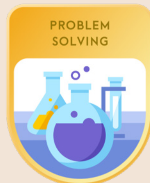
Design Brief

Synthesis of cadaverine in *Escherichia coli* transformed with pET28-a plasmid



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Plastics and fossil fuels are the sources of many environmental problems such as ice cap melting, ocean pollution, and combustion. Fossil fuel combustion is inherently bad for the environment and the public, as it emits toxic sulfur dioxide into the atmosphere. The burning of coal contributes to the occurrence of acid rain as well as the build-up of toxic matter. However, cadaverine, a diamine compound with potential for industrial bioplastic applications, could provide a solution to decreasing toxic waste created by the use of fossil fuels. Cadaverine is a naturally occurring diamine compound, commonly found in decomposing organisms. It is produced from the conversion of the amino acid L-lysine, a form of lysine, during digestion by the enzyme lysine decarboxylase. The gene cadA codes for the expression of lysine decarboxylase. Lysine is not naturally produced in *E. coli*, so a system is needed to bring lysine into the cell. This system, a cadaverine antiporter that imports lysine into the cell in exchange for the export of cadaverine, is coded by the *cadB* gene. One of cadaverine's many functions in *E. coli* is to protect cells in acidic pH environments. In cases of high acid stress, cadaverine induces protein expression, which closes cell porins, preventing interaction with the acidic environment. Cadaverine is toxic to the cell in high quantities, so *E. coli* does not constitutively express it. To increase production of cadaverine in *E. coli*, an insert containing both *cadB* and cadA, preceded by a constitutive T7 promoter and followed by a double terminator, may be inserted into a pET28-a plasmid through a restriction digest. The ligated pET-28a would then be transformed into K12 *E. coli*, and the plasmid would cause the cell to begin synthesizing cadaverine out of the L-lysine. Cadaverine has many industrial applications. Using cadaverine instead of petroleum-based hexanediamine could create a wider range of strong, environmentally sustainable plastics and help lower rates of fossil fuel combustion. Applied on a larger scale, our experiment could generate a cadaverine-producing strain of *E. coli* bacteria, allowing for an environmentally friendly method of producing the polyamides necessary for plastic production.

Keywords: Cadaverine, lysine decarboxylase, plastic production, *Escherichia coli*, restriction digest

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Watch a video introduction by the authors at https://youtu.be/zOL0Po-aO58

Background

Cadaverine is infamous for its pungent odor that exists when it is synthesized in decomposing tissue (Aftermath, 2021). The smell comes from the decarboxylation of L-lysine, which is one of the nine essential amino acids for human growth (National Center for Biotechnology Information, 2021). Cadaverine is a relatively stable, toxic, colorless diamine compound that is also known as 1,5 pentanediamine or pentamethylenediamine. Additionally, cadaverine acts as a plant and animal metabolite, and it is soluble in both water and ethanol (National Center for Biotechnology Information, 2021).

Escherichia coli is a neutrophile, a type of bacteria that maintains its internal acidic homeostasis at a pH of 7.4 - 7.9 (Samartzidou et al., 2003). In *E. coli*, cadaverine helps protect the cell in a lower pH environment. When the cell enters an environment with a pH of 5.6 or less, membrane permeability of the *E. coli* decreases by 20-25% due to molecules blocking the porins (Samartzidou et al., 2003). The cad operon is activated by an acidic signal, which causes the synthesis of lysine decarboxylase. This results in the removal of a carboxyl group from L-lysine, which forms cadaverine. Once the cadaverine molecule has been synthesized, it is released into the cytoplasm where it bonds with porins, obstructing 70% of cell membrane transport proteins (Samartzidou et al., 2003). This inhibits the interaction between the inside of the cell and the highly acidic environment while allowing the intake of nutrients through the 30% of open porins (Samartzidou et al., 2003).

The production of cadaverine is controlled by the *cadB*A operon that contains two separate genes, *cadB* and cadA. The *cadB* gene codes for the lysine/cadaverine antiporter, which exports cadaverine and imports lysine in a ratio of 1:1. In order to synthesize cadaverine for this exchange, *E. coli* produces the single-use enzyme lysine-decarboxylase, coded for by the gene cadA, that is used a maximum of once in the conversion of L-lysine to cadaverine before it degrades (Wang, et. al., 2018).

