Abstract

The UN Intergovernmental Panel on Climate Change issued a *Special Report on Global Warming of 1.5°C* entailing that Earth has twelve years to limit climate change to 1.5°C above pre-industrial levels before irreversible effects take place. The anthropomorphic greenhouse gases that contribute the most to climate change are carbon dioxide and methane. We have devised a way to reduce the amount of these gases in the atmosphere by genetically modifying the bacteria *Micrococcus luteus* strain ATCC 4698 by inserting carboxysomes and soluble methane monooxygenase (sMMO) operons into its genes. Carboxysomes extracted from the cyanobacteria *Synechococcus elongatus* lead to carbon dioxide fixation, while sMMO extracted from methanotroph *Methylocella silvestris* leads to methane fixation. *M. luteus* is able to survive in nutrient-poor environments which makes it an ideal organism to reside in cities and factories that emit carbon dioxide and agricultural areas that produce methane.

Engineering *M. Luteus* to Fix the Greenhouse Gases Methane and Carbon Dioxide

Description

Present Technology

Genetic engineering is still a relatively new yet complicated subject. This technology is mostly being utilized in plants and animals for consumer purposes. Hence the term GMOs being associated with food. Microbes are also being altered for research and potential human benefits. It is now possible to make a microorganism behave differently by replacing certain genes with genes found in another organism.

One such example that contributes to the production of GMOs is Polymerase Chain Reaction (PCR). This process allows scientists to create millions of copies of DNA in a short amount of time. This is done by using a PCR reaction mixture which contains a heat-resistant enzyme called Taq polymerase (isolated from the bacterium *Thermus aquaticus).* It has short sequences of nucleotides that dictate the start and end point of duplication (primers) and free nucleotides. The mixture is heated to a temperature of 95°C thus denaturing the DNA and breaking hydrogen bonds. The temperature is then reduced to 60°C so that the primers can form hydrogen bonds and anneal to their complementary sequences in the target DNA. The temperature is again raised to a temperature of 72°C and the Taq polymerase then begins polymerization and adds the free nucleotides to the end of each primer attached to the DNA, thereby extending the strand and creating an abundance of copies in a short amount of time. This process is usually performed in a thermal cycler which allows for a faster more efficient process. PCR can also be used to detect genetic disorders and diseases by creating copies of an altered gene and diagnosing a patient.

Another relatively new form of technology that is used is CRISPR/Cas9 which targets a gene and allows for it to be altered. It begins with two components: the Cas9 enzyme and a specialized piece of RNA called the guide RNA. The Cas9 enzyme creates the complex which unzips and cuts DNA at a specific area in the genome so that sections of DNA can be added or removed. The guide RNA consists of a small pre-designed RNA sequence about 20 bases in length within a larger RNA piece. The larger piece attaches to the matching DNA and the guide RNA leads the Cas9 to the correct portion of the genome. This process has created much buzz from the medical world recently as many believe it could be an exceptional way to perform gene therapy and maybe even cure single-gene genetic disorders such as cystic fibrosis or hemophilia.

In addition to the CRISPR- Cas9 technology, humans have developed the process of targeting a gene in a bacteria and interchanging it with one of the desired attributes. All bacteria have circular-shaped genetic structures called plasmids within them that can replicate independently of its chromosomes. Plasmids quickly and easily transfer genetic material from one bacterium to another. These structures allow for scientists to cut their restriction sites with enzymes and then add in a gene. The enzymes used to cut are referred to as restriction endonucleases and will cut DNA only when it recognizes the correct sequence. When it cuts the plasmid, the enzyme creates sticky ends and the desired gene is placed in the plasmid along with ligase. If the enzyme in this situation is thought of as a pair of scissors, then the ligase is the glue. The ligase puts the plasmid together and creates a now recombinant piece of DNA. These techniques could potentially be used to modify *M. luteus* so it can produce carboxysomes and soluble methane monooxygenase.

History

Humans have been genetically modifying organisms for over 30,000 years. Back then, we used selective breeding, also known as artificial selection, to create the crops and animals we desired. The corgi is an example of how much humans selectively bred the grey wolf. Evidence of artificial selection of plants can be traced back to 7800 BCE where different varieties of wheat were discovered in archaeological sites in southwest Asia.

