Designing, Cloning, and Testing Genetically Engineered Metabolic Pathways to Produce Therapeutics in Gut Bacteria

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Abstract

Equol is a natural isoflavone metabolite derived from daidzein, an isoflavone found in soybean and other soy products such as soy milk, tofu, and miso soup. Equol has a chemical structure similar to that of mammalian estrogen and thus can bind with estrogen receptors, allowing it to act as a supplement to estrogen in mammals. Research investigating equol has linked it to a reduced risk of breast cancer, prostate cancer, and colon cancer; cardiovascular diseases; osteoporosis diseases; and hormone-dependent illnesses. While some people can naturally convert dietary daidzein to equol utilizing their gut bacteria, a vast disparity exists between populations that can do so due to the presence of that bacteria in their gut. In western countries, the number of people characterized as equol producers is significantly smaller than those of Asian countries. Allowing a non-equol-producer to produce equol from dietary soy products through a series of engineered enzymatic reactions is achievable and has recently been confirmed. Building from this, we would like to genetically engineer a biocompatible filamentous bacteriophage (a virus that infects only *E. coli*) to carry these genes necessary for isoflavone metabolization (specifically daidzein to equol

conversion), such that these may introduce genes to bacteria of the intestines. By using genetically engineered bacteriophage, specially modified to introduce the necessary enzyme for the series of conversions from daidzein to equol, we can create a probiotic that allows consumers to receive the health benefits of soy-product-derived equol.

Introduction

Isoflavones and their respective derivatives are plant-based compounds extensively studied due to their similarities in structure and function to naturally occurring steroid hormones. Due to these similarities, their proposed mechanisms of action and subsequent effects are thought to be due to their binding of estrogen or androgen receptors in the body, giving rise to downstream agonist or antagonistic effects (Matthies et al., 2009). One isoflavone of interest is daidzein which is found exclusively in soybeans and other legumes. Through a series of enzymatic conversions, the reduction of daidzein into equol can occur, although only a fraction of the population possesses the necessary gut bacteria to do so. Equol, in contrast to its precursor and any other isoflavone, exhibits more potent estrogenic agonist activity (Mayo et al., 2019). Those displaying the equol-producing phenotypes have seen beneficial effects on health outcomes, including reduced risk of breast cancer and cardiovascular disease. (Jackson et al., 2011) The disparity between the population capable of metabolizing isoflavones and those unable to do so leads to health inequalities that we aim to address through this work.

The ability to introduce new metabolic pathways to the gut is a relatively novel idea that allows us to customize the biological components that a host can produce and utilize. Previous research employing human fecal transplants has successfully demonstrated the ability to impart

the equol-producing phenotype on those previously unable to metabolize it. (Liang et al., 2020) Supplementing a host with exogenous gut bacteria expressing the necessary enzymes for the desired metabolism of daidzein can allow the host to reap the benefits of daidzein-containing soy. A practical application of this idea is the development of a probiotic strain engineered to code for the necessary enzymes for the metabolic pathway. Research attempting to characterize those enzymes has identified two bacterial strains, *Slackia isoflavoniconvertens*, and *Lactococcus garvieae*, isolated from the human intestine, capable of carrying out the conversions. Further testing profiled four enzymes responsible for the daidzein to equol conversion, known as daidzein reductase (DZNR), dihydrodaidzein reductase (DHDR), tetrahydrodaidzein reductase (THDR), and dihydrodaidzein racemase (DDRC). (Schröder et al., 2013) Related experiments have successfully implemented these enzymes and developed a single-strain probiotic able to carry out the conversions. (Kydd et al., 2022.) Increased efficacy in the equol reduction pathway has been shown to be achievable by increasing the availability of free NADPH. The depletion of this cofactor in the cell hinders its ability to carry out the conversion, resulting in a low production rate. One approach to circumvent this is through the coproduction of glucose dehydrogenase. This addition will allow us to couple these reactions, regenerating the consumed NADPH and increasing our yield (Li et al., 2022). With this in mind, we plan to engineer a single-strain probiotic containing our four previously mentioned enzymes and glucose dehydrogenase. On top of this modification, we plan to introduce our genes through different means. Rather than inserting the genes into a bacterial strain, we plan to transform the modified plasmid containing the enzymes into a filamentous bacteriophage. This newly transformed phage will then interact with bacteria in the gut, where they infect and provide the genes to the bacteria. The goal of using phage is that they're able to infect bacteria already in the gut, minimizing the risk of rejection. To further

determine the efficacy of retention of our bacteriophage and conversion between dietary daidzein to equol, in vitro culture studies will be conducted.

Materials and Methods

Creation of Plasmids: The DNA plasmids (P1 and P2) were engineered through codon optimization of the desired genes from the *Lactococcus garvieae* strain to E. coli and purchased as gene blocks (Integrated DNA Technologies, Iowa, USA). Cyclization of the plasmids was done through PCR of gene blocks using BioBricks prefix and suffix and subsequent enzymatic digest using PstI, XbaI, EcoRI, and SpeI of a pUC19 vector and our purified PCR product insert. After ligation, the plasmid was transformed through heat shock into a competent E. coli strain (DH5alpha) and plated on an ampicillin LB plate for future use.

Creation of modified Bacteriophage: Collection of Fd-tet bacteriophage was accomplished through the purchase and cultivation of phage-infected bacteria and the isolation of resulting plaques. After the mini-prep of the bacteriophage DNA, PCR was utilized to amplify the entire circular plasmid, and DpnI to digest the methylated parental DNA template. The eluted purified product was then cut using restriction enzymes (EcoRI and XbaI) and then purified once again.

