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A Probiotic Organism's Delivery of CRISPR Expression Vectors Engineered with a Low-Magnesium-Induced Biosensor for the CRISPR-Cas13b Knockdown of ACC in EHEC Intestinal Pathogens

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Abstract

Objective: The overuse of antibiotics has led to the worldwide proliferation of antibiotic resistant and pathogenic bacteria, in which antibiotic resistant pathogens have become increasingly problematic for the medical community. Pathogenic bacteria such as enterohemorrhagic Escherichia coli and enteropathogenic E. coli cause bloody diarrhoea and are a byproduct of many gastrointestinal disorders. Overuse of antibiotics has increased the proliferation of antibiotic resistant and pathogenic bacteria. Probiotics have been used to treat and prevent pathogenic E. coli infections; however, the probiotics currently used are limited in the ability to specifically target and treat colonic diseases due to a lack of understanding of probiotic mechanisms of action during their production. As a consequence, using synthetic biological methods to engineer probiotics may allow a more precise targeting and treatment of many colonic diseases. One such synthetic biological tool includes modifying probiotic organisms with recombinant plasmids to more accurately prevent and treat intestinal infections. Probiotic bacterial strains could be engineered with a Clustered Regularly Interspaced Short Palindromic Repeat expression plasmid to help reduce bacterial pathogens in the colon. The CRISPR expression plasmid could be designed to inhibit essential enzymes required for the function of many biological processes in pathogenic bacteria, such as the acetyl-CoA carboxylase enzyme needed for fatty acid synthesis. However, more research is required for discovering new strategies that can more effectively deliver CRISPR-based antibacterials to reduce the pathogenic bacteria present in the colon. ***Results:*** Conclusion: Therefore, this review will discuss the immense potential and the limitations of utilising engineered probiotic organisms to deliver CRISPR-based gene therapies into the gastrointestinal tract. The review will specifically explore a novel gene therapy, of which probiotic organisms deliver recombinant plasmids engineered with synthetic gene circuits that can sense low intestinal magnesium levels and induce the release of CRISPR/Cas13b effectors to inhibit ACC in target EHEC bacterial cells to potentially clear EHEC pathogens from the colon.

Keywords: **ACC inhibitors, Bacterial infection, Biosensor, Cas13b, CRISPR, Diarrhea, Dysbiosis, Hemorrhagic E. coli**

Introduction

Escherichia coli inhabit the digestive tracts of animals and humans. *E. coli* assists with the digestion and with the formation of specific vitamins. There are more than 160 serological strains of *E. coli* that can cause infections of the urinary tract, nosocomial pneumonia, sepsis, infections of surgical sites, infections of the digestive intestinal tract, kidney diseases, inflamed meninges, and meningitis (Sarowska et al., 2019). Because the genome of *E. coli* has plasticity, *E. coli* can evolve into many diverse strains that are pathogenic, which are of immense concern for the public health of humans and animals (Rojas-Lopez et al., 2018). Disease-causing *E. coli* such as intestinal pathogenic *E. coli* strains are linked to chronic diarrhea that are categorised into six pathotypes. These six pathotypes of *E. coli* include enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC). The overuse of antibiotics has led to the extreme proliferation of antibiotic-resistant and pathogenic bacteria such as EHEC, in which antibiotics administered to cancer patients causes the dysbiosis of their gut microflora. This dysbiosis leads to more intestinal infections and to the pathogenesis of chronic colonic diseases. Probiotics have been used to treat and prevent many digestive tract disorders exacerbated by pathogenic *E. coli*. Probiotics have been used to treat intestinal tract disorders, such as ulcerative colitis, IBS, and Crohn's disease. However, existing probiotics lack probiotic supplementation strategies and methods that are consistent, and these probiotics cannot become optimized because there is limited knowledge of the probiotic mechanisms of action used during their production (Bober et al., 2018). In contrast, the use of synthetic biology can provide a direct method to more accurately engineer a probiotic organism (Aggarwal et al., 2020; Bober et al., 2018; Dou & Bennet, 2018; Hwang & Chang, 2020; Landry & Tabor, 2018, Leggieri et al., 2021; Mays & Nair, 2018; Pinero-Lambea & Gallego, 2015; Sola-Oladakun & Culligan, 2017, Zou & Marcotte, 2020). Probiotic products, developed via synthetic biological methods, will more directly treat disease, prevent infections, and benefit the overall health of the human body (Bober et al., 2018). An innovative probiotic product that uses synthetic biology to study the structure-function interactions in the gut microbiota can be used to engineer novel probiotic therapeutics. Because the microbiota have preferences for certain bodily locations, engineered microbial therapeutics can treat specific diseases and increase the health of a host (Bober et al., 2018).

