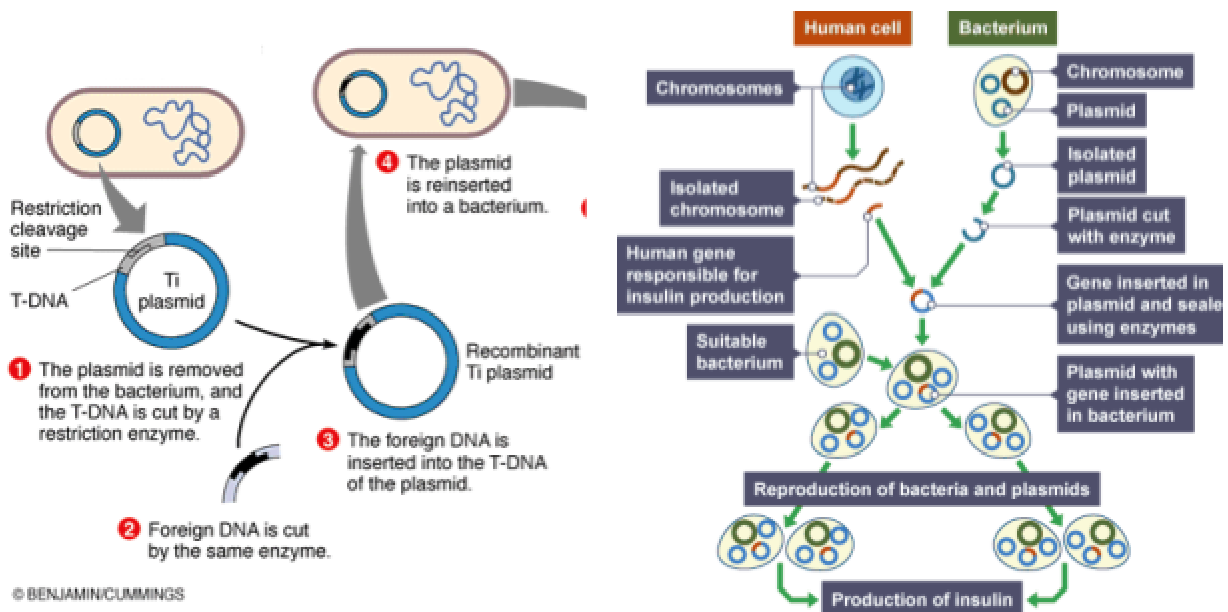
# Background Research

**How are bacteria genetically engineered to produce proteins they wouldn't normally produce?**

Genetic engineering of bacteria is mainly done with the use of plasmids. Plasmids are circular pieces of DNA inside bacteria cells, and they can be modified for the bacteria to produce proteins they wouldn't normally produce. Scientists do this by inserting a gene that codes for that protein.

To do this, special enzymes called restriction enzymes are added to the plasmid to recognize, bind to, and cut specific DNA sequences. This is used to open up and prepare the plasmid for the insertion of the gene. When these restriction enzymes are added, some of them leave the DNA with uneven ends. If the plasmid and the gene they wish to insert are cut with the same enzyme, the uneven ends of both will be able to bond with each other.

After this step, gene fragments, cut plasmids, and an enzyme that connects DNA fragments are put together in a solution. The enzyme, called a ligase enzyme, attaches the uneven ends of the desired gene and the cut plasmids. This makes full circular plasmids that carry the desired gene. This process was done to make the plasmids that I used in my experiment.




( Image from: *Genetic engineering*. (n.d.). BBC. https://www.bbc.co.uk/bitesize/guides/zqgs2nb/revision/1 )

The plasmid used in my experiment consists of the chloramphenicol acetyltransferase gene and the gene that produces Red Fluorescent Protein. (DsRed)

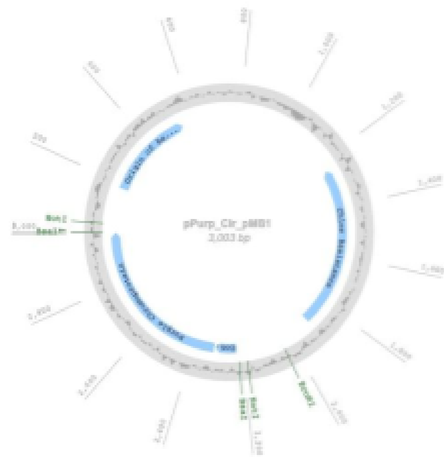
Chloramphenicol acetyltransferase is a bacterial enzyme that gives the bacteria resistance to the antibiotic chloramphenicol, and this gene acts as a selection marker. This makes it so that on the petri dishes with the antibiotic chloramphenicol added, only the bacteria that take the plasmids in will grow, since the regular blank K12 E. Coli cells won't have antibiotic resistance.