Ligation and Insertion into competent cells: PCR and digestion of the P1 and P2 plasmids were done to amplify our target genes and then ligated with our newly constructed Fd phage vector. This modified DNA was then transformed into a competent strain of E. coli (Tg1) and grown on a tetracycline LB plate to confirm the successful transformation of our plasmid. These plasmid-

containing cells were then grown on an LB agar plate to cultivate our desired phage and later isolated for analysis.

Results

The activity of our plasmids and their ability to perform their respective conversions were confirmed through thin-layer chromatography (TLC). An initial comparison of daidzein, dihydrodaidzein, and equol to samples of P1 and P2 culture cell pellets indicated that when provided with daidzein, the extracted cell pellet from our P1 culture exhibited daidzein to dihydrodaidzein conversion (Figure 1). Similarly, TLC testing was conducted to demonstrate the functionality of the enzymatic pathway for conversion of dihydrodaidzein to equol from our P2 culture (Figure 2). Results from these two figures show that the bacteria expressing the pathways from L. garvieae produced the metabolites dihydrodaidzein for P1 and equol for P2 as can be seen by their spot alignment on the TLC plates with that of our control standards.

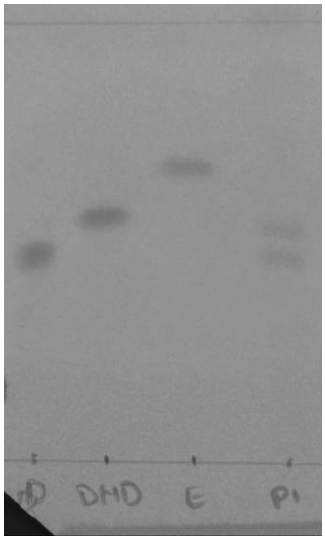

Figure 1. Thin layer chromatography (TLC) of daidzein control (lane 1), dihydrodaidzein control (lane 2), equol control (lane 3) extracted sample of cell pellet from P1 culture provided daidzein (lane 4) and extracted sample of cell pellet from P2 culture provided daidzein (lane 5), showing that the enzymes expressed in P1 carried out the conversion of daidzein to dihydrodaidzein in the isoflavone pathway.

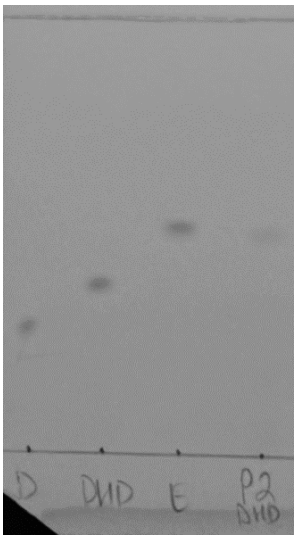

Figure 2. Thin layer chromatography (TLC) of daidzein control (lane 1), dihydrodaidzein control (lane 2), equol control (lane 3), and extracted sample of cell pellet from P2 culture provided dihydrodaidzein (lane 4), showing that the enzymes expressed in P2 carried out the conversion of daidzein to dihydrodaidzein in the isoflavone pathway.

Supplementary data will be collected to establish the validity of our choice of genes using analogous TLC plate analysis in order to determine if our rate of production exceeds that of a strain only expressing the four original enzymes (DZNR, DDRC, DHDR, and THDR), without the glucose dehydrogenase. Further *in vitro* culture studies will also be conducted to determine the efficacy of transmission and retention of our bacteriophage and the cell's subsequent daidzein to equol conversion rate.

Conclusion

In the past decade, numerous scientific articles have been written which emphasize the importance of the gut microbiome and its impact on human health and disease. We now know the beneficial interactions seen between a host and their gut microbiome rely on the type of microorganisms contained within the gut and their ability to coordinate with the rest of the body to provide metabolic processes and immune responses. (Quigley et al., 2013) Because of this, it’s more imperative than ever to build our understanding of the microorganisms within us and learn how to wield them in our favor.

Equol is an isoflavone metabolite that has seen an uptick in relevancy due to its pharmacological capabilities. One such capability is its ability to competitively bind to estrogen receptor beta and activate pathways causing decreases in vascular inflammation, risk of osteoporosis diseases, and hormone-dependent illnesses, among others; however, despite its potential as a pharmaceutical therapy, only a fraction of the population can naturally derive it from its precursor daidzein. (Hong et al., 2012) In response to this health inequality, probiotic strains have been developed to allow consumers the health benefits of soy-product-derived equol. However, with these previously created strains, issues arose surrounding low product yield and ineffective transmission of the probiotic into the body. Using our approach, we hope to address these issues through the addition of another enzyme (glucose dehydrogenase) which increases our product yield by replenishing a cofactor necessary for the conversions, and our use of a bacteriophage which allows us to take advantage of the bacteria already within our body and reduce the risk of the bacteria not being retained within the gut.

To summarize, our current testing has demonstrated the successful conversion of daidzein to dihydrodaidzein using the enzymes expressed in our P1 plasmid, and the conversion of dihydrodaidzein to equol using our P2 plasmid. With this, we ligated and introduced our desired genes to a bacteriophage. Our next step is to conduct in vitro studies and demonstrate the first use of bacteriophage transmission as a vector for providing isoflavone conversion pathway to bacteria. Long term future iterations of this project should focus on analyzing and identifying the specific interactions and pathways that lead to the health benefits observed in those with the equol-producing phenotype. Further insight into these interactions can give us a better idea of how the gut interacts with the rest of the body and may even allow us to replicate the beneficial effects seen by natural equol producers when consuming a daidzein rich diet.

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