However, scientists never specifically studied genes until the term "genetics" was coined in 1905 by William Bateson. The function of DNA was discovered in 1928 by Frederick Griffith. The structure of DNA was discovered in 1953 by Watson, Crick, and Franklin. Tools to manipulate DNA had to be developed. In 1967, DNA ligases, enzymes that join broken DNA, were discovered. Three years later, restriction enzymes that allow DNA to be cut in specific places were discovered by Hamilton Smith. Using both of these enzymes, scientists could copy and paste genes. 1973 was the year of a huge breakthrough in GMO technology which brought many possibilities. The first genetically engineered organism was successfully created. Scientists Herbert Boyer and Stanley Cohen were able to transfer a gene from a strain of bacteria that encodes antibiotic resistance and insert it into another bacterial strain. This procedure successfully gave the non-resistant bacteria antibiotic resistance. Using a similar procedure, a year later Rudolf Jaenisch and Beatrice Mintz introduced foreign DNA into mouse embryos to create the first genetically modified animal. As the technology developed, sequencing DNA was crucial for more complicated modifications. DNA was sequenced in 1977, but the bacterium *Haemophilus influenzae* was the first organism to have its genome sequenced in 1995.

It was the Asilomar Conference of 1975 where scientists, government officials, and lawyers established the guidelines for genetic modification and its research. This brought

tremendous support for genetic engineering research from around the world and initiated an era of genetic modification.

Scientists were able to genetically engineer bacteria to synthesize the growth hormone somatostatin in 1976 and insulin in 1978. The insulin-producing bacteria enabled scientists to produce enough insulin to prescribe it to patients suffering from diabetes.

The first genetically engineered plant was created by inserting an antibiotic-resistant gene into tobacco. It was after 1994 that GMOs in food were being marketed, the Flavr Savr tomato being the first. Crops that had increased shelf life or produced pesticides were getting approved by the U.S. Environmental Protection Agency.

Future Technology

Our idea involves genetically modifying the bacteria *Micrococcus luteus* strain ATCC. This bacteria was recently found in a 120 million-year-old block of amber. Although the exact age of the bacteria was unable to be found, it serves as evidence that this bacteria could survive in oligotrophic environments, environments that have few nutrients. Environments that produce a large portion of carbon dioxide and methane are city and factory environments. These places also have few nutrients so *M. luteus* could potentially grow. This bacteria is harmless and live in environments like water, soil, air, and dust. It has also been sequenced so its genome is already known.

Bacteria have certain metabolic pathways that metabolize different substances and are determined through organelles entirely made of proteins called bacterial microcompartments (BMCs).

Cyanobacteria are bacteria that can fix carbon dioxide, which means they use carbon dioxide as a source of energy and have oxygen as a byproduct. However, they usually live in

extreme temperatures or water abundant areas and will likely not survive in oligotrophic environments. They do, however, have BMCs that metabolize carbon dioxide.

Recently, scientists have discovered the BMCs involving carbon dioxide fixation in the cyanobacteria *Synechococcus elongatus*. These BMCs are termed carboxysomes and this specific set is called β-carboxysomes (beta). These carboxysomes involve BMC-H, BMC-T, and BMC-P acting as a semipermeable protein shell enclosing the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and carbonic anhydrase. These enzymes are responsible for carbon dioxide fixation. Scientists have found the set of genes that code for the icosahedral-shaped structure and inserted it into *E. coli*. This procedure allowed the bacteria to successfully fix carbon dioxide, but in small amounts. Type I DNA topoisomerase in *M. luteus* aligns with 73% identity to the topoisomerase in *E. coli*.

Another metabolic pathway is found in methanotrophs, many of which live in extreme temperatures or uncommon environments that are acidic and geothermal with very high temperatures. Scientists have discovered an enzyme that can oxidize the C-H bond in methane. It is called methane monooxygenase. Specifically, the methanotroph *Methylocella silvestris* has a form of soluble methane monooxygenase (sMMO). This enzyme takes methane, oxygen, NADPH, and hydrogen to get a byproduct of methanol, NADP (which it uses for energy), and water. The main components of sMMO are the reductase, the β unit, and hydroxylase. This complicated structure is around a diiron core. *M. sylvestris* uses the serine cycle and the operon encoding for sMMO has been verified and all the genes needed for methane fixation through the serine cycle.