Genetic tools can modify probiotic organisms, such as *Lactobacillus*, *Bifidobacteria*, and other *Bacteroids*, which can

benefit human health (Bober et al., 2018). A probiotic bacterial strain can be genetically modified to carry a novel genetic tool, such as the Clustered Regularly Interspaced Short Palindromic Repeat interference (CRISPRi) system, which can help target and eliminate intestinal pathogenic bacteria (Bober et al., 2018). The CRISPRi system could be constructed to inhibit the acetyl-coA carboxylase (ACC) enzyme, which catalyzes the first-rate limiting step of fatty acid synthesis (FAS) in bacteria such as pathogenic *E. coli*. In bacterial pathogens such as EHEC, de novo FAS is an essential process of bacterial metabolism (Polyak et al., 2012). Fatty acid synthesis is a significant pathway for bacterial quorum sense signaling and for modifying proteins (Polyak et al., 2012). Because acetyl-CoA carboxylase (ACCase) catalyzes the first step in fatty acid synthesis and is important for membrane synthesis, inhibiting the acetyl-CoA carboxylase enzyme (ACC) can block the fatty acid synthesis needed for restoring and maintaining the cell membranes of bacteria. The enzymes of FAS in eukaryotes are located on a single polypeptide chain, but in bacteria these enzymes are distinct gene products, thus any antibacterial agent that targets these bacterial proteins would not affect eukaryotic proteins, according to Evans (2018).

ACC is critical for FAS and inhibitors of this ACC enzyme can be used as novel antibacterial agents (Evans, 2018). Pfizer determined that a mutation in the carboxyl transferase (CT) subunit of ACC caused a mutant strain of *Staphylococcus aureus* to be temperature sensitive, showing that ACC is critical for mainiaining cell growth (Evans, 2018). GlaxoSmithKline confirmed this by producing a mutant strain of bacteria lacking the ACC gene (Evans, 2018). The fatty acid synthesis pathway in bacteria involves a series of enzymes that are different from the enzymes of mammalian FAS type I pathways, which are conducted by a single and large protein. Because of this distinction between bacterial and mammalian FAS, the bacterial FASII pathway has become a significant target for antibacterial discovery (Marreddy et al., 2018). Developing new and novel antibacterials that target fatty acid synthesis may eliminate bacteria such as pathogenic *E. coli* (Polyak et al., 2012). Utilizing CRISPRi could target and block the transcription and translation of ACC and many other enzymes of the FASII pathway. CRISPR-Cas provides a bacterial immune defense against foreign genetic material that includes extracellular genetic components and bacteriophages. The Cas enzymes are assisted by and guided to the gene target with single-guide RNAs that consist of complementary base pairs. Thus, a CRISPR-cas system for inhibiting ACC gene expression and to eliminate pathogenic bacteria could be developed; however, currently, there is a significant gap in research that lacks the necessary methods for effectively delivering CRISPR-cas complexes into bacteria (Ramachandran and Bikard, 2019). The delivery of CRISPR-based antibacterials presents challenges because the large 160kD CRISPR-Cas protein to RNA complex must permeate through the bacterial cell membrane for increased effectiveness (Greene, 2018). Phages have been used to deliver CRISPR-Cas through its injection of nucleic acids into bacterial cells.