The fluorescent magenta proteins that the E. Coli cells produced in my project were derived from a protein called DsRed, which comes from the sea anemone species Discosoma. The magenta colour instead of a red colour is caused by mutations/slight differences in the sequence of amino acids. The gene that produces the protein was extracted from Discosoma sp., and inserted into the plasmid through the process explained previously.

Protein structure of Monomeric red fluorescent protein, DsRed.M1:

( Images from: *DsRed.M1 :: Fluorescent Protein Database*. (n.d.). FPbase. https://www.fpbase.org/protein/dsredm1/ )

Plasmid map of the plasmid used in my experiment:



After the bacteria are successfully engineered, they will have resistance to chloramphenicol and be able to produce colour proteins.

This plasmid map shows the origin of replication, the chloramphenicol acetyltransferase gene, and the colour protein gene.

The specific plasmid used from the E. Coli bacteria is the plasmid ColE1. The antibiotic resistance gene and the colour protein gene were inserted through the ligation process explained before.

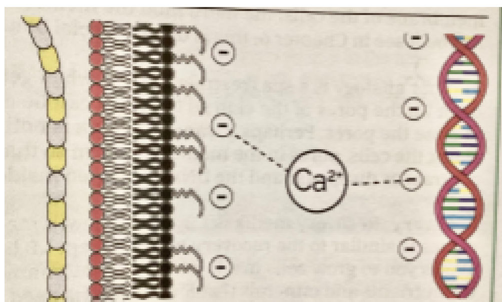
**What is the process of inserting the plasmids into the E. Coli bacteria?**

Following the modification of the plasmid, it is reinserted back into the bacterium, which can now produce proteins that the new gene codes for.

In my procedure, this was done by mixing the blank E. Coli cells in the transformation buffer, then mixing the plasmids in this solution.

**Why the transformation buffer is used:**

The transformation buffer is mostly made up of sterile water and positively charged calcium ions. (Ca**2**+) The DNA plasmids and the bacterial cells' outer membrane are both negatively charged, so they'll be attracted to the positively charged calcium ions. Because of this, the calcium ions can cause the DNA plasmids and bacterial cells to move closer together, and there will be a greater chance of the DNA plasmids getting into the cells during the heat shock.




This is called a coordination complex.

(Image from Zero to Genetic Engineering Hero book)

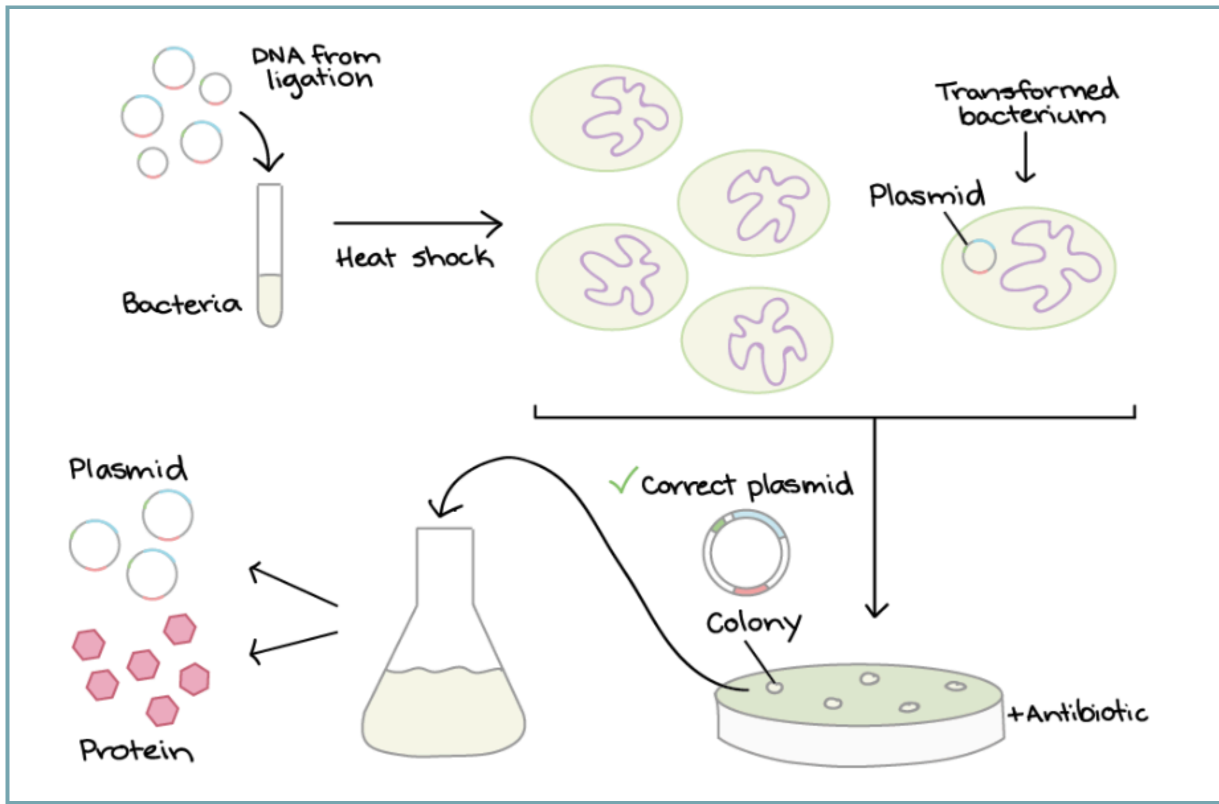
After the DNA plasmids, E. Coli cells, and the transformation buffer are mixed together and left at 4°C for 5 minutes, they are heat shocked at 42°C. This heat shock is done to make the cell membranes of the E. Coli bacteria more permeable, so that there is a greater chance of the DNA plasmids entering the bacteria. (The bacteria with the DNA plasmids inside will be successfully engineered and able to make the magenta colour protein used in the rest of the experiment.)