In twenty years, biotechnology could advance to the point that *M. luteus* can contain carboxysomes which can fix carbon dioxide efficiently. This bacteria has a yellow pigment and

will reside in places with high production of carbon dioxide such as cities and factories. A factory exhaust would have a maze of an exit pathway to maximize contact. *M. luteus* will line the inside of said pathway and will act as a filter for the carbon dioxide. Likewise, to further minimize the amount of the greenhouse gas put in the atmosphere, this bacteria could reside in the spouts of cars, line highways, and even pollution-filled cities.

In the future, the operon for sMMO could be implemented into *M. luteus.* The biggest emissions from methane involve rice cultivation and livestock production. These industries will continue to grow twenty years from now. One positive aspect of *M. luteus* is that it naturally occurs in nature. For livestock, it is their waste that produces methane so methane would be directed to an area with *M. luteus.* Methane from rice paddies could likewise be directed to a designated area.

Breakthroughs

There are many breakthroughs needed for this technology to become a reality. One is that *M. luteus* should be altered by inserting carboxysomes or sMMO so it successfully fixes carbon dioxide or methane efficiently. The modification has been done to *E. coli* with some success, however *M. luteus* is different genetically. *M. luteus* has been sequenced but where to perform the modifications still needs to be discovered.

Also, the modified bacteria should thrive and perform well in the environments of factories, cars, cities, and agricultural areas. Since these involve various temperatures and conditions, the bacteria needs to be able live in the high temperatures of exhausts, low temperatures of cities, nutrient-poor areas of a factory, and the wet environment of rice paddies. This technology does not exist because *M. luteus* has not gone through major experiments so its capability in these living conditions has not been tested. Similarly, there has not been a major

experiment involving its genetic modification, so modifying something large, like its metabolic pathway, seems out of reach.

Certain investigations and experiments have to take place to test *M. luteus* and its ability to express carboxysomes. First, a synthetic operon for a carboxysome structure that can perform carbon dioxide fixation would have to be produced. To do that, we have to modify the twelve 12 β-carboxysome genes from *S. elongatus* in order to express the construct in *M. luteus.* These genes are located in five chromosomal loci, but the main genes are in the locus that has the operons rbcLS and ccmKLMNO. The synthetic operon will combine the major operons rbcLS and ccmKLMNO into one. This creates an operon that codes for the carbon dioxide-fixing organelle with the fewest amount of genes. The next step is to find the correct expression construct to introduce this operon into *M. luteus* so it can produce carboxysomes. Since the organelle is made entirely out of proteins, a vector for protein synthesis should be used. The plasmid pNM2-2 is responsible for protein synthesis and could be used as a vector for this procedure.

After much trial and error using different vectors and modified genes, once the synthetic operon for the carboxysomes is successfully inserted into *M. luteus,* testing its efficiency should take place. Using an electron microscope, we should look for polyhedral-shaped organelles. These organelles are the BMCs for carboxysomes. After they are identified, the bacteria should multiply into a culture. Tests should be performed to indicate its carbon dioxide-fixing capacity. By enclosing it in a small area with carbon dioxide and waiting a period of time to check the carbon dioxide and oxygen levels, we can test its efficiency. This experiment will ultimately measure its Rubisco activity. The more activity, the higher capacity to fix carbon dioxide.

Another breakthrough that is needed is discovering how to insert the operon for sMMO, which performs methane fixation, into *M. luteus*. Unlike the operon for particulate methane monooxygenase, the operon for soluble monooxygenase is spread out over different genes. This complicates things as to where to do the modifications in *M. luteus* because no experiments have been done involving such drastic modifications.

If this modified version can metabolize methane, it has to be in an efficient way which takes in the most amount of the greenhouse gas, yet little byproduct. Certain experiments need to be conducted in different environments to get the best possible result.

Design Process

We went through various phases in planning before reaching this as the most viable solution. They involve using different bacteria instead *of M. luteus,* expressing structures other than carboxysomes and a different type of methane monooxygenase.

At first, we were thinking of using *E. coli* as the medium for the modification because there have been many experiments involving its modification. It already successfully had carboxysomes expressed in one experiment. However, there are many strains of *E. coli* and the bacteria multiplies very quickly. This leads to many mutations and the possibility of a harmful mutation forming. Most *E. coli* are harmless, however, there are some strains that can get humans very sick. There are strains that cause kidney failure, anemia, or bloody diarrhea. Since it multiplies so quickly, it would be hard to keep it under control if there is an outbreak. This bacteria isn't something that should be put in cities or around food. Granted, *M. luteus* could have a genetic mutation that could be harmful, but this bacteria has a relatively slow doubling rate so it would be easier to handle.