Cadaverine is a polyamine monomer that is used as a base chemical in the production of various polyamides and is currently manufactured using a petroleum-based synthesis (Nærdal et al., 2015). Polyamides are polymers made from molecules linked by amides and are produced either by amine groups interacting with a carboxyl group or the polymerization of amino acids or their derivatives. These polyamides are used in the production of nylon plastics. The first nylon plastic, called nylon 6 or polycaprolactam, was made from amines that were derived from hexamethylenediamine and adipic acid (Wells & Wells, 2021).

When crude oil is extracted from the earth, it is convertedinto different petroleum products through a process known as refinement. During refinement, heavy molecules in the oil are broken down into monomers, such as hexamethylenediamine (Baheti, 2021). Hexamethylene monomers are polymerized into hydrocarbons such as naphtha, which is crucial to plastic development. Naphtha is a mixture of C5 and C10 hydrocarbons, and when decomposed at high temperatures, it splits into compounds such as butane, propylene, and ethylene. These compounds are combined into liquids, which are heated and transformed into plastic pellets that make up the plastic products we use every day (Baheti, 2021). A common method to obtain petroleum is hydraulic fracking, a process in which large amounts of water and various chemicals are used to extract oil from rock formations. Hydraulic fracking can have effects on water availability in the area, effects on local marine life, and has potential for leaks (EIA, 2020). If the current system in which cadaverine is produced using petroleum could be replaced by *E. coli* biosynthesis, there would be decreased water use, negation of spills that can affect local wildlife, and a reduction in the disturbance to the local environment that comes from petroleum refinement. Refining petroleum has many environmental consequences, including the build-up of greenhouse gases and the release of sulfur and other byproduct compounds into the environment. These environmental consequences include disturbance of local wildlife, clearing of native vegetation, contamination of water that affects marine life, and excessive use of water during production which leads to lack of access to water for the residents of the area (EIA, 2020). This process then yields a molecule similar in structure to cadaverine but yields more environmental damage than cadaverine synthesis in *E. coli*. By using *E. coli* instead of a petroleum base to create the cadaverine polyamine, the amount of petroleum in use is reduced.

Systems Level

In this experiment, the pET28a plasmid, containing the *cadB*A insert, will be transformed into BL21-DE3 *E. coli*. A population of *E. coli* will be grown to an optical density of 0.6 in LB broth, at which point it will be induced with IPTG, then allowed to grow to an optical density of 0.8 (Sezonov, 2007). Once the *E. coli* population reaches the appropriate density, it will be induced with a pre-tested measurement of IPTG ranging from 0.1 to 0.5 mM to synthesize the T7 RNA polymerase which will then attach to the T7 promoter of the *cadB*A insert to start the constitutive production of lysine decarboxylase (Kortmann et al., 2014; Collins et al., 2013). The cells will then be chemically lysed by using lysis buffers to disrupt the cell membrane in order to produce a cell lysate solution. Next, the lysine decarboxylase will be

purified out of the cell lysate solution using a nickel sepharose column in order to bind the His-tag. This will then be added to a 200 g/L L-lysine solution to begin the synthesis of cadaverine. The cadaverine will be extracted using the selective solvent methyl ethyl ketone (Hong, et al., 2018).The final product, cadaverine, can then be used in the production of nylon plastics.

Device Level

BL21 (DE3) *E. coli* was chosen for this experiment because it contains the gene for the synthesis of T7 RNA polymerase under inducible control of the lac promoter (New England Biolabs, 2016). Once the population of *E. coli* has grown to an optical density of 0.8, IPTG will be introduced into the media to induce the lac promoter and the synthesis of T7 RNA polymerase. Once the pET28a plasmid has been transformed into the *E. coli*, this polymerase can begin transcription.

