Tackling Atherosclerosis from the Gut Up

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Atherosclerosis is the number one cause of death in the world today. Dietary factors are a significant contributor to the development of the disease. The role of trimethylamine N-oxide (TMAO) has recently been pointed out as a factor contributing to the initial stages of atherosclerosis. TMAO is produced in the liver from trimethylamine (TMA), a reduced form of the compound that arises from chemicals found in meat, dairy and eggs. We identify several metabolic pathways that exist or have been recently discovered and characterized for use in a bacterial system that could break down TMAO and TMA in the gut thus reducing development of atherosclerosis in people. We lay out different pathways as separate devices that can be introduced to *Escherichia coli* or other gut bacteria and used to reduce overall levels of TMAO and therefore atherosclerosis development in humans. We lay out an experimental plan as well for testing the effectiveness of TMA and TMAO breakdown in genetically modified strains.

Keywords: atherosclerosis, TMA, TMAO, methanogen, medicine, treatment, TMAO monooxygenase, TMAO reductase (TOR), corrinoid methyl transferase (MttB), pyrrolysine

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Cardiovascular heart disease is the leading cause of death in many countries. Atherosclerosis is a disease which causes the hardening and thickening of coronary arteries. Atherosclerosis results in narrowing of the blood vessel lumen and can lead to thrombosis (blood clot development) and if blockage occurs in coronary arteries, myocardial infarction (heart attack). Damage to blood vessels that occurs because of atherosclerosis is linked to the two largest causes of death worldwide: ischemic heart disease and stroke. Arteries thicken and harden due to the buildup of fats, mainly low density lipoprotein (LDL) cholesterol within the arterial blood vessels. As the fat continues to build-up, the arterial walls become thicker and cause the lumen to become narrower. This prevents blood from flowing to cardiac cells, which would eventually cause them to die off. The death of a significant amount of cardiac muscle will eventually lead to a heart attack.

Individuals who have diets that are high in fat content have a higher chance of developing cardiovascular heart disease. This is due to the higher concentration of LDL cholesterol in their blood. Individuals who smoke and have high blood pressure have a higher chance of damaging their arterial walls, which causes LDL cholesterol to build up.

While the mechanism of atherosclerosis is complex, the process starts with damage, inflammation or dysfunction of the endothelial cells lining arteries. Endothelial cells send chemokine signals recruiting monocytes to the area and also release reactive oxygen species (ROS) and other compounds that cause LDLs to be oxidized (oxLDL). Diet and gut bacteria metabolism are believed to play a role in starting this process. The compound TMAO has been correlated with increased risk of such cardiovascular events (Jonsson & Backhed 2016).

Sources of TMAO

TMAO is formed by the conversion of TMA in the liver. This process is a reduction process. Some TMA precursors include certain nutrients such as choline and L-carnitine. Choline, a water-soluble vitamin-like nutrient, is abundant in red meat, egg yolks, and dairy products. L-Carnitine on the other hand is an amino acid found in red meat, as well as certain energy drinks and supplements that are commonly used by athletes. When these nutrients are ingested, gut bacteria then break them down to convert them into TMA. This TMA is then reduced in the liver to form TMAO.

TMAO is found in some foods such as fish but is mainly formed

in the liver by the enzyme flavin monooxygenase 3 (FMO3) from TMA. Gut bacteria convert many compounds such as L-carnitine, phosphatidylcholine (PC) and choline found in foods like red meat, shellfish, eggs and milk into TMA. Once absorbed into the bloodstream, TMA is converted to TMAO in the liver, resulting in decreased levels of reverse cholesterol transport, blood platelet activation and increased formation of foam cells from macrophages engulfing oxLDL (Jonsson & Backhed 2016). If TMA is not oxidized to TMAO by FMO3 in the liver it can build up in the sweat, breath and urine resulting in trimethylaminuria (TMAU) also known as 'Fish-Odor Syndrome' (Brugere et al. 2014).