Another thing we were originally thinking of using was particulate methane monooxygenase. This kind of MMO is popular among the majority of methanotrophs. In fact, *Methylocella* is one of the few genera that uses sMMO instead of pMMO. However, after doing some research, there was a lot more information and research on sMMO. Also, sMMO might have the ability to oxidize other hydrocarbons. This gives sMMO the upper hand in regards to its potential and the information on it. Furthermore, pMMO uses copper and various metals instead of iron as its core. It is easier to utilize one type of metal instead of multiple.

Originally, in order to deal with carbon dioxide, we thought of modifying bacteria to express chloroplasts instead of carboxysomes. They are similar because they both take in carbon dioxide as a form of energy and release oxygen as a byproduct. However, chloroplasts require a lot of water and sunlight in order to function properly. These conditions are vital to the identity of chloroplasts and cannot be modified out. The necessity of water and sunlight could be a problem considering that the environments they will reside in—factory and car exhausts—will not be exposed to said necessities. Carboxysomes, however, are a different organelle recently discovered. These don't require sunlight or much water. They perform their reactions using enzymes and carbon dioxide which makes them a better choice for our plan.

Consequences

Scientific and technological advances impact our society greatly. However, technology has its pros and cons. Society must reap the benefits and face the challenges.

Consumer demands are responsible for the abundance of greenhouse gases in our atmosphere which consists of water vapor, carbon dioxide, methane, nitrous oxide, and ozone. The ones we decided to direct our focus on and are threatening our planet the most are carbon dioxide and methane. Carbon dioxide is constantly being produced as a result of our dependence

on fuels like oil, coal, and natural gases. The United States gets 81% of its energy from these fuels. Methane is a major heat-trapping gas that is also responsible for rising global temperatures and is more potent than carbon dioxide.

There are pros and cons concerning genetically modifying *Micrococcus luteus* with carboxysomes to take in carbon dioxide. A problem with *M. luteus* conducting methane fixation is that it would produce methanol, which is toxic if ingested and very flammable. The plan is that the bacteria will reside in a designated area to perform methane fixation so methanol vapors will avoid being ingested or catching fire. On the plus side, methanol has some potential uses and can be used as an alternative fuel for vehicles.

An issue with using genetically modified *Micrococcus luteus* is that it absorbs UV light. UV light is also known to cause mutations in bacteria by altering the DNA so the thymine base pairs next to each other in a sequence bond to become pyrimidine dimers, which is harmful because reproductive enzymes cannot copy them. In the worst case scenario, the bacteria could be the cause of a disease or multiply uncontrollably because of a specific mutation.

The positives would be a reduced amount of carbon dioxide in the atmosphere causing global temperatures to see a slight change. The *M. luteus* will reproduce and gradually increase in amounts, so there would be less carbon dioxide in our atmosphere over time. Another positive is that the *M. luteus* that contains sMMO performs methane fixation, which will reduce the amount of methane that reaches the atmosphere. Hopefully, this will make a difference in the fight against climate change and pollution.

Also, having this yellow-pigmented organism around people could serve as a reminder to make smart decisions for the planet. Society could become united against the fight against climate change. If not, generations in the near future will suffer its effects. Although the greatest

catalyst for change could be done by taking initiatives to actively reduce emissions through government regulations and renewable forms of energy, it may be an inconvenience for many to alter their lifestyle. It is this generation's responsibility to preserve the planet and the future of generations to come.

Hopefully, climate change will become a thing of the past in the future.