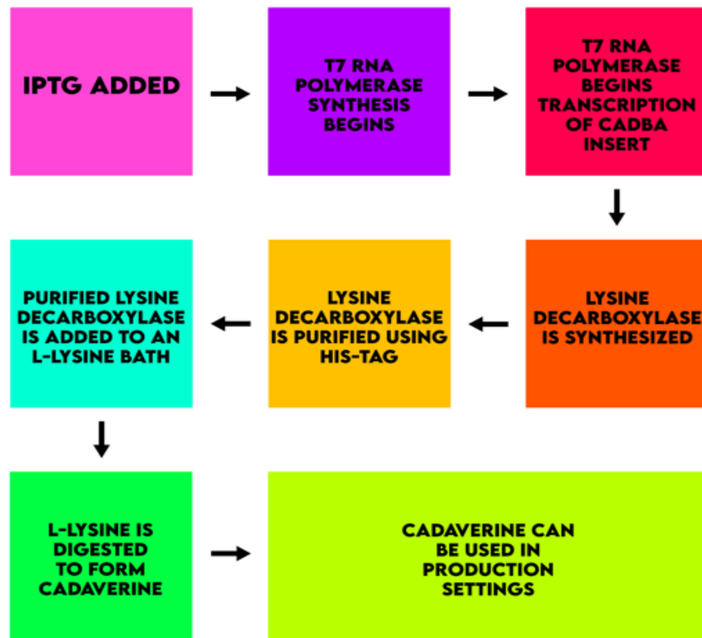

Figure 1. - *A flow chart depicting the process of cadaverine production.*

The pET28a plasmid, which contains the *cadB*A insert, is under the control of the T7 promoter (GSL Biotech LLC, 2020). Using a species of *E. coli* with an operon that has the ability to induce this polymerase will eliminate an unnecessary step in the method. As BL21-DE3 *E. coli* is also somewhat resistant to toxic substances, this species will be able to better sustain itself in the presence of increased levels of cadaverine (Agilent Technologies, n.d.).

In order to simplify the experiment method, a nickel sepharose His-tag procedure will be used. Without a His-tag, lysine would need to be mixed into the agar medium, and cadaverine would have to be extracted from the lysed cell debris. With a His-tag, the lysine decarboxylase created in the cell can be purified out of the cell before synthesizing cadaverine and can be immersed in a lysine bath in a different container. Cadaverine can then be synthesized through lysine decarboxylation without having to remove purified cadaverine from the lysed cell.

Parts Level

The plasmid used in this design is the pET-28a plasmid. The relevant restriction sites within the plasmid are BamHI and NotI, found before and after the insert, both of which are surrounded with PCR amplification sequences. On the pET-28a plasmid, shortly before the NotI restriction site, there is a T7 promoter (DNASU Plasmid Repository). The *cadB*A insert, engineered by

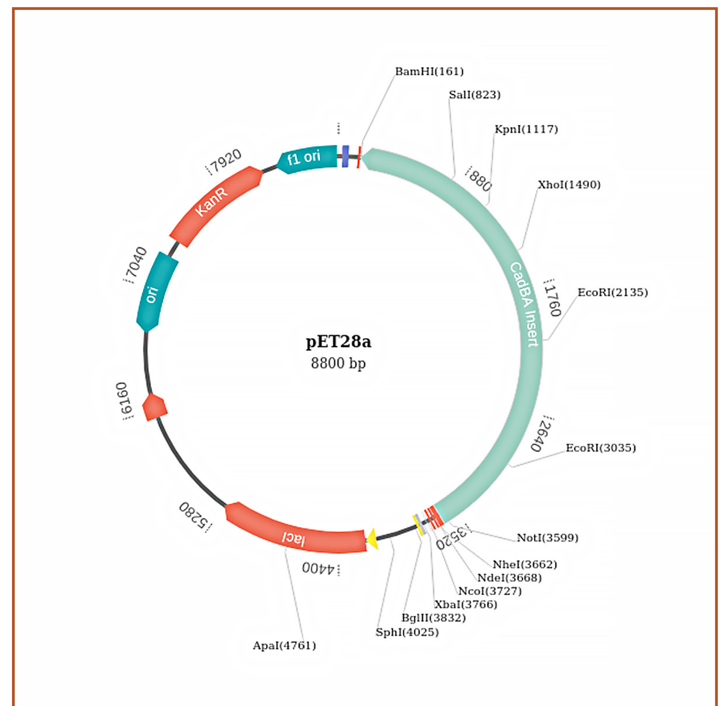

Figure 2. - *A modified pET28a plasmid containing the Renaissance lab's CadBA insert.*

