An *E. coli* biosensor for the detection and monitoring of environmental copper concentration

Introduction

Over the last century, mass technological and industrial advancements have led to the widespread anthropogenic release and distribution of toxic metals in the environment, posing danger to both local ecosystems and human health [1]. Copper, one of the most common heavy metal elements, presents a particularly interesting duality to all living cells in that it is both essential for survival and potentially lethal. Indeed, research shows that excessive copper is toxic for all living organisms [2]. On a cellular level, copper ions can catalyze harmful redox reactions resulting in oxidation of cell membranes, damage to nucleic acids and generation of free radicals. In humans, copper is a major neurotoxin, causing cerebellar motor dysfunction and neurodegenerative diseases such as Parkinson Disease [2]. Further, copper exhibits additional hepatotoxicity, including Wilson's Disease, a hereditary disease in which copper elimination is impaired [3]. Given the evergrowing bioaccumulation of copper and due to its biohazardous nature, the global monitoring of environmental Cu levels has become more important now than ever before.

E. coli is armed with an endogenous CusS/CusR copper detection and expulsion system [4] [7]. When a high concentration of copper is present in the extracellular environment, the CusS transmembrane protein detects this change and phosphorylates the intracellular CusR protein, effectively activating it. CusR in turn binds to the pCusC promoter, initiating a cascade of downstream cellular events that aid in the efflux of copper ions, thereby expelling them and preventing cellular toxicity.

This paper hypothesizes that *E. coli* bacterial cells can be engineered to provide a useful indicator of copper ion concentration. By utilizing specific synthetic biology techniques to insert the blue amilCP chromoprotein downstream the intrinsic CusS/CusR detection system, the hypothesis is that *E. coli* cells will gain the ability to turn blue in proportion to the extracellular concentration of copper.

Methods

PCR AMPLIFICATION

First, PCR amplification of the pCusC template DNA was performed. Two 200µL tubes were prepared and filled with the appropriate reagents, including water, DNA primers and *Taq* 2x MasterMix (NEB, Item #M0270L), which contains *Taq* DNA polymerase. The pCusC template DNA was pipetted into one of the tubes, while the other was labeled as control. Both tubes were subsequently held on ice and loaded into a Thermocycler (Axygen MaxyGene II) where the following PCR program was executed: 95 °C for 5 min 30 sec, 58.3 °C for 30 sec, 72 °C for one minute and repeat, finally mounting to 72 °C for 10 min and holding at 4 °C.

PLASMID MINIPREP

The pAmilCP-promotorless plasmid DNA was extracted from an overnight pAmilCP-promoterless *E. Coli* cell culture. A series of buffer additions and microcentrifugations at 12,000 g were performed to obtain the ultimately eluted pAmilCP-promotorless plasmid DNA. The initial culture broth was placed into a spin column and spun down via microcentrifugation for 2 minutes. The resulting cell pellet was salvaged and resuspended in 250µL of resuspension

buffer. 250µL of lysis buffer and 350µL of precipitation buffer were subsequently added, causing the *E. coli* cells to lyse and the plasmid DNA to precipitate out, respectively. Finally, nuclease-free water was added to elute the plasmid.

GEL ELECTROPHORESIS

Gel electrophoresis was performed to verify the success of the PCR amplification and plasmid miniprep, ruling out contamination. First, agarose gel was loaded with 2µL of genRuler 1 kb DNA ladder. 2µL of loading dye was pipetted into three fresh tubes, each of which receiving 10µL of either the pAmilCP-promoterless plasmid miniprep (1), the pCusC insert PCR product (2) or the control PCR product (3). The resulting PCR and miniprep solutions were subsequently loaded into corresponding gel electrophoresis lanes and the procedure was performed at 100V for 45 minutes.

STANDARD BIOBRICK DOUBLE DIGEST

Next, a standard BioBrick double digest was performed on the pCusC insert PCR product and the pAmilCP-promotorless plasmid miniprep. Upon purchase, both parts already contained the standard BioBrick prefix and suffix sequences (figure 1). As is the standard, the pAmilCP-promotorless plasmid miniprep was digested with XbaI and ECORI-HF in a 1:1 ratio, while the pCusC insert PCR product was digested using a 2:1 ratio of SpeI and EcoRI-HF.