Scientists have thought about how reducing levels of TMAO could be accomplished and therefore lower risks of atherosclerosis, major innovations have focused on either producing inhibitors of enzymes and then delivering them to people orally or trying to cultivate bacteria that have the natural ability to break down these methylamine compounds, like archaebacterial methanogens (Brugere et al. 2014). While several different species of methanogens exist that have the ability to breakdown TMA and related compounds, their ability to survive in the human gut is not confirmed and likely not possible to the extent that they would be able to break down significant levels of TMA or TMAO on their own. The solution proposed here relies on using enzymes that exist in methanogenic bacteria as well as others and designing genetic devices that can be used to transform *Escherichia coli* or other commonly found gut bacteria that currently find their niche in the human intestines.

Probiotics

Probiotics are microorganisms (such as bacteria) that are introduced into the body for their beneficial qualities. The popular Indonesian drink Yakult, made by fermenting a mixture of skimmed milk with a special strain of the bacterium *Lactobacillus casei*, is a good example of a probiotic product. You may not think about your digestive system when you think about your overall well-being, but that's where good health and proper nutrition begin. From childhood to old age, good digestion is important because around 70% of our overall immunity (immune cells) lies there. So a healthy digestive system is the key to good health and longevity. *L. casei* is a species of genus *Lactobacillus* found in the human intestine and mouth. This particular species of *Lactobacillus*, which is documented to have a wide pH and temperature range, complements the growth of *L. acidophilus*, a producer of the starch-digesting enzyme amylase. Methanogenic bacteria are possible probiotics which are able to reduce the amounts of TMA and TMAO in the human intestines; however, conditions inside the human intestines are not suitable for their growth. Our proposed design gives *E. coli* the ability to reduce TMA and TMAO levels in the human gut by transferring several methylamine degrading devices and others needed to make rare amino acids found in the enzymes themselves.

Systems Level

The idea of using methanogenic bacteria to reduce TMA and TMAO levels in the human body is not new. Brugere et al. (2014) proposed the idea and called it 'Archaebiotics' to indicate the approach was probiotic using archaebacterial methanogenic bacteria. They carefully identified the biochemical pathways that methanogens use to break down TMA into methane (CH4) and showed that this breakdown would reduce TMA concentrations, which would reduce the amount of TMA that gets into the bloodstream and liver where it would be converted to TMAO by FMO3. The main challenge they present is that the methanogens capable of doing this are anaerobes and possibly not capable of surviving in large numbers; they would also be in competition with other similar bacteria. *E. coli*, however, is found in 100% of people tested and is one of the most common bacteria in the human intestines (Todar 2012). *E. coli*'s genome is known as well, and this makes it an excellent chassis for use in this design.

The basic idea of the proposed design is to destroy TMA that is produced in the intestines by intestinal bacteria from the meat, dairy and fish components of the human diet. These foods contain compounds such as L-carnitine, PC, choline and lecithin, which gut bacteria convert to TMA. Fish and some other foods also contain TMAO, which can be converted to TMA or absorbed into the bloodstream. Destruction of TMAO and TMA should result in less TMAO in the blood and therefore less inflammation or damage to endothelial cells of arteries, which would inhibit the onset of atherosclerosis. *E. coli* can be transformed using a plasmid that has the genetic devices needed to break down TMAO and TMA before either enter the bloodstream.

Device Level

Device 1: TMAO Reductase (TOR) Converts TMAO to TMA

While TMA reduction is the primary goal of our system, reducing TMAO found in the gut either from food or because of gut bacteria metabolism is also desirable, and adding a device with the enzyme TOR ensures that any TMAO found is turned into TMA for destruction (Figure 1). Even though TOR has been found in both *E. coli* and *Roseobacter denitrificans*, the addition of the TOR device will help to further target any TMAO that comes from foods like fish or is converted by gut bacteria from TMA.