A probiotic organism could be engineered to carry and transport CRISPR-Cas expression plasmids through the digestive tract into the pathogenic *E. coli* cells. Probiotic organisms could be genetically modified to transfer these conjugative plasmids, including a synthetic gene circuit with an inserted biosensor and the CRISPR-Cas system, to detect and eliminate pathogenic *E. coli*. A CRISPRi expression plasmid can be designed and assembled with a biosensor to sense changing intestinal conditions and aid the delivery of CRISPR-cas complexes into pathogenic bacteria. The inclusion of synthetic genetic components can expand the capabilities of an engineered probiotic to sense, record, and respond to the local environmental conditions in the gut (Bober et al., 2018; Chua et al., 2017; Claesen & Fischbach, 2015; Mimee et al., 2016; Tanna et al., 2021). Biosensors can detect the ions, small molecules, nucleic acids, and proteins of whole bacteria and viruses. A biosensor includes a biomolecule to detect and measure an analyte such as a small molecule or a nucleic acid. The biomolecule can be a nucleic acid, an antibody, or an enzyme (Buerk, 2014; Burlage and Tillmann, 2017; Goers et al., 2013; Goldschimidt, 1993; Hobson et al., 1996; Mulchandani & Rogers, 1998; Zourob et al., 2008). Developing sensors and diagnostics to help prevent and treat bacterial pathogenic infections of the intestines can favorably enhance the gut microbiome (Bober et al., 2018). The use of biosensors may also provide another rapid and frugal method for detecting bacterial pathogens (Ahmed et al., 2014; Amiri et al., 2018; Cesewski & Johnson, 2020; Furst & Francis, 2018; Lazcka et al., 2007; Palchetti & Mascini, 2008; Riu & Giussani, 2020; Simoska & Stevenson, 2019; Sin, 2014; Song, 2006). Additionally, many environmental conditions in the human intestines affect the adherence and virulence of *E. coli* pathogens, such as low pH, butyrate, fucose, ethanolamine, and low magnesium (Liu et al., 2020). For these reasons, this review will discuss the development of a biosensor that can detect, aid in targeting, and assist the delivery of CRISPR-Cas expression plasmids transferred by probiotic organisms into EHEC *E. coli* cells under low-magnesium intestinal conditions.

Discussion

LEE gene expression in EHEC during low magnesium intestinal conditions

The high potential for *E. coli* O157:H7 cells to adhere is determined by the expression of virulence factors, which cause inflammation and colonic wall lesions (Hwang et al., 2021). These virulence factors of EHEC are produced and secreted by the Type III secretion system that include EspD, EspB, and EscF, and EspA. The Type III secretion system secretes proteins that are encoded in the LEE gene cluster, causing pathogenicity. EHEC strains attach and efface to induce these lesions known as A/E lesions on the apical surfaces of host enterocytes. The EHEC and EPEC pathogens can adhere to the plasma membranes of host cells, damage the microvilli of the enterocytes and alter cytoskeletal orientations attached to the bacteria below. Every A/E pathogen has storage of pathogenicity islands called the locus of enterocyte effacement (LEE) that encodes the gene regulators of virulence factors such as intimin, adhesin, chaperones, many secreted proteins, intimin receptors, Tir, and a type III secretion system (Wong et al., 2011). The LEE-coded regulator (Ler) regulates the overexpression of the LEE region and also upregulates LEE (Hwang et al., 2021). LEE genes are increasingly regulated, in which LEE genes are only expressed during favorable environmental conditions such as low pH, fluxuations in butyrate levels, and low-magnesium levels (Carlson-Banning & Sperandio; Hernandez & Sperandio, 2013, Jia et al, 2021; Liu et al., 2019; Liu et al., 2020; Nijoroge et al., 2012; Wang et al., 2021).

In humans, essential magnesium extrinsic sources are only acquired via gastrointestinal absorption and digestion. The small intestines mainly absorb the magnesium because magnesium absorption does not occur in the large intestines, and as a result, the human large intestines have low levels of magnesium (Liu et al., 2020). Bacteria use a PhoQ/PhoP two component regulatory system to respond to magnesium level signals. When magnesium levels are low, PhoQ assists with phosphorylating PhoP (PhoP-P) that then increases the transcription of magnesium transport genes termed *mgtA* and *mgtB* (Liu et al., 2020; Salazar et al., 2010). Under high levels of magnesium, PhoQ promotes the unphosphorylation of PhoP. This PhoQ/PhoP regulatory system is essential and necessary for amplifying the virulence of many bacterial pathogens (Kato et al., 2008; Lippa, 2012; Liu et al., 2020; Salazar et al., 2010; Yuan et al., 2017). Liu et al. (2020) found a regulator of virulence called Z4267 or LmiA, which is a low-magnesium-induced regulator,