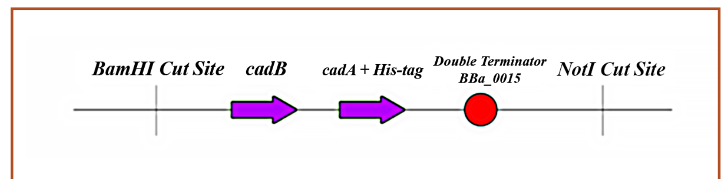

Figure 3. - *The CadBA Insert designed by the Renaissance School lab. The purple arrows represent coding regions, the red circle represents the double terminator, and the vertical lines represent enzyme cut sites.*

the Renaissance lab, contains the *cadB*A gene, which is naturally occurring within *E. coli*; a sequence of six histidine codons; three stop codons; and the BBa_B0015 double terminator (iGEM Foundation, 2003). The *cadB*A insert is composed of the *cadB* gene, followed by an intermediate sequence of nucleotide bases, which precedes the *cadA* gene (DDBJ Center 2016). This *cadB*A insert also includes a His-tag shortly before the stop codon of *cadA*. This is because the *cadB* gene codes for a lysine antiporter, and the *cadA* gene codes for lysine-decarboxylase and the Renaissance School lab wishes to isolate lysine-decarboxylase (Wang et al., 2018).

Safety

Although most of the individual materials used in this experiment are not directly harmful to humans, a cautious sterile procedure should be followed. One of the main safety concerns in this experiment is the molecule cadaverine. Cadaverine is corrosive and can irritate the skin and is also toxic if consumed, inhaled, or absorbed (National Center for Biotechnology Information, 2021). Additionally, when heated to the point of decomposition, cadaverine releases highly toxic nitric oxide fumes (National Center for Biotechnology Information, 2021). For this reason, it is important to use safe procedures when handling cadaverine and to keep the compound away from flames and combustible materials. The procedures include personal protective equipment (gloves, masks, lab coats), working in a fume hood, and properly sterilizing and disposing of all tools used. When cleaning up cadaverine in a lab setting, it should be soaked up with an absorbent material and treated as hazardous waste; it then needs to be stored under an inert gas to prevent oxidation and interaction with the environment (National Center for Biotechnology Information, 2021).

The second large safety concern in this experiment is the potential misuse/escape of engineered *E. coli* strains from the lab. The *E. coli* transformation will be performed in a controlled lab environment to prevent any contact with non-engineered *E. coli* species. If the transformed *E. coli* cells were released into the environment, the plasmid would express kanamycin resistance, which could create widespread resistance throughout the native *E. coli* population. However, the cells will continue to produce cadaverine at an overwhelming rate and will be unable to survive for very long before the build-up of cadaverine becomes toxic and the cell ruptures. This ensures that the environmental hazard does not exceed controllability. As a second protection from potential environmental hazard, the cells cannot produce excess cadaverine without the presence of both L-lysine and IPTG.

The third and final safety consideration with this experiment is maintaining proper COVID-19 protective procedure. This includes masks, social distancing, and frequent cleaning of surfaces.

Discussions

The Renaissance lab attempted to order our customized plasmid insert from Integrated DNA Technologies (IDT). However, the cost of the plasmid was greater than the laboratory's annual budget. Time was also a notable constraint; we were limited to three hours a week and had frequent disruptions from holidays and school events. Thus, there were not many slots of continuous time to work on the experiment without hindering the efficacy of some of the more time-sensitive elements.