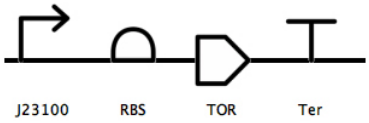

Figure 1. TOR converts TMAO into TMA

Device 2: Methyl Removal Pathway

There are metabolic routes identified in the KEGG Methane Metabolism Map (KEGG Map 00680); however, the corrinoid methyltransferase pathway is characterized by Brugere et al. (2014). This is a multi-step pathway where the TMA produced in the first step is the starting material for the second step. The three proteins involved are corrinoid methyltransferase (MttB), TMA corrinoid protein (MttC) and coenzyme M methyltransferase (MtbA). The first protein, MttB, removes methyl groups (-CH3) from TMA and transfers them to MttC and MtBA before being converted to methane (CH4). Hydrogen gas is required

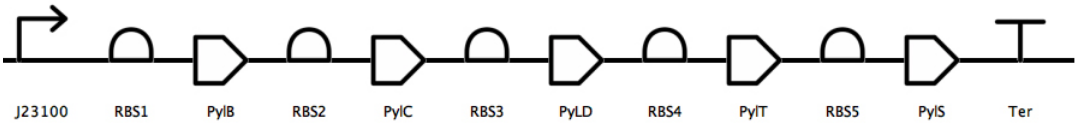

Figure 2. Methyl Removal Pathway Involving Three Proteins (MttB, MttC and MtbA)

and this would be supplied by hydrogen producing bacteria in the gut. Ribosome Binding Sites (RBSs) are found upstream of each protein to improve translation through ribosome binding (Figure 2).

Device 3: Pyrrolysine Production

The MttB enzyme is unique because it relies on an unusual amino acid called pyrrolysine in the place of lysine. This amino acid is found in methanogenic bacteria but not in humans. This additional device is needed to produce the pyrrolysine, pyrrolysine transfer RNA and also pyrrolysine tRNA activating enzyme needed to attach pyrrolysine to the tRNA (Figure 3).

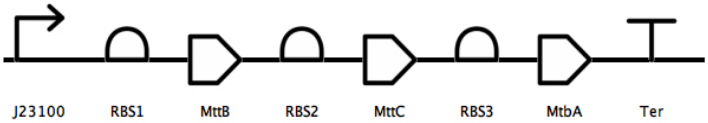

Figure 3. PylB, PylC and PylD are enzymes needed for pyrrolysine production, PylT codes for the tRNA and PylS attaches pyrrolysine to tRNA

Parts Level

TOR

The microbial enzyme TOR has the ability to reduce TMAO to TMA. It is part of the electron transport chains found in mitochondria and is capable of accepting electrons from proteins called cytochromes (Gon et al. 2001). TOR is found in *E. coli* and also in *R. denitrificans*, which is a pink pigmented bacteria (Arata & Takamiya 1992). High concentrations of TMAO are found in the tissues of fish. The bacterial reduction of TMAO into foul smelling TMA is the major process on the spoilage of fish (Fennema et al. 2016).

MttB

MttB is a protein that removes methyl (-CH3) groups from TMA. Hydrogen is required but this is a common product of some intestinal bacteria called hydrogenotrophs. MttB works with TMA, MttC, and MtbA to produce methane (CH4) and ammonia (NH3) from TMA. It contains the amino acid pyrrolysine , which is found in methanogenic bacteria but not in humans or other eukaryotes. In order to produce MttB, additional genes are needed not only for pyrrolysine production but also to activate pyrrolysine transporting tRNAs to bring pyrrolysine into ribosomes. The biosynthesis of pyrrolysine requires three separate enzymes: 3-methylornithine synthase (PylB), 3-methylornithine-L-lysine ligase (PylC) and 3-methylornithyl-N6-L-lysine dehydrogenase (PylD). In addition to the gene coding for tRNAs that transport pyrrolysine (PylT), the tRNA-activating enzyme-pyrrolysine tRNA ligase (PylS), is used to attach pyrrolysine to tRNA molecules for use in translation. The codon for pyrrolysine is the 'amber' stop codon, UAG (Brugere et al. 2014).

