AliveSCENT: A method for engineering *E. coli* to produce limonene as a natural mosquito repellent

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As a vector for disease transmission, mosquitoes are responsible for over 700,000 deaths each year, making it one of the most dangerous animals on the planet. Current methods of repelling mosquitoes, such as DEET, can cause their own problems like skin and eye irritation and allergic reactions. We propose a solution to this public health problem called AliveSCENT, a biologically-inspired mosquito deterrent that relies on the native methylerythritol 4-phosphate (MEP) pathway of *Escherichia coli* in the production of limonene as a natural, odor-releasing system for commercial use. Limonene is an aromatic compound that makes up a large portion of most citrus scents including lemons and is a safer alternative to sprays and flames and is cleaner than the insect mess created by bug zappers. The smell of the compound by itself has been compared to that of citrus and turpentine, known insect repellents. This product will attempt to reduce the incidence of malaria and other mosquito-borne illnesses. This system will be constructed through plasmid design, assembly, and integration, followed by verification of limonene product using gas chromatography, odor detection by wafting, and evaluation as an effective repellent using a mosquito choice chamber. Here, work towards plasmid assembly will be shown. This phase will require the assembly of a stationary phase promoter, with a ribosome binding site and limonene synthase translational unit into a red fluorescent protein-encoding vector.

Keywords: *Escherichia coli*, mosquito, limonene, repellent, MEP Pathway

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Watch a video introduction by the authors at https://youtu.be/QbxoF9eEDFA

Background

The world's deadliest animal is incredibly small, but that makes it no less concerning (Centers for Disease Control and Prevention 2019). The mosquito is responsible for thousands of deaths each year, making it one of the most dangerous animals on the planet. In 2017, nearly 500,000 people died of malaria (Centers for Disease Control and Prevention 2017). Malaria is an epidemic in over 100 countries (Haque, Scott, Hashizume, et al. 2012) and 3.2 billion people are at risk for contracting it (ChildFund International 2019). In the United States there are about 1,500 – 2,000 cases of malaria reported annually. The majority of these individuals traveled to a country with high rates of malaria (Mosquito Squad of Greater Washington 2014) including Honduras, French Guiana, Guatemala, Mexico and Ecuador (Behrens, Carroll, Beran, et al. 2007). When a mosquito bites a person with malaria, some blood contaminated with a Plasmodium parasite is taken up. These will mix with the mosquito's saliva. When the mosquito bites its next victim the saliva enters the person's bloodstream. This causes them to contract the illness. Malaria is only one of several mosquito borne illnesses that affect humans. In this way, mosquitoes are referred to as vectors for disease (Vedaste 2018).

There are over 3,000 species of mosquitoes. About 175 species are found in the U.S. They can live in almost any environment except extreme cold and can be found in most countries. Most mosquitoes lay their eggs in stagnant water. Both male and female mosquitoes feed on sugar, which they obtain through nectar and plant juices. Females, however, are the only ones that drink blood. This is because in order to lay eggs, they need proteins and lipids which are found in blood. Mosquitoes feed off of animals as well as humans. They are attracted to movement, smell, and carbon dioxide emissions. While mosquitoes are vectors for disease, not all mosquitoes transmit diseases. The mosquito genuses *Anopheles, Culex*, and Aedes are responsible for nearly all of the spread of mosquito-borne illnesses (National Geographic 2020). Mosquitoes can be repelled by certain scents because the strong odor interferes with their senses and makes it difficult to locate food.

The most common way to prevent mosquito bites and therefore disease transmission is by using products containing the colorless oily liquid with a mild odor, *N*, *N*-Diethyl-*meta*-toluamide, otherwise known as DEET. It is the United States Centers for Disease Control and Prevention (CDC) and Environmental Protection Agency (EPA) recommended product for insect repellent to protect against the spread of diseases, such as eastern equine encephalitis and malaria. Every year, approximately one-third of the U.S. population uses the chemical in some form to protect itself specifically from mosquito-borne illnesses and an estimated 130 products containing DEET are currently registered with the EPA. However, DEET products can cause skin and eye irritation, and sometimes major allergic reactions. Children, as well, are not supposed to use the product, leaving them with little to no protection from mosquito-borne illnesses (National Pesticide Information Center 2008). Although DEET is a commonly used product in the United States, in areas where malaria is not only more prevalent, but where malaria poses a real life or death risk, the usage of DEET products is not typical. Therefore, DEET may not be the best approach to repelling mosquitoes in the most geographically vulnerable areas. Our alternative, AliveSCENT, will be a less expensive, scalable, and safer alternative.

