 → Invert (5–10 times) → Sit max 1 min → Add 300 µL P3 → Invert (10 times).
7. Incubate on ice for 10 min.
8. Centrifuge at maximum speed for 10 min at room temperature.
9. Aliquot the supernatant into a 1.5 mL microfuge tube (~600-800 µL) (Benchling.com, 2018b)
10. Add 650 µL isopropanol (room temperature) → Invert → Incubate for 10 min at room temperature.
11. Centrifuge at maximum speed for 10 min at room temperature → Remove supernatant.
12. Wash pellet with 70% cold (-20°C) Ethanol / Reagent Alcohol (~500 µL).
13. Centrifuge at maximum speed for 2 min at room temperature → Remove supernatant.
14. Air-dry pellet (place tubes upside down) for 10 min.
15. Resuspend (by flicking) in 40 µL MilliQ water or TE Buffer (for long-term storage).
16. Leave standing at room temperature for a few minutes to facilitate dissolving of the plasmid.
17. Run 3–4 µL on agarose gel to check quality AND/OR measure concentration (A260/A280).

Notes

Avoiding the white precipitate! Stab a 200 µL pipette tip through the top of the liquid in the tube quickly to avoid the white precipitate scum on the supernatant surface.

DO NOT disturb the translucent pellet.

Some buffers can interfere with subsequent reactions, so use water if doing these reactions immediately; otherwise, resuspend DNA in TE Buffer for long-term storage.

Protocol 3: Testing and Characterizing the Effectiveness of the Construct

To verify the synthesis has worked, it must be tested on real-world plastic. The 3 cm by 3 cm square pieces of sterilized trial material should be placed in a beaker and left at room temperature, then viewed three different times throughout the process. It will need to be tested to determine whether or not the PET-ase and mCherry construct adheres selectively to PET plastics only, and whether or not the construct is visible, either to the naked eye or using spectrophotometry. The bio-tag is expected to be seen with a UV light, based on whether or not the proteins fluoresce (Figure 2).

The trials in place are to demonstrate the effectiveness of the solution. PET plastic and non-PET plastics, as well as glass and metal, have been chosen to test the four different constructs on. The expectation is that the mCherry PET-ase construct will only bind to PET plastic, the mCherry hydrophobins will bind to any material, and the mCherry PET-ase with the separate, unmarked hydrophobins will adhere only to PET. However, we predict that PET-ase without the mCherry will produce a slight discoloration because it does not contain the fluorescent protein. The following protocol will test this hypothesis:

Procedure

1. Cut the PET plastic, non-PET plastic, glass, and metal into 3 cm by 3 cm squares.
2. Place each of the 4 different supernatants into 4 separate sterilized Erlenmeyer flasks.
3. Place a supernatant of untransformed *B. subtilis* into another 4 sterilized Erlenmeyer flasks as a control.
4. Place 5 squares of sterilized PET into the first Erlenmeyer flask of each supernatant, one square in each.
5. Repeat with five squares of sterilized non-PET plastic into the second Erlenmeyer flask of each supernatant,one square in each.
6. Repeat with 5 squares of sterilized glass into the third Erlenmeyer flask of each supernatant, only one square in each.
7. Repeat with 5 squares of sterilized metal into the fourth Erlenmeyer flask of each supernatant, only one square in each.
8. Store each Erlenmeyer flask at room temperature with the top covered in tin foil.
9. Check after 30 min, 1 h, and 24 h.
10. Remove the squares with a sterilized instrument.
11. Observe the color of each with the human eye and UV light.
12. Record the results.

Notes

With the hydrophobin mCherry construct, because of its qualities and because it isn't engineered to target one specific material, it is predicted that it could possibly bind with the beaker as well as the materials inside.