After this step, recovery media is added to the cells. The recovery media consists of nutrients and minerals similar to the LB agar used in petri dishes, except that it contains no agar. It's used so that the bacteria can recover after the process of becoming engineered and taking in new DNA. Now, the bacteria are transformed and have antibiotic resistance to chloramphenicol, while also being able to able to make colour proteins.
When they're grown on selective petri dishes containing chloramphenicol, they will be producing colourful pigments while being the only bacteria growing.

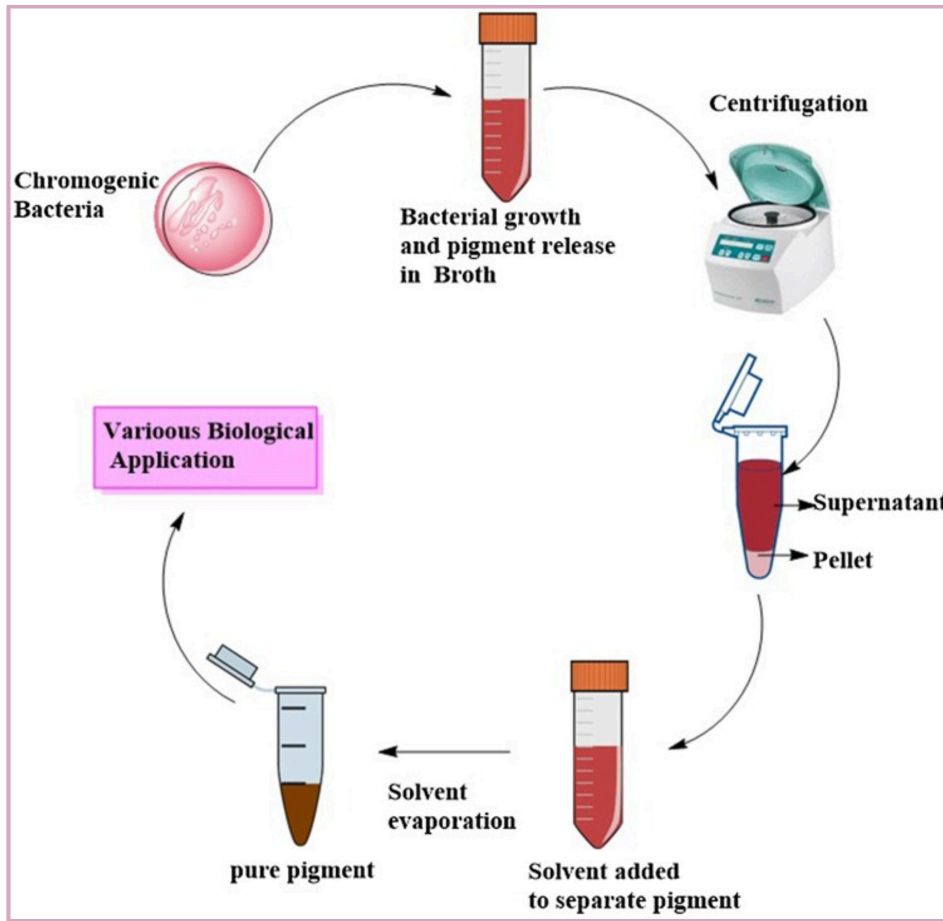
**How do you get the colour proteins/colourful pigments out of the bacteria?**
In order to get the colour proteins out of the bacteria, the cells need to be lysed. (so the cell membrane breaks down.)
In my experiment, surfactants Triton X-100 and Hen Egg White Lysozyme (HEWL) were used to do this. Triton X-100 is able to break down cell membranes while not destroying the proteins that the cells were engineered to produce. HEWL is a protein enzyme that is able to bind to and break apart the peptidoglycan in the intermembrane space of E. Coli cells.
By using both of these surfactants, the membranes of the cells will break down and the colour protiens/pigments will come out from the bacteria.

