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# Saving the Honeybees: A Synthetic Biology Approach

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## Abstract

Since 2006-2007, beekeepers in the US have seen dramatic losses in honeybees, which could potentially cost billions of dollars in agriculture. The phenomenon has been termed Colony Collapse Disorder (CCD), and is characterized by rapid losses of adult honeybees that do not return to the hive to die. Even more puzzling is that damaging levels of normal bee parasites are not present at the time of collapse. Researchers have yet to determine a specific cause of the disorder, although most agree that CCD is caused by multiple factors that either work individually or in combination. A newly suspected cause of CCD is the fungus-virus combination of *Nosema* and invertebrate iridescent virus. This research project will focus on solving this possible cause of CCD using a synthetic biology approach. The use of genetically engineered microorganisms to test the theory could be an important step in solving the mystery of CCD.

## Background

Honeybees have an estimated value of about \$15 billion to agriculture in the United States and over \$215 billion worldwide, and it is estimated that one-third of the food we eat comes from honeybee pollination, either directly or indirectly. Honeybees also play an important role in pollinating other plants that are vital to maintaining ecological balance.

Colony Collapse Disorder (CCD) is characterized by rapid loss of adult honeybees that do not return to the hive to die, leaving excess immature bees in the combs. The queen bee is usually still present and there is an absence of damaging levels of typical bee pathogens or parasites. In June 2010, the CCD Steering Committee reported an estimated 34% overall honeybee loss in the US, with similar losses reported in 2007, 2008, and 2009. There are many suspected causes of CCD, including malnutrition, pathogens, immunodeficiency, pesticides, antibiotics, genetically modified crops (with pest control characteristics), migratory beekeeping practices, stresses from environmental change, and even electromagnetic radiation. Most researchers agree that while the exact cause of CCD remains unknown, it must be multiple factors working individually or in combination.

One recent study on CCD, "Iridovirus and Microsporidian Linked to Honey Bee Colony Decline," strongly correlated levels of an invertebrate iridescent virus (IIV) and *Nosema*, a unicellular fungus, with CCD. The study reinforced this correlation with cage trials using IIV-6 and *Nosema ceranae*. The trials showed that "co-infection with these two pathogens was more lethal to bees than either pathogen alone." The results of the study are compatible with results from previous studies, which helps support the hypothesis that CCD is caused by the combination of IIV and *Nosema*.

If this pathogen combination is, in fact, the cause of CCD, then CCD prevalence should be decreased if one of the pathogens is eliminated. It would be beneficial to test the hypothesis that CCD is caused by the combination of *Nosema* and IIV by developing a system to eliminate *Nosema* using synthetic biology techniques.

Beekeepers currently control *Nosema* with a compound called fumagillin, which is produced by a fungus called *Aspergillus fumigatus*. The genome of *A. fumigatus* has been sequenced and the molecular structure of fumagillin is known (Figure 1), though the genes responsible for fumagillin synthesis have not been identified. Once these genes are identified, engineering a microbe to produce fumagillin may be a more efficient and permanent method of fumagillin distribution.

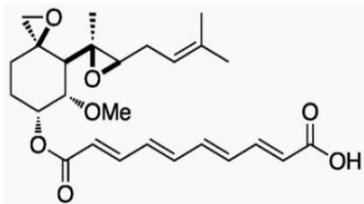


Figure 1 - Fumagillin Structure

## Approach

### Step 1: Identify the genes for fumagillin production

In order to identify the genes responsible for the production of fumagillin, transposon mutagenesis can be used. This technique involves inserting an antibiotic resistance gene into a transposon, mutating *A. fumigatus* spores with the transposon, then screening the mutants. To screen the mutants, they are grown on media with the antibiotic so that only mutants containing the transposon will survive. Screening for fumagillin production can be accomplished using HPLC. Mutants that do not produce fumagillin have transposons inserted into their genome such that genes for fumagillin synthesis are interrupted. PCR amplification and sequencing can then be utilized to sequence portions of the interrupted genes. Those sequences can then be analyzed against the genome of *A. fumigatus* and the genes for fumagillin production can be identified. Statistical analysis can then be performed to verify that all genes in the pathway have been identified.