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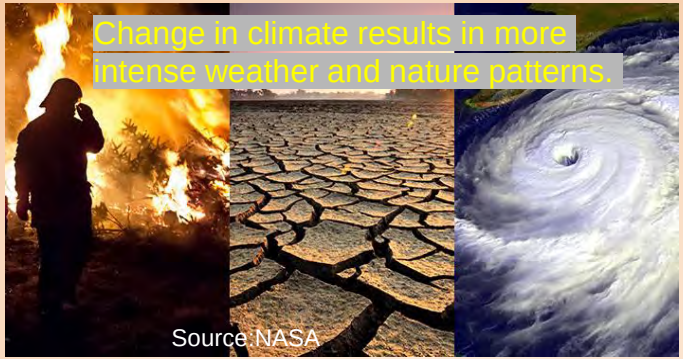
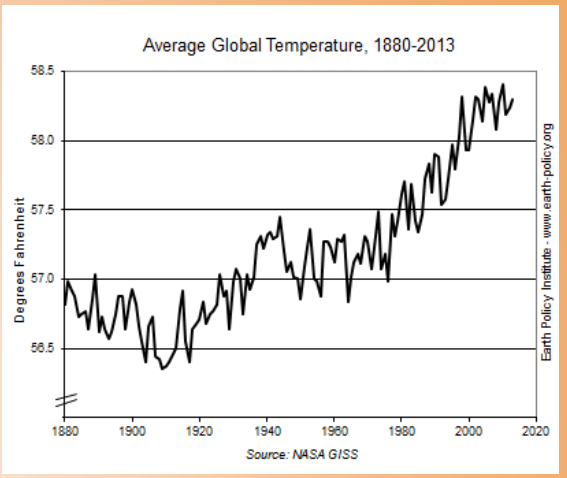
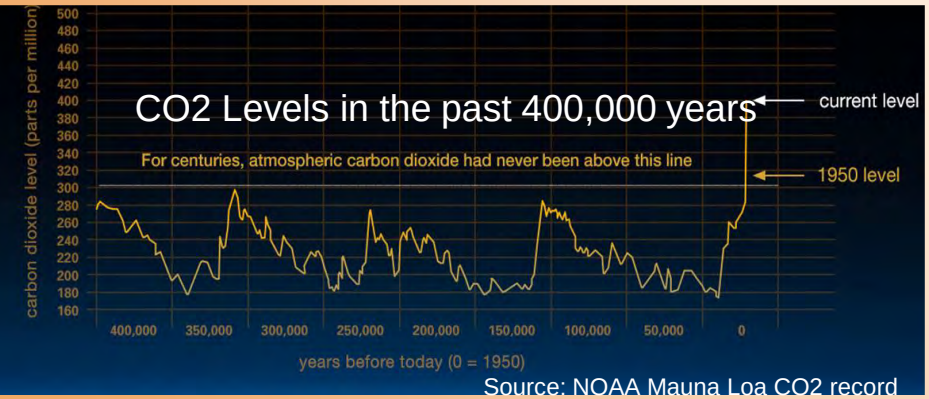
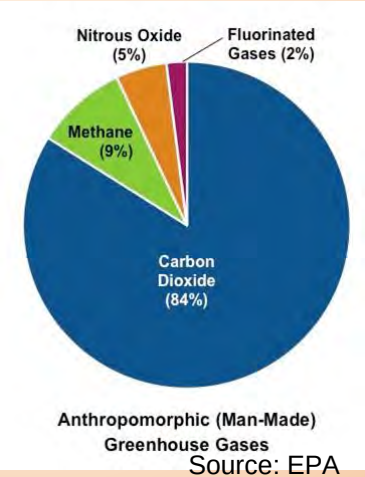
The Intergovernmental Panel on Climate Change has released statements of how ongoing climate change poses a serious threat.
There is a 95% chance that this threat is man-made







Source: NOAA Mauna Loa CO2 record



Source: EPA

Methane and Carbon Dioxide compromise a large portion of man-made greenhouse gases

As the temperature keeps rising, the ice caps melt, increasing sea levels and harming wildlife around the world.