Afterwards, the solution is centrifuged, which separates cell debris from the pigments, and sterilized through a 0.22μm filter, which makes sure no bacteria is in the final solution.

Images showing this process:




( Image from: *Bacterial transformation & selection*. (n.d.). Khan Academy. https://www.khanacademy.org/science/biology/biotech-dna-technology/dna-cloning-tutorial/a/bacterial-transformation-selection )

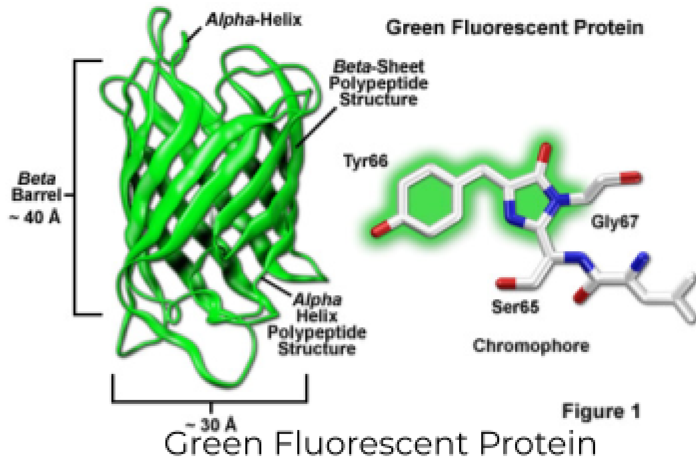



( Image from: Numan, M., Bashir, S., Mumtaz, R., Tayyab, S., Rehman, N. U., Khan, A. L., Shinwari, Z. K., & Al-Harrasi, A. (2018, April 4). *Therapeutic applications of bacterial pigments: a review of current status and future opportunities*. Europe PMC. https://europepmc.org/article/pmc/5884752 )

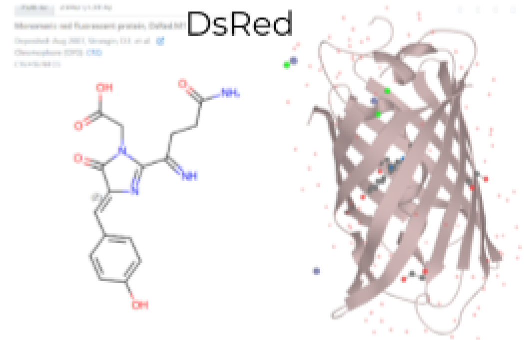
**How do bacterial pigments have resistance to environmental conditions?**

DsRed, the colour protein that the bacteria in my experiment produced, has been tested and shown to have better photostability than other similar pigments. DsRed's protein structure contains a beta-barrel structure, which creates a protective environment for the chromophore. A chromophore is the part of the protein that reflects colour. (causes the colour)

In colour proteins similar to DsRed, like Green Fluorescent Protein, this beta-barrel structure has been shown to contribute to the overall stability of the protein. It acts as a shield, protecting the chromophore from environmental damage and preventing photobleaching of the pigment.




The structure of Green Fluorescent Protein is very similar to the structure of DsRed.




( Image from: Piston, D. W., Campbell, R. E., Day, R. N., & Davidson, M. W. (n.d.). *Introduction to Fluorescent Proteins*. Carl Zeiss Microscopy Online Campus. https://zeiss-campus.magnet.fsu.edu/print/probes/fpintroduction-print.html )

**What are the potential negative health effects that acrylic paint (and paint powder for kids) can have?**

Some acrylic paints and pigments used in paint contain harmful additives that are carcinogenic and toxic to human health. For example, pigments used in paint may contain heavy metals, which can cause respiratory irritation, kidney disease, and many other health problems if there's exposure over long periods of time. Also, some acrylic paints contain additives like phthalates and formaldehyde, which have been associated with causing cancer, disrupting hormones, and liver toxicity. These ingredients specifically become more harmful when they're on skin.