The most common insect repellents include bug zappers, citronella candles, and sprays. All of these come with hazards as they use electricity, fire, and chemicals. The main problem with the topical repellent DEET is that it is partially taken into the body through the skin. An alternative to using DEET is limonene, an aromatic compound that makes up approximately 90% of most citrus rinds, including lemons and oranges (Ladaniya 2008). Emitting limonene in a form reminiscent of an essential oil diffuser is a much safer and more efficient method than the topical application of DEET. Limonene is commonly said to smell of citrus, although some have likened it to turpentine, which is an effective, natural, environmentally-friendly insect repellent. Orange peel extract is about 90%-95% limonene. Mosquitoes are increasingly repelled at concentrations of 15%, 20%, and 25% orange peel extract (Effiom, Avoaja and Ohaeri 2012). If our engineered *E. coli* culture can successfully generate limonene, then a citrus smell should be detected. To confirm that the limonene produced by our system is sufficient to repel mosquitoes, a mosquito choice chamber will be used. One end of the choice chamber will contain the limonene generated by the reprogrammed bacteria and the other end will contain water as a control. A population of female mosquitoes will be introduced to the choice chamber and the number of mosquitoes at each end of the chamber will be counted every 30 seconds for 10 minutes. This will provide us with initial data to confirm whether or not our product effectively repels mosquitoes.

While other scents were considered, limonene seemed to be the best option for this system because *E. coli*, the selected chassis, naturally contains the genes to carry out the 2-C-methyl-d-erythritol 4-phosphate pathway which produces the precursor IPP needed to generate it. This seven step pathway converts pyruvate to (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP), an intermediate in isoprenoid biosynthesis. HMPP is further reduced to IPP and its isomer, dimethylallyl pyrophosphate (DMAPP). In our system, IPP will need

to be converted to geranyl pyrophosphate (GPP) with the help of geranyl diphosphate synthase (GPPS). GPP will then be able to be converted to limonene by LIMS (Figure 1). To mimic the biosynthesis of limonene in *E. coli* will require a two-tiered genetic program that diverts IPP from the native MEP pathway for use in the non-native limonene generating pathway. Since *E. coli* do not naturally manufacture either GPPS, necessary for GPP synthesis or LIMS, necessary for limonene synthesis, the genetic construct will need to contain the instructions for generating both enzymes. To that end, two devices will be built. One will be programmed to make GPPS controlled by a constitutive promoter described by Device A, thereby allowing for GPPS to be made continuously in order for available IPP to be converted to GPP. A second will be programmed to make LIMS controlled by a stationary phase promoter described by Device B. When the culture has reached its stationary growth phase, it will begin to manufacture LIMS. By coordinating the production of these two essential enzymes, limonene output will be optimized.

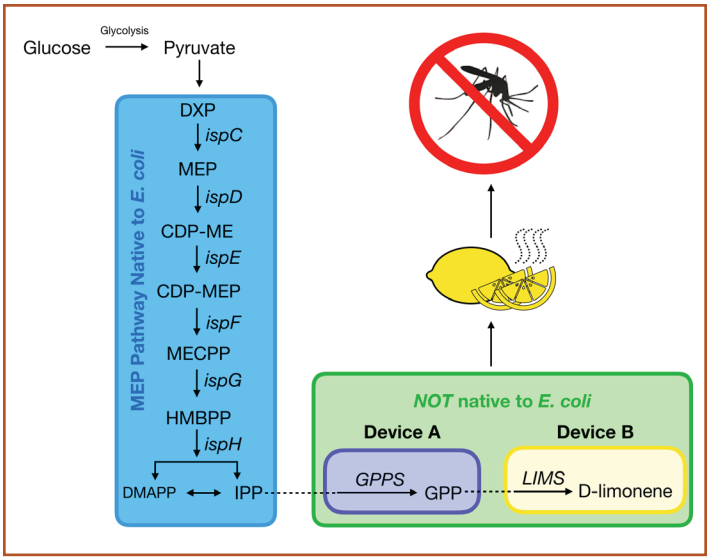

Figure 1. *General description of AliveSCENT showing the native MEP Pathway (shaded blue) for IPP biosynthesis and non-native pathway (shaded green) for limonene production.*

Systems level

The proposed AliveSCENT system has been designed to be a natural insect repellent for the mitigation of mosquito-borne illnesses. The oils in citrus fruit rinds contain limonene, known to be a potential mosquito repellent. This system intends to exploit the MEP pathway found naturally in NEB 5-alpha Competent *E. coli* for the production of limonene. IPP, the end product of this native pathway is a necessary input for the desired limonene output (Figure 2). In summary, for the aromatic monoterpene limonene to be detected by the olfactory machinery of mosquitoes will require its diffusion into the environment in a manner similar to an oil diffuser. Like a citronella candle without the flame, the purified limonene can be placed indoors or outdoors in areas where mosquito-borne illnesses are prevalent.