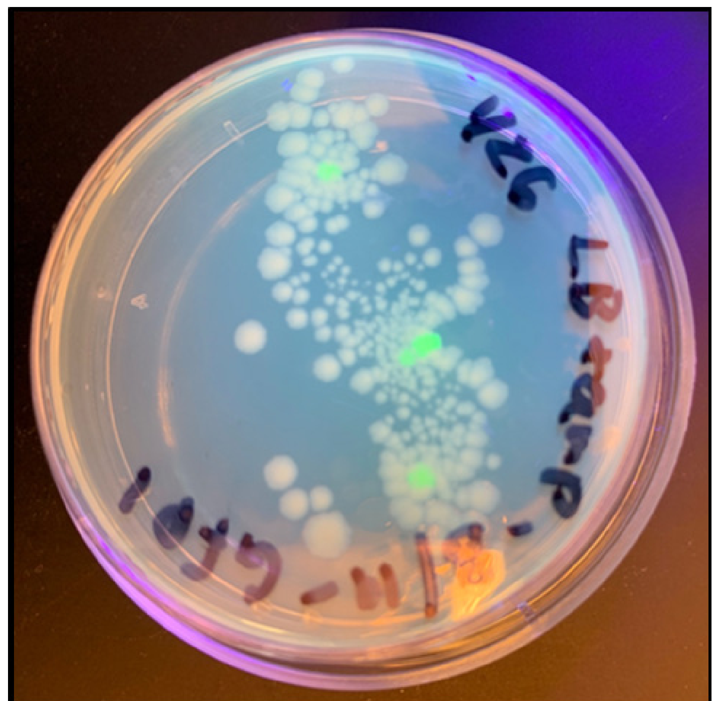

Figure 4. - *Transformed MM294 E. coli with green apt-g protein colonies. Fluorescing colonies show E.coli cells which have accepted and expressed the apt-g protein in their phenotypes.*

Due to these financial constraints, our lab was unable to complete this specific experiment. However, in order to practice the methods and procedures used in this experiment, several proxy labs were performed. We were able to successfully transform plasmids into MM294 *E. coli* cells several times, which resulted in blue and green fluorescing apt-g colonies. We used MM294 strains of *E. coli* for our proxy experiments because it was readily available; BL21-DE23 was chosen for the cadaverine experiment because it already contained the T7 promoter, a property which was not necessarily as important in our previous practice experiments.

Our first transformation lab had a calculated transformation efficiency of 52.25%, with 1 colony per every 0.0194 µg of plasmid. We then attempted to purify out the fluorescing protein with a methylated HIC resin protocol but were unsuccessful. In the coming weeks, we will perform a lambda phage digest with agarose gel electrophoresis, a similar procedure to that required in the cadaverine experiment. Finally, we will complete another experiment in which we will purify a plasmid out from cells, digest it, and run it on an agarose gel. By completing these proxy experiments, the Renaissance lab can mimic the key processes performed in this experiment and understand the important procedures that would be needed.

In designing the insert used in this paper, our lab found it difficult to locate multiple consistent and reliable sources for the nucleotide base sequence of the *cadB*A operon. Across multiple databases, there were large differences in what should have been the same gene, causing it to be very difficult to corroborate the information found in each database. The Renaissance lab queried GenBank, the DDBJ, the BioBrick Database, and the EMBL-EBI. While other sequences, such as the double terminator and his-tag, were stored in multiple databases, we were only able to find the complete *cadB*A operon in one database — the DNA Data Bank of Japan (DDBJ). Other databases contained *cadB* and cadA separately, however the DDBJ was the only database we found that contained the entire operon. This was necessary because, as we discovered, there is an intermediary sequence of base pairs between *cadB* and *cadA* on the naturally occurring *cadB*A operon.

During the process of designing the gene insert, we discovered that there is a naturally occurring segment of DNA between the genes on the cad operon. At first, we assumed it was junk DNA, because it was a non-coding region not serving as a promoter, but upon further exploration we discovered that the segment may serve other purposes. In our exploration, we found there was little to no literature on this intergenic non-coding region of DNA. Even looking at the lac operon, which has been widely studied for genetic engineering purposes, the intergenic code was just glossed over. In a call with a custom gene design company, IDT, we hypothesized that the code may serve regulatory purposes for the upcoming gene(s) or it may just provide space for RNA polymerase to attach to the DNA strand.

Author Contributions

All authors participated in general research and proxy experiments. SB wrote the background. WB primarily researched and designed the plasmid insert and wrote the parts section. CO wrote the systems level section. ES worked on writing the background, and researched the gene insert and the methodology. HY wrote the device level and safety sections, and organized project materials. All authors wrote the discussion section and contacted related organizations.

Next Steps

The next step for this project is to raise the funding to purchase the plasmid insert and then perform the process as outlined in this paper. After producing *cada*verine, we would then work on researching an efficient way to utilize cadaverine in the production of nylon plastics. This *E. coli* based cadaverine production could potentially be the basis of a "green" plastic start-up company.

Acknowledgements

We would like to thank Ms. Anna Minutella for supporting us throughout this process. We would also like to thank Integrated DNA Technologies for helping us with the design of the gene insert.

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