Problem

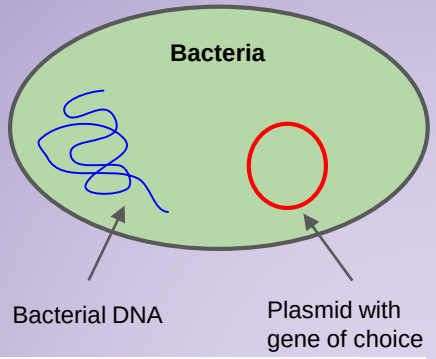
Technology

Biology

M. luteus

Application

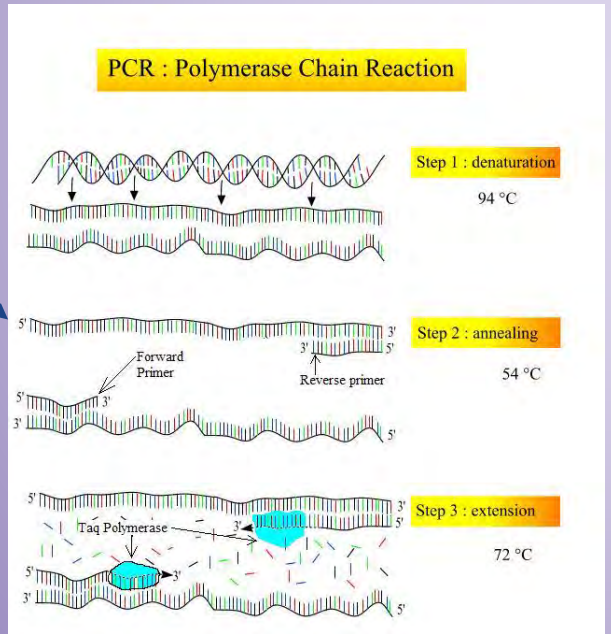
The most common type of genetic modification is the combination of two different types of DNA.

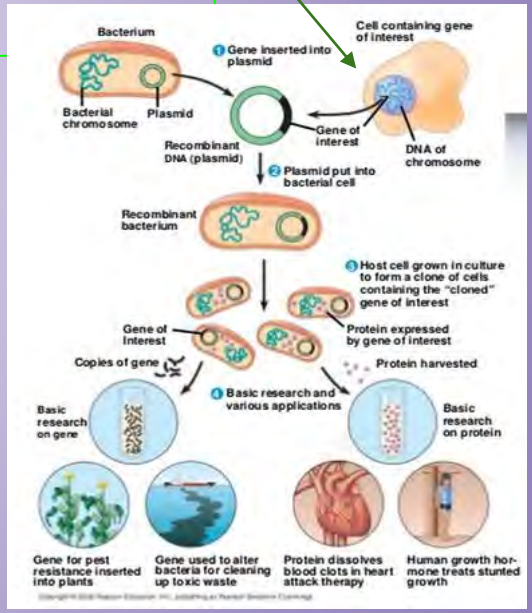


The second step is to insert the gene into the cell. With bacterial cellsnormally share DNA with each other. This can be done by inserting the chosen gene into a vector, like a plasmid. Then the vector can be used to place the gene into the bacterial cell. To allow the plasmid to enter the cell, you expose the cell to electroporation which involves shocking the cell. This does not kill the cell, but creates tiny holes in its membrane that plasmids can pass through. Eventually the holes will seal up and the cell will not be vulnerable to anything harmful.

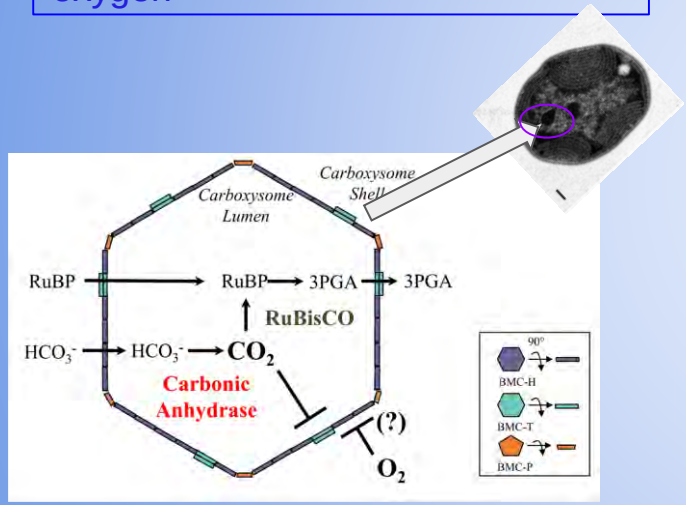
Scientists have discovered a way for these genes to enter into a cell without interfering with existing genes.

The first step in this is selecting the gene of interest and creating multiple copies of it using Polymerase Chain Reaction.



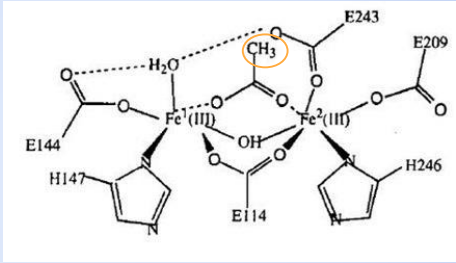


Carboxysomes are organelles in cyanobacteria that take in carbon dioxide for energy and release oxygen