>generic biobrick fwd primer (prefix)	>generic biobrick rev primer (suffix)
GTTTCTTCGAATTCGCGG	GTTTCTTCCTGCAGCGG

Figure 1: Standard BioBrick prefix and suffix (primers).

DNA PURIFICATION

Following digestion, both DNA products were purified using the PureLink PCR Purification Kit™. In accordance with the sellers' protocol [reference], each sample was centrifuged a total of four times with the appropriate buffer insertions.

DNA LIGATION

Once purified, the pAmilCP-promotorless backbone was combined with the pCusC PCR insert to generate a ligated DNA product. Water, T4 ligase and 10X ligase buffer were additional, necessary reagents in this ligation process. Further, a negative control was set by preparing a tube without the pCusC insert, thereby eliminating the possibility of backbone-insert ligation in this sample, offering some indication of the proportion of pAmilCP-promotorless plasmids that are self-ligating.

BACTERIAL TRANSFORMATION

E. coli cell cultures were placed in two separate microcentrifuge tubes. The ligation product was added to one tube while the control tube received the control ligation. Superbroth medium was further added to each sample and they were incubated in a cell shaker for 30 minutes. Meanwhile, LB agar plates were supplemented with 25 µg/mL chloramphenicol and 1mM copper sulfate. Following incubation, the ligated and control *E. coli* bacterial solutions were streaked onto corresponding agar plates.

ABSORBANCE ASSAY (EFFICACY TEST)

The newly transformed *E. coli* strains from both the pAmilCP-promoterless control plate and the pCusAmil-IBM ligated plate were then resuspended in mediums containing 0.2, 1.0 and 2.0 μM copper sulfate. The resuspended cultures were subsequently spun down into cell pellets, effectively removing any copper sulfate that could interfere with the sample's color.

The pCusAmil-IBM ligated *E. coli* culture was pipetted into eight different test tubes with concentrations of copper sulfate ranging from 0.0 to 1.9 μM. These were centrifuged, eliminating any residual copper sulfate, and resuspended in pure water. At last, a triplicate absorbance assay was performed at 589 nm for each of the eight samples.

FINAL DIGEST/ELECTROPHORESIS

Finally, pAmilCP-promoterless and pCusAmil-IBM plasmids were extracted from the control and ligated bacteria respectively. These plasmids were digested using EcoRI-HF and SpeI before undergoing gel electrophoresis.

Results

The first round of gel electrophoresis assessed the effectiveness of pCusC amplification by PCR and control PCR, as well as the preparation of pAmilCP-promotorless plasmid miniprep (figure 2). The pCusC PCR product (lane 6) showed a tight intense band at about 250 bp, representing the multiple copies of short, linear pCusC sequences that migrated together under the same conditions, thus ending in the same spot. The negative control PCR product (lane 7), on the other hand, showed no DNA bands, effectively indicating that PCR reagents were likely uncontaminated. Finally, the plasmid miniprep (lane 2) revealed several smeared bands peaking at 2.5 kpb, larger than the pCusC product. This is as expected, since individual plasmids often contort and smear as they move through the agarose mesh-work [7].

