Research Article

Escherichia coli Splinter Killer System: Degradation of Plant Material Using a Transformed *E. coli* Chassis

SCIENTIFIC RIGOR

Lee Dong, Jana Latayan, Mayank Mali, Ashna Raiker, Caleb Trotz, Peter Wilson, Roy Yoon

Acton-Boxborough Regional High School, Acton Massachusetts, United States

Splinters are a common everyday injury, and current home remedies remain insufficient. Tweezers can be painful, difficult to use, and can leave fragments of wood behind. It is also important to remove a splinter promptly for two reasons: first, to reduce inflammation, and second, to reduce the chance of infection. To solve this problem, *Escherichia coli* can be genetically engineered to enzymatically digest the cellulose and lignin present in a wood splinter, which can then be washed out of the wound or reabsorbed by the body. Using Gibson Assembly, competent *E. coli* were transformed with a ligninase to digest the lignin in wood, with plans to add an endoglucanase, exoglucanase, and a beta-glucosidase to digest the cellulose in wood after the lignin is removed. However, when we assayed our transformed bacteria for ligninase production, we found that our bacteria were not expressing it extracellularly. Going forward, we plan to troubleshoot our lack of expression of ligninase and then transform our bacteria with cellulases to complete our system. For commercial purposes, this system can be used as the source of enzymes for a future enzymatic cream or bandage.

The goal of our system is to create bacteria that can digest lignin to make the removal of splinters in human skin less painful. In flowering plants, lignin evolutionarily supports rigidity for vascular tissue structure and therefore is an important component to consider in softening plant material. Bacterial digestion of wood has been extensively investigated for the production of sustainable fuels (Kumar et al. 2008), however, while systems that can digest pure cellulose exist, the lignin contained in wood prevents its digestion as the cellulose is held inaccessible to cellulolytic enzymes (Mosiera et al. 2004). Our ultimate goal is to transform a bacteria that is able to digest wood. Mosiera's paper outlines the process of enzymatically digesting lignin in plant material.

Materials and Methods

Preparation of Ligninase gBlock dsDNA. An insertable dsDNA sequence was designed to contain the desired ligninase coding region (BBa_M36188), preceded by a highly efficient isopropyl β-D-1-thiogalactopyranoside (IPTG) promoter (BBa_R0010), a strong ribosome binding site (RBS; BBa_B0034), a peIB leader sequence (BBa_J32015), and followed by a double terminator (BBa_B0010). The sequence also contained intentional 20 bp overlaps with the EcoRI and HindIII sites on vector pUC19 for simpler assembly. This sequence was manufactured and purchased from Integrated DNA Technologies (IDT, Coralville, IA) in the form of powdered dsDNA. The ligninase gBlock was reconstituted in Tris-EDTA (TE) buffer and incubated at 50°C for 20 min.
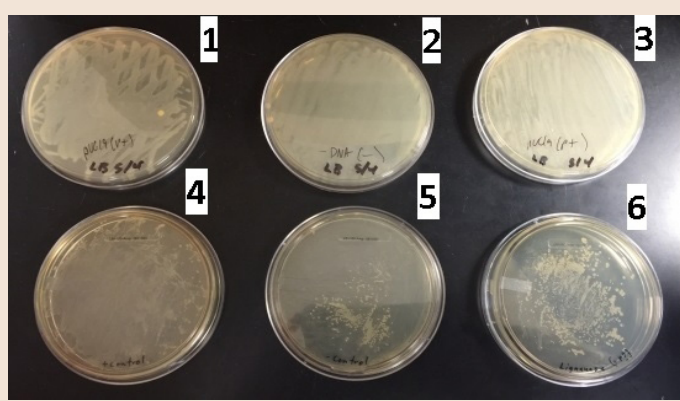
Figure 1. Panel 1: When plated with pUC19 transformants and no ampicillin (amp), the bacteria produced a contiguous lawn of growth on the agar. Panel 2: This plate did not contain any amp when it was inoculated with bacteria that lacked plasmid DNA. There was a "lawn" of E. coli, as expected, considering the absence of an antibiotic permits strong bacterial growth. Panel 3: When bacteria containing pUC19-Ligninase were added to plates without amp, they produced a lawn of growth. Panel 4: As expected, this plate had individual bacterial colonies from the addition of amp and bacteria transformed with pUC19. Those bacteria that accepted the pUC19 grew, while those that did not failed to grow. Panel 5: Without the pUC19, there should not have been any growth of E. coli due to the presence of the antibiotic. However, there was minor growth, which suggested a lab error. Panel 6: This plate contained pUC19 transformants as well as the ligninase. There were individual colonies, which indicated that some E. coli accepted both the pUC19 and the ligninase.

The gBlock solution had a final concentration of 10 ng/µL.

Linearization and Preparation of pUC19. A 1 µg/µL pUC19 solution, nuclease-free water, New England Biolabs (NEB, Ipswich MA) CutSmart Buffer, NEB Hi-Fidelity EcoRI solution, and NEB Hi-Fidelity HindIII solution were mixed by gentle flicking in a microcentrifuge tube to linearize the pUC19 transformation vector. The solution was thermocycled at 37°C for 30 min. to digest pUC19, and then at 65°C for 20 min. to inactivate EcoRI and HindIII. The solution was then diluted with nuclease-free water to achieve a final concentration of linearized pUC19 at 50 ng/µL.