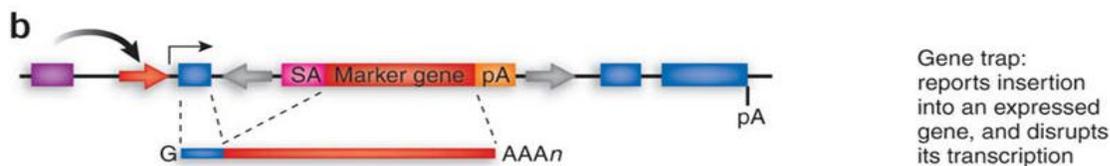


Figure 2 –Transposon Mutagenesis

### Step 2: Clone genes into a standard vector

The first step in cloning the genes of interest into a plasmid is to design primers for said genes. Then PCR amplification can be utilized to create many copies of the genes, which are then individually ligated into a standard high copy biobrick plasmid backbone. Ligation products are then transformed into *E. coli* cells. Possible ligation products include the gene ligated to itself, the plasmid ligated to itself, and the

gene ligated to the plasmid. The gene ligated to the plasmid is the desired product and can be screened for using the antibiotic resistance of the plasmid. Colonies that contain the plasmid with the gene will be selected. This process is completed for each gene in the pathway.

Once each gene is cloned into a standard vector, the genes must be combined into one plasmid. The general process for combining genes into a plasmid is depicted in Figure 3.

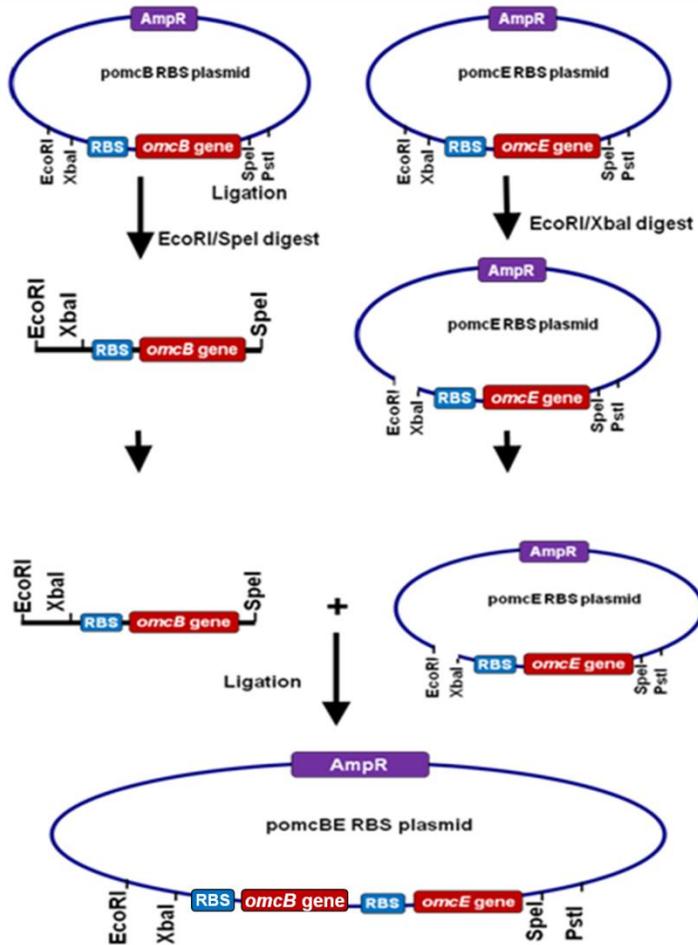


Figure 3 – Combining Genes

### Step 3: Test the system

After obtaining *E. coli* that is transformed with a plasmid that contains all of the genes in the fumagillin pathway, the system must be tested. The modified *E. coli* can be tested for fumagillin production by using HPLC, and levels of production would be compared with that of *A. fumigatus*. Testing for the control of *Nosema* can be accomplished with an antibiotic sensitivity test. If *Nosema* is controlled by the modified bacteria, the petri dish containing *Nosema* would have no growth around *E. coli* colonies. An example of this is pictured in Figure 4, where *Nosema* would be yellow and *E. coli* would be white.