Chemicals in paint can release into the air while drying, and can also enter the bloodstream within 60 seconds of being on skin.

Additionally, powdered paints, like the tempera paint powder used in my project, have a greater inhalation risk. Therefore, there's also a greater risk of respiratory irritation, and a greater risk of toxicity from ingredients in the pigment and paint fillers/additives.

The production of pigments used in commercial paints is also very harmful to the environment. The process of mining, milling, extraction, and grinding releases toxic substances into the air, water, and soil. This negatively affects ecosystems, and aquatic life are particularly vulnerable to these harmful effluents.

**How has engineering of bacteria been used commercially?**
To be used commercially, there has to be a way for proteins made by bacteria to be produced in large quantities. This has been done in many biotechnological applications, like the creation of insulin from genetically engineered E. Coli bacteria. The engineered bacteria, containing a plasmid with a human insulin gene added, are put into large fermentation tanks. In the fermentation tanks, the bacteria mass-produce insulin that can be harvested and used.

This method using fermentation tanks has also been successfully used for the production of pigments. For example, scientists at Amgen, Inc. have been able to engineer E. Coli to produce large amounts of indigo pigment in fermentation tanks.
By using fermentation tanks, the cost of large amounts of petri dishes and large incubators is removed.

**Sources:**


Pahara, J., & Legault, J. (2021). *Zero to Genetic Engineering Hero* (P. Rea & P. Anta, Eds.; 1st ed.). Make: Community LLC.

*Protocol - Bacterial Transformation*. (2017, November 13). Addgene.
https://www.addgene.org/protocols/bacterial-transformation/

*Genetic Engineering: Using Plasmids to Induce Cells to Produce Proteins*. (n.d.). PBS LearningMedia.
https://www.pbslearningmedia.org/resource/biot11.sci.life.gen.genengdna/genetic-engineering-and-working-with-dna/

*DsRed :: Fluorescent Protein Database*. (n.d.). FPbase.
https://www.fpbase.org/protein/dsred/

Strongin, D.E., Bevis, B., Khuong, N., Downing, M.E., Strack, R.L., Sundaram, K., Glick, B.S., & Keenan, R.J. (2007, November 6). *Monomeric red fluorescent protein, DsRed.M1*. RCSB Protein Data Bank.
https://www.rcsb.org/structure/2VAD

Mukiri, K., Lau, T., & Alcock, B. (2017, January 25). *chloramphenicol acetyltransferase (CAT)*. The Comprehensive Antibiotic Resistance Database.
https://card.mcmaster.ca/ontology/36261

Matthews, R. (n.d.). *Where does the colour go when a material fades in the Sun?* BBC Science Focus Magazine.
https://www.sciencefocus.com/science/where-does-the-colour-go-when-a-material-fades-in-the-sun

Piston, D. W., Campbell, R. E., Day, R. N., & Davidson, M. W. (n.d.). *Introduction to Fluorescent Proteins*. Carl Zeiss Microscopy Online Campus.
https://zeiss-campus.magnet.fsu.edu/print/probes/fpintroduction-print.html

Yarbrough, D., Wachter, R. M., Kallio, K., Matz, M. V., & Remington, S. J. (2001, January 16). *Refined crystal structure of DsRed, a red fluorescent protein from coral, at 2.0-Å resolution*. Proceedings of the National Academy of Sciences.
https://www.pnas.org/doi/10.1073/pnas.98.2.462

*Is Acrylic Paint Really Toxic? – Natural Earth Paint*. (2024, June 26). Natural Earth Paint.
https://naturalearthpaint.com/blogs/blog/is-acrylic-paint-really-toxic

Duggirala, R., Mikkilineni, P., & Croghan, I. (2024, October). *De Novo Synthesis of Biopaint Using Transformed Bacteria: Analysis of Spectral Intensity Trends and Comparison to Commercial Paint*. Scientific Research - An Academic Publisher.
https://www.scirp.org/journal/paperinformation?paperid=136808

Charkoudian, L. K., Fitzgerald, J. T., Khosla, C., & Champlin, A. (2010, October 5). *In Living Color: Bacterial Pigments as an Untapped Resource in the Classroom and Beyond*. National Library of Medicine.
https://pmc.ncbi.nlm.nih.gov/articles/PMC2950131/

*How did they make insulin from recombinant DNA?* (n.d.). National Library of Medicine.
https://www.nlm.nih.gov/exhibition/fromdnatobeer/exhibition-interactive/recombinant-DNA/recombinant-dna-technology-alternative.html