Assembly of pUC19-Ligninase. The assembly reaction was set up on an ice bath. Linearized pUC19 solution, ligninase dsDNA solution, and NEB Hi-Fidelity DNA Assembly Master Mix were mixed in a microcentrifuge tube. The sample was then thermocycled at 50°C for 15 min. to complete the assembly reaction. The sample was then stored on ice.

Expression of pUC19-Ligninase. NEB chemically competent *Escherichia coli* K-12 cells were thawed to liquid state and transferred to a microcentrifuge tube. pUC19-Ligninase solution was then added to the microcentrifuge tube. The mixture was then mixed by gently flicking 5-6 times. The mixture was placed on ice for 30 min. The cells were heat-shocked in a hot water bath at 42°C for 30 s. The cells were then stored on ice for 2 min. Room temperature super optimal broth with catabolite repression (SOC) media was added to the cells. The cells were then incubated at 37°C for 60 min. The Luria Bertani-ampicillin (LB-amp)

(100 µg/mL) plates were heated to 37°C. pUC19-Ligninase cells were then plated, inoculated, and incubated overnight at 37°C. The cells were then replated on a second set of LB-amp plates due to failure of the first set of LB-amp plates. pUC19-Ligninase cells were also plated on LB plates.

Preparation of Positive Control Plates. NEB chemically competent *E. coli* K-12 cells were thawed to liquid state and transferred to a microcentrifuge tube. Unassembled pUC19 solution was then added to the microcentrifuge tube. The mixture was then mixed by gently flicking 5-6 times. The mixture was placed on ice for 30 min. The cells were heat-shocked in a hot water bath at 42°C for 30 s. The cells were then stored on ice for 2 min. Room temperature SOC media was added to the cells. The cells were then incubated at 37°C for 60 min. The LB-amp (100 µg/mL) plates were heated to 37°C. 100 µL of cells were then plated, inoculated, and incubated overnight at 37°C. The cells were then replated on a second set of LB-amp plates due to failure of the first set of LB-amp plates. pUC19 cells were also plated on LB plates.

Preparation of Negative Control Plates. NEB chemically competent *E. coli* K-12 cells were thawed to liquid state and transferred to a microcentrifuge tube. Room temperature SOC media was added to the cells. The cells were then incubated at 37°C for 60 min. The LB-amp (100 µg/mL) plates were heated to 37°C. 100 µL of cells were then plated, inoculated, and incubated overnight at 37°C. The cells were then replated on a second set of LB-amp plates due to failure of the first set of LB-amp plates. Untransformed cells were also plated on LB plates.

Ligninase Assay. The assay procedure described by Magalhães et al. (1996) was performed on pUC19-Ligninase *E. coli*. 0.2 M acetate buffer at pH 4.0, 8 µM methyl blue solution, 80 µM hydrogen peroxide, and a liquid LB-amp culture of pUC19-Ligninase *E. coli* were prepared. A reaction solution of methyl blue solution in a 1:1 ratio with hydrogen peroxide was prepared in the acetate buffer and added to the liquid media in order to assess lignin peroxidase activity. The assay solution was blue, so we looked for a color change and bubbling.

Results and Discussions

Upon the first trial and plating, it became apparent that the amp utilized had not been potent enough to kill untransformed *E. coli*. Therefore, a replating with a different amp was necessary on the plates that contained amp in order to garner valid results (Figure 1).

The assay described by Magalhães (1996) was performed according to the protocol above. Particularly, we looked for the color change in a dilute solution of Methyl blue. No color change occurred when the *E. coli* was added to the methyl blue solution, so we conclude that lignin peroxidase was not present.

The overall results of the experiments were successful. The *E. coli*, which accepted the pUC19, showed successful growth against the amp, indicated by the spots of bacterial growth on plate 2-1, which served as a positive control. Plate 1-2 showed a vast amount of *E. coli* growth, as no amp was added to the plate. Plate 2-3 contained *E. coli* with both the pUC19 and ligninase. The *E. coli* that grew on plate 2-3 showed similar patchy spots of bacterial growth, as in plate 2-1, indicating that the *E.*

coli that accepted the pUC19 and ligninase were able to successfully grow. Plate 2-2 contained ampicillin, and strains of *E. coli* that did not have the pUC19. Unexpectedly, there was a minute growth of *E. coli*. By theory, no strains of the *E. coli* should have been expressed, as none of the bacteria on plate 2-2 had resistance to amp. The results of plate 2-2 suggest the possibility of contamination by improper lab protocol. Some foreign material or other strains of bacteria may have entered plate 2-2 during the process of making the agar plate, the handling of the *E. coli*, or the inoculating process. Alternatively, an insufficient amount of amp may have been used or ineffective amp may have been used, but this is very unlikely, as plate 2-2 had minimal growth of *E. coli*, and plates 2-1 and 2-3 did not show the same growing patterns of the *E. coli* as plate 1-2, suggesting that the amp was effective.

The assay to confirm extracellular expression of ligninase failed, indicating no extracellular expression of the enzyme. Our leading hypothesis for why this was the case is that our bacteria were transformed with pUC19 without our ligninase insert. However, it is also possible that our design as a whole does not work. In the future, a restriction digest using electrophoresis to analyze components of the plasmid can indicate whether the ligninase sequence was spliced at all. If the results from this test show that we successfully transformed the plasmid that had our gene in it, we will need to modify our system before incorporating our original desired enzymes: exoglucanase, endoglucanase, and beta-glucosidase.

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