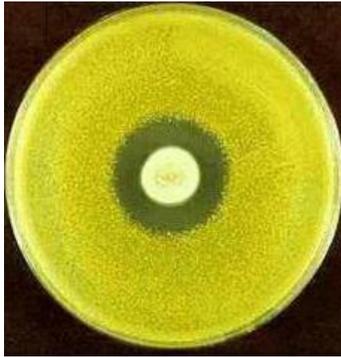


Figure 4 – Antibiotic Sensitivity Test

In order to test the hypothesis that CCD is caused by *Nosema* and IIV, cage trials must be performed. If the hypothesis is supported, there would be decreased death rates of CCD honeybees when exposed to the modified *E. coli*. Cage trials could be modeled after the method used in the study “Iridovirus and Microsporidian Linked to Honey Bee Colony Decline.” It may be beneficial or necessary to characterize a bacterium native to the honeybee and modify it with the plasmid created in Step 2 in order to decrease honeybee deaths (*E. coli* may not be compatible with the honeybee). A microbe compatible with the honeybee may not exist, be easily attainable, or be easily engineered.

### Conclusion

It is important to recognize that there are a few limitations or drawbacks to this research proposal. First, identifying all genes in the fumigillin pathway could prove difficult, as there is little previous research on the pathway; it may even be impossible to create a functioning system if all necessary genes are identified. However, if the cage trials show that CCD is still prevalent in hives exposed to modified bacteria, or if the modified bacteria do not produce fumagillin, then further research into IIVs would be beneficial. Second, constant exposure to fumagillin could cause *Nosema* to develop resistance to the antibiotic, though that does not seem to have happened with current fumagillin delivery techniques. Third, use of fumigillin has the potential to favor displacement of *N. apis* by *N. ceranae*. This is a problem because *N. ceranae* is already more prevalent and is more virulent than *N. apis*; *N. ceranae* causes immune suppression in honeybees which could allow other pathogens to take advantage of the weakened immune system. More research needs to be done to test the resistance of the two *Nosema* species to fumagillin. Finally, identifying and characterizing a microbe that is compatible with honeybees would be necessary for cage trials or real application. A compatible microbe might not exist or be easily attainable. The microbe also might not be easy to work with using synthetic biology or genetic engineering.

Most of the possible limitations explained above are not unique to this project. In genetic engineering projects, there is the danger of creating drug-resistant mutants when microbes are modified to produce chemicals, and a microbe compatible with the project must be found, if not characterized.

Even with limitations, this project is a promising alternative to spraying hives with fumagillin. The more invasive technique of having a microbe produce fumagillin within or around the honeybee may kill *Nosema* more effectively than dosing the hive with the antifungal. It would be cheaper for beekeepers in the long run, since they pay for part of the research and order immortal bacteria; one application of fumagillin-producing bacteria should infest several bee colonies indirectly (those exposed could spread the bacteria to other hives). If the design is successful, nationwide honeybee decline would be greatly decreased, and the cost of treatment would likely be lower than current methods. This method of CCD treatment is also sustainable, as bacteria can be kept alive by beekeepers with ease, and the bacteria distributes on its own.

Though the main focus of the project is stopping the decline of honeybees everywhere, there are other benefits to finding the genes responsible for synthesizing fumagillin. According to "Fumagillin Inhibits Colorectal Cancer Growth and Metastasis in Mice: in Vivo and in Vitro Study of Anti-angiogenesis," fumagillin discourages the formation of new blood vessels, resulting in fewer metastases in cancer-infected mice. This could mean that engineering an organism to produce fumagillin is useful to cancer research and treatment, not just CCD.

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