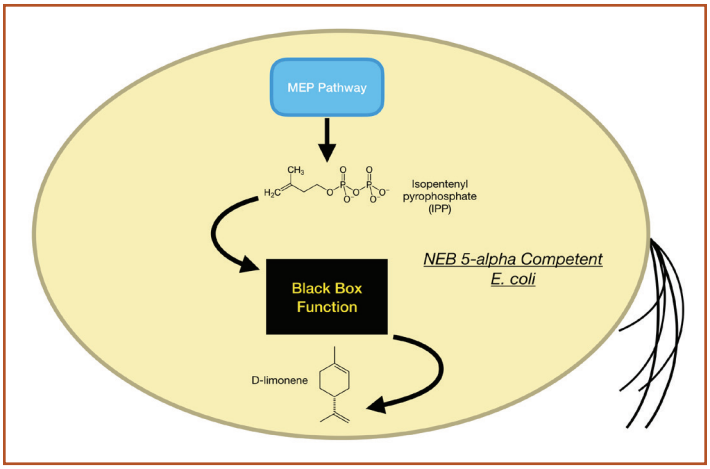

Figure 2. *Systems level description of AliveSCENT. The exploitation of isopentenyl pyrophosphate (IPP) from the native MEP pathway serves as the input for production of the monoterpene, D-limonene output in an E. coli chassis. The black box shown is intended to convey the introduction of two, novel genetic devices that will lead to limonene output. Specific device characteristics are described below.*

Device level

The proposed genetic program will be run in NEB 5-alpha Competent *E. coli* to produce limonene, an aromatic compound said to resemble the smell of lemons or turpentine. This system takes advantage of *E. coli*'s native MEP pathway. The biosynthesis of IPP, the necessary limonene precursor compound begins when glucose is converted to pyruvate. Five enzymes (DXR, ispD, ispE, ispF, and ispG) catalyze the conversion of pyruvate into HMBPP which is finally reduced to IPP. The MEP pathway native to E.coli can be exploited for the synthesis of isoprenes like IPP in limonene production, but will require the introduction of two novel devices - Device A and Device B. Device A will be designed to express the enzyme GPPS for the conversion of IPP to GPP (Figure 3). Device B will be designed to express limonene synthase (LIMS) for the conversion of GPP to limonene, thus producing the target scent.

Parts level

This system will first take advantage of the natural production of IPP through the MEP pathway and then introduce two non-native genetic cassettes (Device A

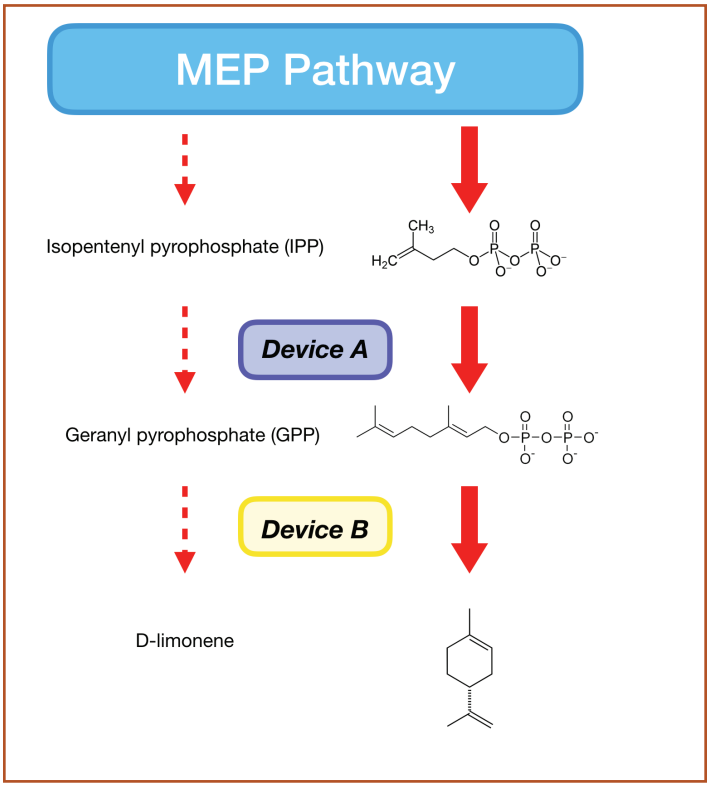

Figure 3. *Device level description of AliveSCENT. Isopentenyl pyrophosphate introduced from the native MEP pathway, will be converted to geranyl pyrophosphate catalyzed by geranyl pyrophosphate synthase expressed by Device A. Geranyl pyrophosphate is converted to D-limonene catalyzed by limonene synthase expressed by Device B.*