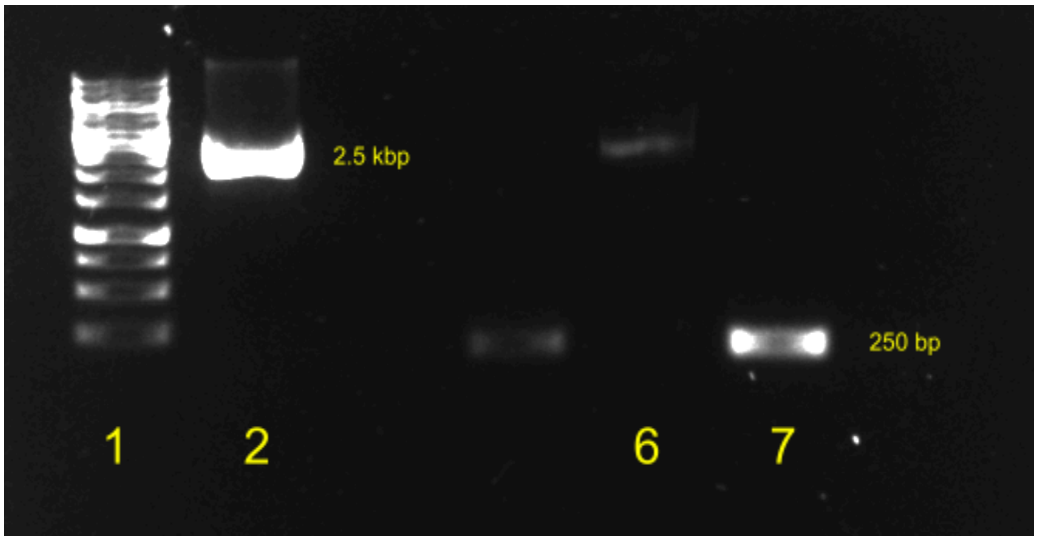

Figure 2: Gel electrophoresis results from the miniprep, PCR and control PCR products. Lane 1: genRuler 1kb DNA ladder (3 μL) . Lane 2: pAmilCP-promoterless plasmid miniprep (10 μL). Lane 6: pCusC insert PCR product (10 μL). Lane 7: control PCR product (10 μL).

Next, it was necessary to check whether the transformed *E. Coli* would indeed turn blue in proportion to the presence of copper sulfate. That is, the success of the newly engineered pCusAmil-IBM device in detecting Cu ions had to be assessed. It was expected that the agar plate containing pCusAmil-IBM (left) would exhibit blue *E. Coli* colonies since amilCP should be expressed following copper detection. However, this result did not occur and traditional, white bacterial colonies were observed on both plates (figure 3). This outcome shows that, while plasmid-containing *E. Coli* colonies were successfully cultivated and indeed chemically competent, the amilCP chromoprotein was, for some reason, not expressed. Though an error is most definitely at play here, it doesn't necessarily mean that the biological device is intrinsically faulty, and is more likely indicative of the fact that ligation is an imperfect process and often requires several attempts. Note that even under perfect conditions, even seasoned researchers might have to try again and again to achieve successful ligation [7].

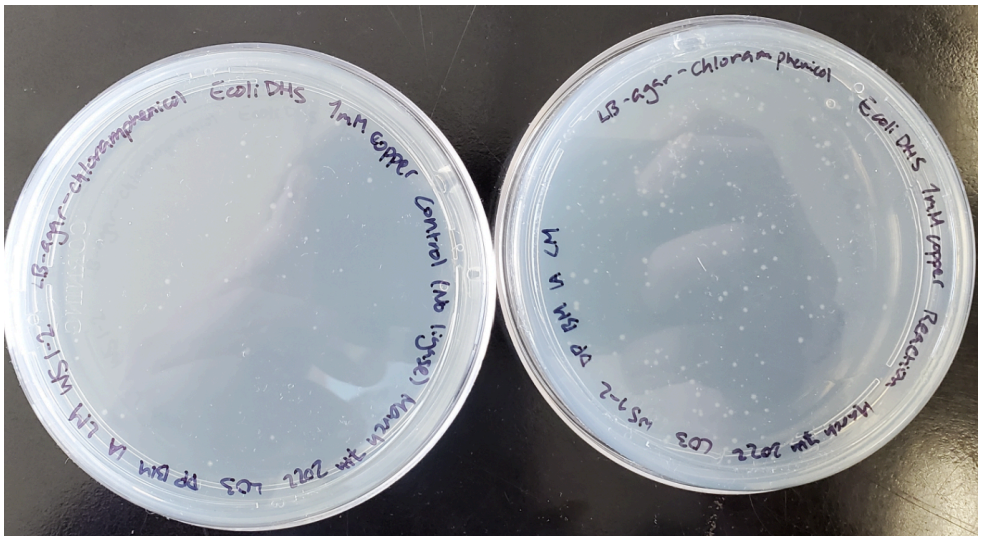

Figure 3: *E. Coli* modified cultures streaked onto agar plates, visible as white dots. Left: plate containing *E. coli* cultures with successfully ligated pCusAmil-IBM plasmid. Right: control plate containing mock ligation transformants (containing no pCusAmil-IBM).