encoded by OI-119, that binds to the *ler* promoter and stimulates *ler* transcription during conditions of low-magnesium. The transcription of *ler* leads to the expression of LEE genes. The PhoQ/PhoP regulatory system guides the LmiA response to low magnesium signals in the human large intestine (Liu et al., 2020). For these reasons, Liu et al. (2020) investigated whether magnesium levels affect *lmiA* transcription levels.

In low magnesium levels of 50 μM, *lmiA* transcript levels were the same and unaffected, but in levels of high magnesium at 500 μM, *lmiA* transcript levels were substantially decreased (Liu et al., 2020). Through bacterial adherence assays the magnesium levels of 500 μM reduced the adherence of EHEC O157 in Caco-2 cells (Liu et al., 2020). The expression levels of the LEE genes were significantly decreased in high magnesium concentrations of 500 μM when compared against magnesium levels at 0M. These results confirmed that EHEC O157 can sense low levels of magnesium to signal the activation of LEE gene expression, which increases adherence (Liu et al., 2020). Because the adherence nor the LEE gene expression levels of the ΔlmiA, ΔphoQ, and ΔphoP mutants were altered by high magnesium levels, this indicates that LmiA and the PhoQ/PhoP two-component system are required for inducing this magnesium dependent virulence-regulatory pathway (Liu et al., 2020).

Additionally, mice were fed a normal to high magnesium diet for 7 days (Liu et al., 2020). When under the magnesium-rich diet, there was a 48.94-fold lower level of adherence by the bacteria than from the exact same sites when fed under a normal diet (Liu et al., 2020). The mice that were fed a magnesium rich diet displayed an 18.27-fold increase in magnesium levels of the luminal colon at a colonic concentration of 500 to 900 μM (Liu et al., 2020). The colonic concentration of magnesium at a normal diet of magnesium was 25 to 50 μM (Liu et al., 2020). These results show and confirm that at low-magnesium levels the adherence of EHEC O157 is increased in the mouse intestine, in which a high-magnesium diet significantly lowers adherence in vivo. The amount of bacteria collected from the ΔlmiA, ΔphoQ, or ΔphoP mutants was lower than the number of bacteria recovered from the mice infected with WT EHEC O157 (Liu et al., 2020). After feeding the mice a magnesium-rich diet, none of the mutants showed significant adherence in the intestinal tracts of the mice. Therefore, an agreement between the *in vitro* and *in vivo* results established that the effect of magnesium on EHEC O157 adherence is intermediated by LmiA and the PhoQ/PhoP two-component system.

The EHEC LmiA-PhoP virulence regulatory pathway under low magnesium intestinal conditions

Many bacteria have a PhoQ/PhoP regulatory system that responds to magnesium levels. During conditions of low-magnesium, PhoQ enhances the phosphorylation of PhoP (PhoP-P), which increases the transcription of magnesium translocation genes (Liu et al.,2020). Under high magnesium levels, PhoQ induces an unphosphorylated PhoP (Liu et al., 2020). The PhoQ/PhoP regulatory system is also present in pathogenic bacterial cells and also affects the virulence of these bacterial pathogens.