and Device B) enabling limonene production. Device A (Figure 4) will be constructed from four parts including a constitutive promoter, a strong ribosome binding site, the GPPS translational unit, and a double terminator. The benefit of having a promoter that is always active is that *E. coli* will be programmed to always produce GPPS, thereby constantly converting available IPP into the chemical intermediate GPP. When the cells reach the stationary phase, they will have enough GPP input for limonene synthesis. To achieve this goal, we propose the introduction of limonene synthase, the enzyme required to convert GPP to limonene (see Figure 3). Device B (see Figure 5) will be constructed from six parts beginning with a stationary phase promoter. We used this promoter in order for the bacteria to produce the most limonene during the stationary growth phase, which describes the point during growth when the total deaths are equal to the number of new cells, keeping the population constant and limonene production at its maximum. The promoter is followed by a ribosome binding site, involved in the translation of mRNA at the site of the ribosome where limonene synthase will be built. The next part of the sequence is the LIMS open reading frame which codes for the production of limonene synthase responsible for catalyzing the conversion of GPP to limonene. Limonene is an aromatic compound in citrus rinds that has been found to repel mosquitoes when released into the surrounding environment. After the LIMS open reading frame, a second RBS is included to assist in the binding of the mRNA coding for red fluorescent protein (RFP). The second open reading frame codes for red fluorescent protein and has been introduced as a biological marker used to detect successful operon function. Finally, the double terminator ends the sequence.

Safety

When designing and experimenting with the product AliveSCENT, necessary precautions need to be taken in order to maintain a safe lab environment. All appropriate lab equipment will be worn and utilized during any experiments. *E. coli* used will be treated with 10% bleach prior to disposal. When the chemical

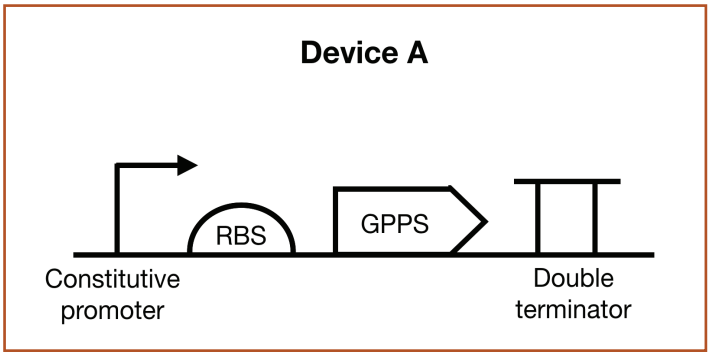

Figure 4 . *Parts level description of Device A for AliveSCENT. This genetic program will be constructed from four parts including a constitutive promoter, a strong ribosome binding site (RBS), the GPPS translational unit, and a double terminator.*

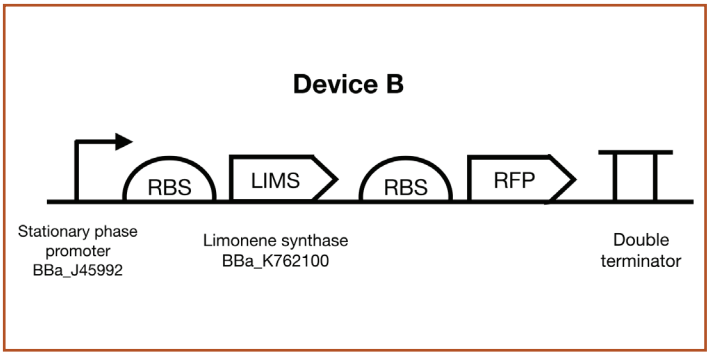

Figure 5 . *Parts level description of Device B. This genetic program will be constructed from six parts including a stationary phase promoter, ribosome binding site, a LIMS translational unit, a second ribosome binding site, a red fluorescent protein (RFP) open reading frame, and a double terminator.*

limonene is produced or tested, all experimentation will be conducted under a fume hood. It is important to keep in mind that limonene may cause irritation of the skin, eyes, or respiratory tract. This chemical can also be flammable in liquid and vapor forms, with an upper limit of 6.10% as described by the Materials Safety Data Sheet (MSDS). It is also important to know that if consumed, limonene could potentially cause injury and affect health with a National Fire Protection Association health rating of 3 (Thermo Fisher Scientific 2018). In turn, safe lab practices will be employed when dealing with the substance, specifically in large quantities or high concentrations. The MSDS for limonene will be made available upon request.