***It is important to note that the following results were obtained under the assumption that the agar plates presented the expected (correct) data, as provided in the coursepack [7].

E. Coli colonies containing either pAmilCP-promoterless or pCusAmil-IBM plasmid were extracted and placed into tubes containing growth mediums of 0.2, 1.2 or 2.0 uM copper sulfate. The negative control tubes containing the pAmilCP-promoterless plasmid displayed a constant light blue color as the concentration of Cu ions increased, most likely due to the color of the copper ions themselves. On the other hand, the tubes containing successfully transformed pCusAmil-IBM *E. coli* cells displayed vibrant shades of blue that seemed to increase in strength with increasing copper sulfate concentration, suggesting that the detection circuit was functioning as intended.

That being said, since it is impossible to objectively distinguish between the two sources of blue coloration (copper sulfate and amilCP expression), the transformed *E. Coli* cells were spun down to remove any copper sulfate that remained in solution. The control cell pellets

appeared a dull orange-brown while the pCusAmil-IBM *E. Coli* pellets exhibited a dark blue color, verifying that the amilCP expressed in the pCusAmil-IBM plasmid was responsible for the intracellular blue coloration.

Finally, the absorbance assay was used to verify the pCusAmil-IBM plasmid's ability to quantify copper concentration via amilCP expression, which emits the color blue. The relative amount of amilCP protein present in each pCusAmil-IBM amilCP sample was thus determined by measuring the absorbance at 589 nm, to which amilCP is highly sensitive. Theoretically speaking, amilCP production should be upregulated with increasing Cu concentration and therefore 589 nm absorbance rates should also be greater. As seen in Figure 4, this was indeed the case. There is a linear trend in absorbance versus copper concentration, proving that amilCP is an effective quantitative signaller.

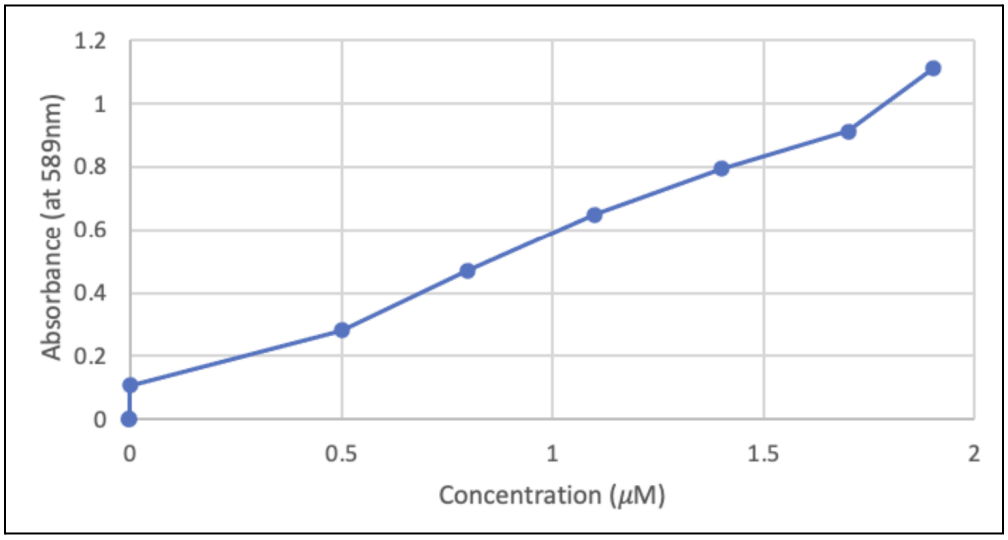

Figure 4: Absorbance of *E. coli* modified cultures at 589 nm vs extracellular concentration of copper sulfate (blue). Included is also the absorbance of the water used (origin point).