In order for Liu et al. (2020) to confirm whether LmiA regulates the expression of LEE genes, Liu et al. (2020) investigated the binding of LmiA to the LEE promoters, which include PLEE1, PLEE2/3, PLEE4, and PLEE5, in vitro. Liu et al. (2020) used electrophoretic mobility shift assays (EMSAs) and competition assays to determine the binding of LmiA to LEE promoters. When LmiA concentrations were increased, the bands for the LEE1 promoter were slow migrating bands (Liu et al., 2020). Additionally, adding an unlabeled LEE1 promoter competed for the binding of LmiA to the promoter region of LEE1. Liu et al. (2020) then confirmed the fold enrichment of the LEE promoters in LmiA-chromatic immunoprecipitation (ChIP) samples when compared against the mock-ChIP control sample groups. PLEE1 displayed a 9.87-fold enrichment in LmiA-ChIP samples versus the mock-ChIP control sample groups, which means LmiA binds to PLEE1 in vivo (Liu et al., 2020). These ChIP results were in agreement with the EMSA results. *ler* is the first gene encoded within LEE1, which initiates the expression of the LEE1 and LEE5 genes. Liu et al. (2020) then determined whether LmiA binds directly with the LEE1 promoter and then amplifies *ler* expression, and they confirmed whether LmiA controls EHEC O157 adherence and LEE gene expression through Ler. Liu et al. (2020) found that less expression of ler in EHEC O157 substantially reduced the EHEC O157 capability to adhere and decreased the expression of the LEE genes, in which, this confirmed ler regulates bacterial virulence.

Liu et al. (2020) used DNA affinity pulldown assays by labeling fragments with the lmiA promoter with biotin as a probe. Liu et al. (2020) did this to identify proteins a part of the transcriptional regulation of *lmiA*. Through this screening and assay, PhoP, as the regulator from the two-component transcriptional regulation, was discovered. They further investigated the binding capacity through EMSA and competition assays of the *lmiA* promoter with PhoP. When PhoP protein concentrations were increased, the *lmiA* promoter appeared as slow migrating bands. Adding unlabeled *lmiA* promoters effectively competed with PhoP for binding with the labeled *lmiA* promoters, in which this indicates that PhoP binds to the *lmiA* promoter in vitro (Liu et al., 2020). After ChIP-qPCR assays, the binding of PhoP to the *lmiA* promoter was further confirmed in vivo. In PhoP-ChIP samples *lmiA* promoters were highly present with a 7.45-fold increase in *lmiA* quantities versus the mock-ChIP control sample groups, in which these results indicated that PhoP binds exclusively to the *lmiA* promoter (Liu et al., 2020). These results show that PhoP binds specifically to *lmiA* promoters both in vitro and in vivo (Liu et al., 2020). Liu et al. (2020) continued investigating whether the PhoQ/PhoP two-component system controls EHEC O157 virulence via LmiA by forming ΔphoQ ΔlmiA and ΔphoP ΔlmiA double-mutant strains. Liu et al. found that the adherence capacity and the expression of LEE genes were increasingly reduced in all the mutants when compared against the wild-type (WT) strains. The ΔlmiA, ΔphoQ ΔlmiA, and ΔphoP ΔlmiA mutants displayed a decreased adherence and less LEE gene expression (Liu et al., 2020). When the ΔphoP mutant or the ΔphoP ΔlmiA double mutants were paired with a trc promoter-controlled *lmiA*, the virulence was reinstated similar to that of the WT levels. Therefore, these results confirmed that the PhoQ/PhoP two-component system positively modulates the expression of LEE genes to increase EHEC O157 adherence, in which LmiA mediates this regulation.

Possible effects of CRISPR-Cas system transferred by probiotic organisms into EHEC *E. coli* cells in low-magnesium intestinal conditions

The LmiA promoter can act as a biosensor to bind to PhoP during low-magnesium intestinal conditions. Under low-magnesium intestinal conditions, the PhoP produced can bind to the LmiA promoter of an expression plasmid to activate the CRISPR-Cas13b-gRNA RNase degradation of ACC (Figure 1). The CRISPR-Cas13b system could also be fused to an Enhanced Green Fluorescent Protein (EGFP) that would act as the reporter protein. Labeling the Cas13b protein with EGFP can allow the detection of the Cas13b degradation of ACC mRNA. The samples would appear bright green if there is substantial Cas13b activity, reporting amplified degradation of ACC target mRNA. Because LmiA is only present as a positive virulence regulator in EHEC O157, and in many other EHEC and enteropathogenic *E. coli* serotypes, the conjugative plasmid will only become expressed in these pathogenic *E. coli* cells. The conjugative plasmid would then be expressed when transferred into the pathogenic EHEC *E. coli* cells, which utilize this LmiA-mediated virulence regulatory pathway, and only under low magnesium conditions in the intestines. Under low intestinal

magnesium conditions, the *E.coli* pathogens would increasingly produce PhoP that would bind to the LmiA promoter of the conjugative plasmid (Figure 2). After PhoP binds to the LmiA promoter of the conjugative plasmid, this would signal and induce the production of LmiA to bind the LEE1 promoters contained in the CRISPR expression plasmid (Figure 1). The LmiA would bind to the LEE1 promoters to induce the expression of the CRISPR-Cas13b-gRNA-ACC system, which would knockdown the gene expression of ACC, causing the degradation of the cellular membranes of the EHEC pathogens.