Prior to testing the effectiveness of AliveSCENT at repelling mosquitoes using a choice chamber, a colony of mosquitoes will need to be raised. Mosquito larvae native to our region will be obtained from stagnant water sources and will be bred in plastic containers with clean water until adulthood. Transferring mosquitoes to a choice chamber tube will require temporarily anesthetizing a sample of adult mosquitoes and sorting them by gender specific phenotypes. Female mosquitoes will be carefully transferred to the chamber using a soft hair paint brush and males will be returned to the colony. After experimentation, live mosquitoes will be properly euthanized by freezing for 48 hours. Personal protective equipment including goggles, lab aprons, gloves, and clothing that provides coverage of arms and legs will be worn when handling mosquitoes. Extra screen doors will be added to rooms housing the mosquitoes as an extra precaution against possible escapes.

AliveSCENT will provide a safer alternative against mosquitoes compared to other popular methods such as sprays, flames, and insect nets, which could all cause irritation or injury. However, AliveSCENT employs the use of the compound limonene. Individuals who suffer from an allergy or sensitivity to limonene should avoid this product to reduce possible allergic reactions. It is also important to avoid releasing the modified *E. coli* used in AliveSCENT to the outside environment. To achieve this we will include a kill switch with our genetic circuit to ensure bacteria that escape the testing or AliveSCENT environment will not be viable (Aguiton, N.D.). This kill switch is necessary to ensure the safety of this system, as well as the individuals and environments it may find itself in contact with.

Discussions

The purpose of this project is to engineer a genetic program that produces limonene, an aromatic hydrocarbon found in the oil of citrus fruit peels for use as an effective mosquito repellent. In order for limonene to be expressed as a final product, its precursor compound IPP generated from the native MEP pathway of Escherichia coli will require GPPS and LIMS not naturally produced by Escherichia coli, but necessary for limonene synthesis. Given this constraint, we have designed a 2-device system to address the issue.

At present, progress towards meeting this goal has been challenging. To date, the THS-BBC has transformed NEB 5-alpha competent *E. coli* (NEB#C29871) with the translational unit limonene synthase (Hollingshead 2007) and has reported mixed results. After a 24 hour incubation period, cells plated on a 1:500 dilution of 34 mg/mL chloramphenicol stock showed a lack of growth. Similar results were observed for cells grown in a 1:10 dilution of the 34 mg/mL chloramphenicol stock solution. While chloramphenicol was intended to determine transformation it was impossible to ascertain which cells had been successfully transformed because the concentration of chloramphenicol was not only too low, but the cell concentration was also too high. To account for this complication, an additional plate was prepared with 25µl of undiluted 34 mg/mL chloramphenicol stock solution and inoculated with 2 µL of cells. After a 24-hour incubation period, there was no visible growth with the exception of colonies along the plate's periphery. Given that the LB plate was superficially treated with chloramphenicol and may not have reached the plate edges, we are hesitant to conclude that the few colonies growing at this location are successful transformants.

Future workflow to achieve our goal of reprogramming *E. coli* to produce limonene will first require a successful transformation of the limonene synthase translational unit. Once we have confirmed that this part has been successfully taken up, transformants will be grown into a liquid culture and the part will be subsequently purified. The pBbE5a-RFP vector along with LIMS will be amplified using polymerase chain reaction (pcr). The pcr products will be confirmed using gel electrophoresis and purified for final assembly. Gibson assembly will be employed to ligate the stationary phase promoter BBa_J45992, LIMS and the pBbE5a-RFP vector into the final AliveSCENT product which will be transformed into NEB 5-alpha Competent *E. coli* cells. Confirmation of AliveSCENT will be accomplished by gas chromatography, odor detection by wafting, and evaluation as an effective repellent using a mosquito choice chamber. Furthermore, experimentation will include the construction and an additional transformation of *E. coli* with a second plasmid containing GPPS required to close the MEP pathway for limonene production. Successfully transformed cells will be tested for limonene production by lysis followed by detection through gas chromatography. Evaluation of limonene effectiveness as a mosquito repellent will be accomplished by using a choice chamber to compare mosquito response to limonene extract from

E. coli versus limonene extract from lemon peels. The mosquitoes should demonstrate negative chemotaxis to the limonene. Lemon grass and citronellol are well known mosquito repellents and turpentine has been shown to deter insects such as ticks. We envision AliveScent to be a non-topical product that can be used both indoors and outdoors as a better alternative to the current products on the market. It is our hope that our product can provide a sustainable, affordable, and accessible option to be used by people all over the world.

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