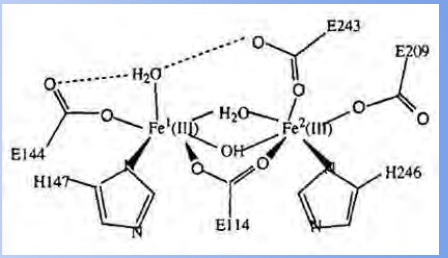


In carboxysomes, RuBisCO and carbonic anhydrase are enclosed within a semipermeable protein shell. The shell is composed of BMC-H, BMC-T and BMC-P proteins.
RuBisCO: Ribulose-1, 5-bisphosphate carboxylase/oxygenase

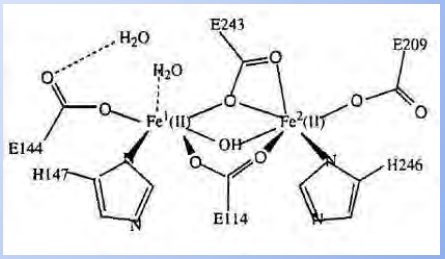
Soluble methane monooxygenase is an enzyme in methanotrophs that takes apart methane and produces methanol.



Resting state



Oxidized state



Reduced state

Micrococcus luteus is a harmless bacteria that lives in nature. It resides in soil, dust, and plants. A recent discovery was made that showed how *M. luteus* was able to survive in oligraphic environments. These are environments that have little nutrients.

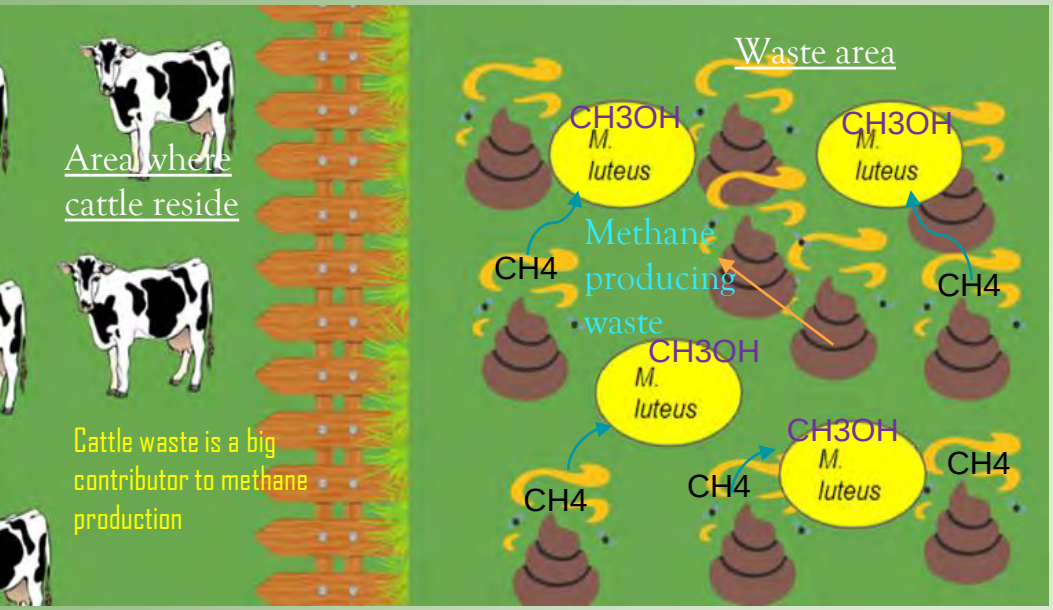
Through biomedical technology, the metabolic pathways can be altered.

There is a possibility for inserting genes that code for soluble methane monooxygenase. This enzyme will use methane as a source of energy. The modified *M. luteus* can extract methane from the atmosphere

The entire genome of this bacteria has been mapped so it is possible to genetically modify it.

The operon for carboxysomes could be inserted into the DNA. This organelle uses carbon dioxide as energy. With this organelle, *M. luteus* could extract carbon dioxide from the atmosphere.

Problem | Technology | Biology | *M. luteus* | Application



Most of the methane in our atmosphere is due to livestock farming and rice farming. *M. luteus* should reside in manure areas in cattle farms, along with implementing them in rice paddies.

Cities and factories are the main producers of carbon dioxide. *M. Luteus* could grow in the ventilation chimneys in factories and in the mufflers and exhausts of cars.

O2 being released into atmosphere

M. *luteus* fixing carbon dioxide and producing O2

Maze-like exit pathways from exhausts

CO2 emissions