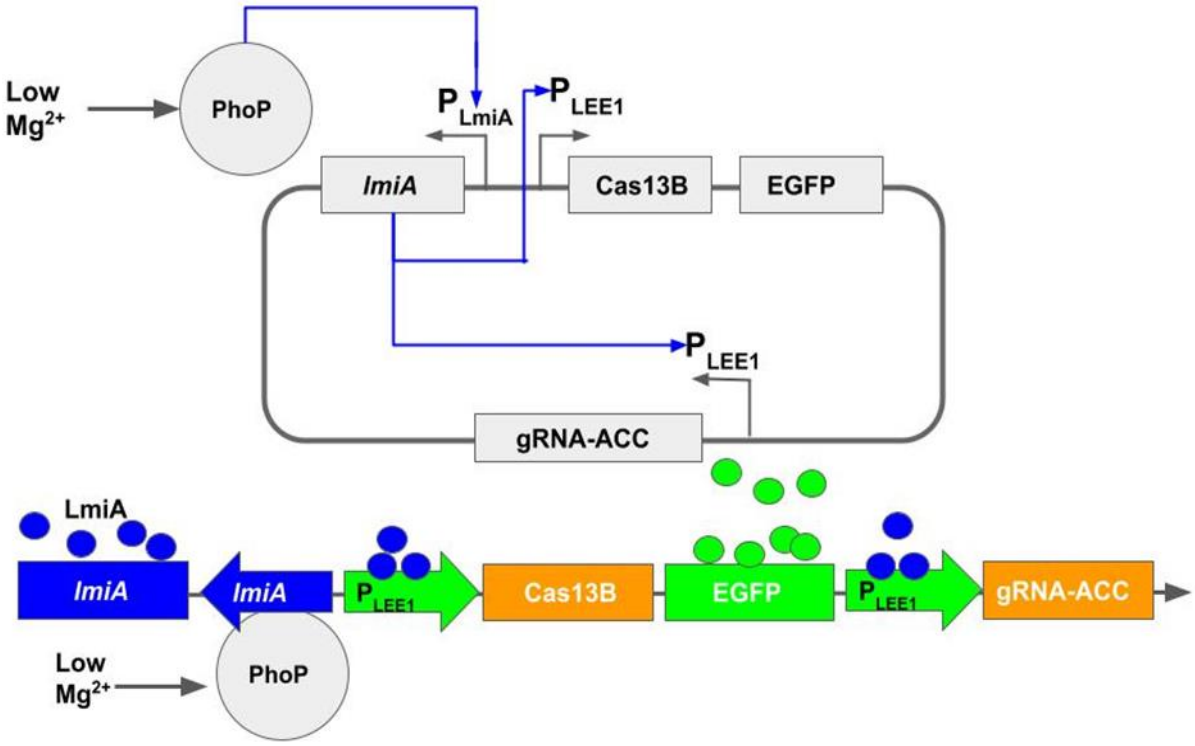

Figure 1. Plasmid Map and Model When the intestinal levels of magnesium are low in the intestines, PhoP production is amplified and the PhoP can bind to the LmiA promoter of the expression plasmid vector. The binding of PhoP to the LmiA promoter activates the expression of the LmiA gene, and then the LmiA binds to the LEE1 promoters, which induces the downstream expression of the CRISPR-based system for Cas13b-GFP-gRNA-ACC.