Finally, gel electrophoresis of the promoterless miniprep and pCusAmil-IBM digests was performed once more to confirm whether or not the ligation of the BioBrick part was successful. The results appear to indicate a success, but it is hard to judge given the small size of the Biobrick part (157 bp) in comparison to the complete plasmid. Nevertheless, lanes 4 and 5, which contained digested pAmilCP-promoterless and pCusAmil-IBM plasmids respectively, showed two bands of seemingly appropriate lengths. The topmost, thicker band adequately portrays the plasmid backbones of equal length, while the lower, much lighter bands represent the additional pCusC insert (lane 5) or lack thereof (lane 4), separated by the appropriate length of about 157 bp, as indicated by the DNA ladder.

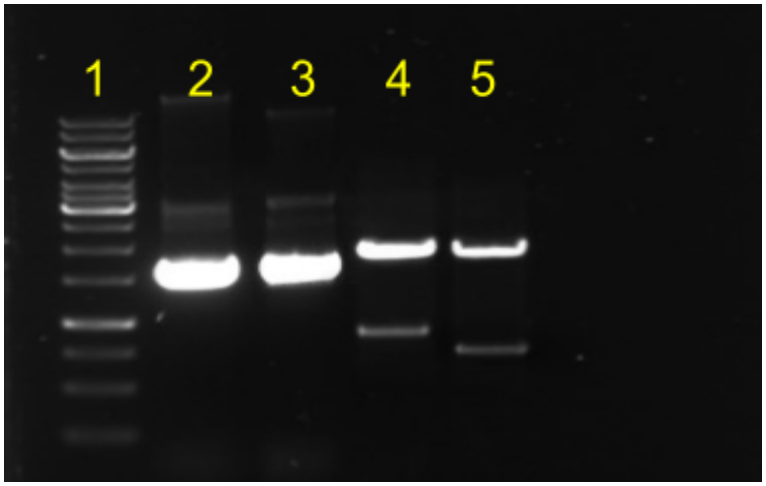

Figure 5: Gel electrophoresis of digested and undigested pAmilCP-promoterless and pCusAmil-IBM plasmids. Lane 1: genRuler 1 kb DNA ladder (3 μL). Lane 2: undigested pAmilCP-promoterless miniprep (10 μL). Lane 3: undigested pCusAmil-IBM miniprep (10 μL). Lane 4: digested pAmilCP-promoterless plasmid (10 μL). Lane 5: digested pCusAmil-IBM plasmid (10 μL).

Discussion & Conclusion

E. coli cells were successfully transformed with the constructed pCusAmil-IBM plasmid, assuming successful plasmid-insert ligation in accordance with the substitute data provided in the coursepack [7]. Therefore overall, despite this lab group's faulty ligation, this experiment verified that the CusS/CusR copper ion detection system endogenous to *E. coli* cells can be used in conjunction with the pCusC promoter product to effectively detect changes in copper ion concentration. Quantification of such changes is further achieved by upregulating downstream amilCP chromoprotein expression which successfully emits a blue color in proportion to Cu concentration.

The first step in reducing morbidity from copper exposure (or any other issue, for that matter) is the establishment of gold standard methods to measure the degree of the problem. Currently, detection of heavy metal pollutants in the environment mainly relies on physicochemical methods, like spectroscopy and mass spectrometry. Although these methods are accurate, they require expensive instrumentation and professional operation [1]. Therefore, developing an inexpensive, portable alternative method to determine the biological footprint of copper is paramount. This whole-cell amilCP bioreporter is an exciting new methodology that has proven its efficacy and high sensitivity. Widespread adoption of such bioreporters has the potential to mitigate the growing problem of copper toxicity both in humans and in the natural environment.

References

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