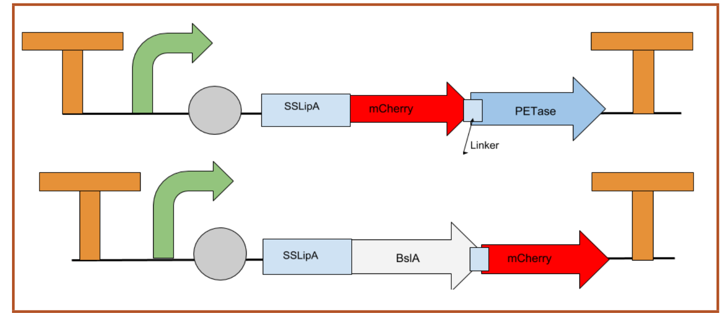

Figure 2. *Schematic diagram for trial assays for construct effectiveness and expected results.*

Prototype

If successful, the bio-tag would be a proof of concept for a novel technology that could be easily implemented in existing sorting and recycling facilities. A prototype for industrial application would use a streamlined, linear process that involves both existing technology and new robotics to effectively sort plastics. The first process will be a bathing station in a contained/closed system as shown in Figure 3A. This would be the area where the protein would adhere to the PET plastic. As it goes along the linear pathway, an optical scanner that will detect the plastic will cause a door to open on the conveyor belt, dropping the plastic into the designated area (Figure 3B). This project will be implementing the protein construct solution into an already existing technology.

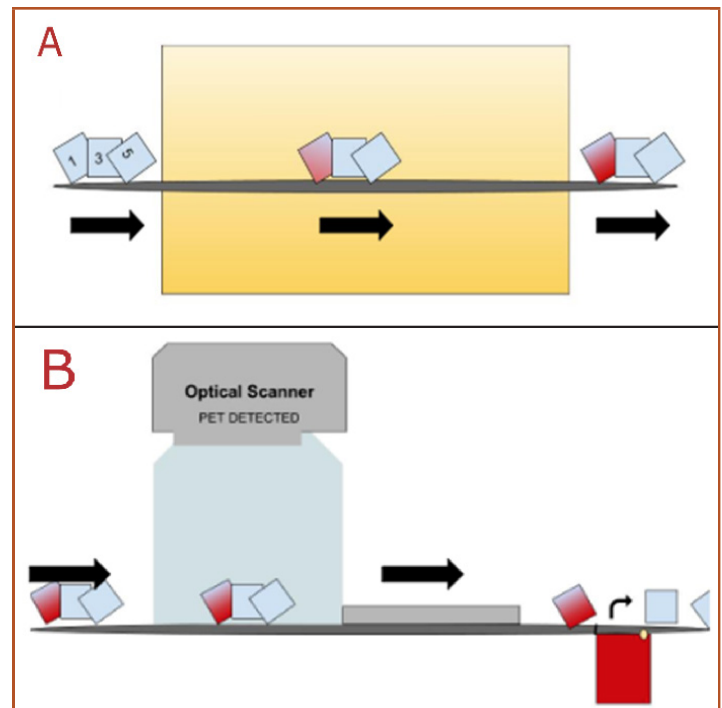

Figure 3. *(A) Prototype 1 - Containment. (B) Prototype 2 - Scanning and sorting.*

Safety

Along with universal lab safety (such as lab goggles, lab

coats, long pants, and proper footwear), and aseptic technique, there are a lot of safety protocols to address regarding this project. Firstly the construct is designed to be secreted, isolated and purified from active bacteria, therefore the plastic will not be exposed to live bacteria, but instead to a stable protein product. For large scale industrialization, the persistence and stability of these proteins in the environment would need to be ascertained. Most recycling facilities include high heat and sometimes UV irradiation, which would theoretically denature the bio-tag proteins and eliminate the risk of environmental contamination. Before a product like this proposed bio-tag could be used in a real-world situation, these questions would need to be answered and careful consideration of potential protein contamination is necessary.

Discussion

To date, the OLS Synbio team has ordered the synthesized genetic constructs, but has not yet completed any labs or experiments. We do not have any results indicating a positive or negative outcome. We will begin to get results after receiving the DNA constructs and starting lab protocols. However, since no procedures have yet been started, there has been no determined outcome of the project.

Acknowledgments

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Lastly, the OLS SynBio team would like to thank the Canmore community for all of their support; Peter Duck (zero waste manager), Simon Robins (recycling supervisor), the Rocky Mountain Outlook Newspaper, Alberta Recycling Council, as well as the parents who got involved and supported OLS SynBio. Without all of the help and support, from so many different people, the team would not be where we are today.

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Our team