Probiotic bacterial organisms could be genetically modified with this pUC-PLmiA-PLEE1-CRISPR-Cas13B-GFP-PLEE1-gRNA-ACC recombinant plasmid for the plasmid's conjugative delivery into the EHEC pathogens of the intestinal tract. The modified probiotic organisms when transited through the intestines would transfer the conjugative plasmids through cell-to-cell contact with the EHEC cells (Figure 2). The recipient EHEC cell of the conjugative plasmid would express the Cas13b RNase knockdown of ACC during low magnesium intestinal conditions when low magnesium induces the amplified production of PhoP that would bind to the P_{LmiA} promoter of the conjugative plasmid (Figure 2). After PhoP binds to the LmiA promoter, the LmiA expressed would bind to the LEE1 promoters of the conjugative plasmids and activate the Cas13b RNase degradation of ACC target mRNA (Figure 2). Because more frequent horizontal gene transfer (HGT) events occur in the human gut microbiome than in a diverse ecosystem of nonhuman microbes (Liu et al., 2012), the probiotic organisms can transfer a significant amount of the conjugative plasmids. This physical proximity contributes to the increased frequency of HGT in the gut via crossdomain gene transfer (Shterzer & Mizrahi, 2015). Conjugation via HGT is significantly affected by physical proximity (Shterzer & Mizrahi, 2015). Additionally, the intestinal tract has conditions that are highly favorable for HGT since there is a continuous flux of nutrients and an access to food, a higher density of microbiota, a stable temperature, an increased formation of biofilm, and there is a large diversified array of enteric microbiota. According to Lerner et al. (2017), the gastrointestinal tract is a hot spot for HGT events where ingested probiotics can also become engaged in these events of HGT.

Additionally, the transfer efficiency of the probiotic organisms carrying the conjugative plasmids can be optimized and enhanced. Neil et al. (2021) optimized the transfer efficiency of conjugative probiotics by modifying the conjugative plasmid TP114 through accelerated laboratory evolution techniques. Neil et al. (2021) was able to eliminate more than 99% of the targeting antibiotic-resistant *E. coli* in the gut microbiota of mice by administering a single dose. This Neil et al. (2021) method also cleared *Citrobacter rodentium* infection, in which full clearance was achieved within four consecutive days of their optimized conjugative probiotics (COP) treatment. The work of Neil et al. confirmed and proved a succinct demonstration that bacterial conjugation can be optimized and leveraged for efficient CRISPR-Cas delivery. Their experiments with COP proved that COP is equally effective as streptomycin to clear *C. rodentium* infection in mice (Neil et al., 2021). The CRISPR-Cas13b RNase degradation of ACC can also inhibit the fatty acid synthesis needed for restoring and maintaining the cell membranes of EHEC pathogens. Possible effects of inhibiting fatty acid biosynthesis include membrane destabilization, the formation of pores, increased cell permeability, less bacterial cell growth, and the cell death of bacterial pathogens (Yoon et al., 2018). Utilizing CRISPR-Cas13b to inhibit fatty acid synthesis in EHEC can stagnate the electron transport chain and lessen the frequency of oxidative phosphorylation, which are required for producing the energy pathogenic bacterial cells need to function (Yoon et al., 2018). A lack of available fatty acids can impede the electron transport chain process because less access to fatty acids degrades the membrane integrity and decreases oxidative phosphorylation by lowering the membrane potential and decreasing the gradient of protons (Yoon et al., 2018).

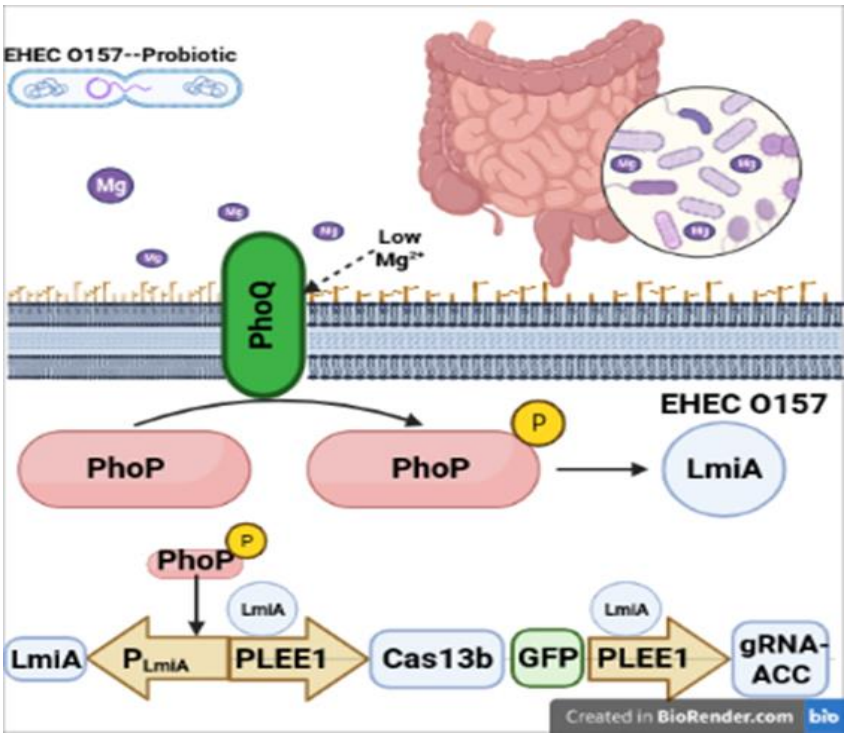

Figure 2. The Modified Probiotic's Conjugative Plasmids transferred to EHEC O157 during Low-Magnesium Intestinal Conditions The ingested probiotics transit through the intestines and transfers the conjugative plasmids via cell to cell contact with the EHEC O157 E. coli serotype. The EHEC O157 conjugation recipient, during low magnesium intestinal conditions, increases PhoP-P levels that bind to the LmiA promoter of the conjugative plasmid, which activates amplification of LmiA gene expression. The LmiA produced binds to the LEE1 promoters, inserted into the synthetic CRISPRi gene circuit of the conjugative plasmid, and induces CRISPR-Cas13b RNase degradation of ACC mRNA.

In conclusion, a probiotic organism could be modified with a recombinant plasmid inserted with an inducible signal biosensor that can consist of a promoter for LmiA that would bind to PhoP in low magnesium conditions. When the conjugative plasmid is transferred from the modified probiotic bacterial cell into the recipient pathogenic E. coli cell, low magnesium levels would trigger the binding of PhoP to the LmiA low magnesium biosensor of the conjugative plasmid that would produce LmiA molecules for binding to the LEE1 promoters and activate the CRISPR-Cas13b-gRNA RNase degradation of ACC mRNA in the targeted EHEC cells. This CRISPR-Cas13b inhibition of ACC would inhibit fatty acid synthesis in these bacterial pathogens, which would cause less cell growth, disrupt the electron transport chain with reducing oxidative phosphorylation, and lead to the cell death of these EHEC intestinal pathogens. The use of a biosensor such as a LmiA promoter to detect PhoP in low magnesium levels would more specifically sense, target, and transfer CRISPR-based antibacterials into EHEC bacteria while not significantly impacting the commensal bacteria in the gut microbiome since PhoP exclusively binds to LmiA in low magnesium concentrations *in vitro* or *in vivo*. However, future research is required for the *in vitro* and *in vivo* testing of the safety, effectiveness, and toxicity of applying CRISPR-Cas13b ACC inhibitors as antibacterials. Off-target effects may occur; however, Cas13 enzymes have significantly lower off-target effects where Cas13b does not express as many off-target effects as Cas13d (Xu et al., 2020). Designing gRNAs that are more specific for the target gene can eliminate many of the off-target effects. Including spacers of 30 nucleotides in the guide RNAs can limit the occurrences of off-target effects (Tang et al., 2021). Ultimately, utilizing biosensors to sense the environmental conditions, such as low magnesium levels, within the human intestines can more effectively detect bacterial pathogens and accurately deliver CRISPR-based antibacterials into bacterial pathogens such as EHEC O157.

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Conflict of interest declaration

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Contributors

Tatiana Hillman, LAL4Bsynbiotics L.L.C
The author T.H contributed to the conception, design, production, and writing of the present study.

Ethical Clearance

No animals or humans were included in this study.

Trial details

This study was not a part of a clinical trial.

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