Constitutive Promoter (J23100)

The constitutive promoter J23100 is from the iGEM Registry of Standard Biological Parts combinatorial library and promotes strong expression of parts used, described as 'Anderson Strong'. Constitutive promoters are not affected by transcription factors and are active in all circumstances. Given the complex nature of devices in our system this is preferred; however, testing a range of possible promoters from the Anderson Promoter Collection to test which gives the best result is a valuable approach.

RBSs (Anderson RBS Family)

RBSs from the Anderson RBS family from iGEM's Registry of Standard Biological Parts have been shown to be suitable for prokaryotic ribosome binding in translation. RBSs are found upstream to start codons and increase the likelihood of ribosome binding during translation, increasing the output of protein. The best combination of constitutive promoter and RBS can be determined experimentally by trying several different combinations. The Anderson RBS Family also contains start codons downstream of the RBS.

Terminator (BBa_B0015)

The double terminator BBa_B0015 is found on iGEM's Registry of Standard Biological Parts and is a double terminator that can terminate in both the forward and reverse directions. It has been shown to function in *E. coli*, which is our biological chassis. The BBa_B0015 double terminator is composed of the 64 base pair BBa_B0010 T1 and BBa_B0012 TE coliphage T7 terminators both from *E. coli*.

Safety

The safety of the proposed device would have to be confirmed using germ-free mice or other germ-free animals (Round & Mazmanian 2009). With such animals, the maximum effect of the genetically modified TMA-reducing *E. coli* could be determined by feeding them strict diets of meats and dairy products and observing the levels of ammonia and methane in them. One possible way around any high buildups of dangerous products like ammonia might be an additional device that converts the nitrogen source into something useful like amino acids.

Discussion

Design Testing

While the design presented uses genetic pathways in methylamine metabolism and breakdown with enzymes that have

known sequences it has not been shown whether or not the system will work as a whole in the biological chassis, *E. coli*. The next step of the project is to conduct laboratory experiments measuring the ability of *E. coli* transformed with plasmids containing the devices to reduce levels of TMA and DMA in agar cultures or using optical density measured at 600 nm in liquid cultures. The compound Folin-Ciocalteu Phenol has been used in the past to measure TMA levels and could be used in our experiment as well (Ikawa et al. 2003). The compound changes color and a spectrophotometer or colorimeter could be used to quantify changes in methylamine concentrations in the two treatments of transformed and untransformed *E. coli*.

Further Questions

There are several issues in addition to being able to successfully reduce the amounts of TMA and TMAO in the lab that might affect the success of the design. These include DMA back conversion to TMA, pyrrolysine competition and the amount of hydrogen required for methyl group transfer.

DMA Back Conversion to TMA

Fennema et al. (2016) describe how methanogens and other bacteria have the ability to convert TMA into TMAO using the enzyme TMA monooxygenase, and there are possibly other metabolic pathways that bacteria may utilize to convert dimethylamine (DMA) or monomethylamine (MMA) back into TMA. If gut bacteria are able to convert DMA formed back into TMA, then an additional device will have to be added to deal with this. DMA could also be dangerous or further converted by some unrecognized pathway.

Pyrrolysine Competition

The codon for pyrrolysine is the 'amber' stop codon, meaning that there may be competition between stop codon binding and pyrrolysine tRNA attachment. Recoding of bacterial genomes by removing certain codons and also factors involved in binding may be necessary due to interactions with this unusual amino acid.

Roles of Hydrogen

The methyltransferase pathway requires a source of hydrogen and it is not known whether levels of hydrogen in the gut are sufficient to allow this reaction to proceed at a high level. Future devices could be built to supply the hydrogen needed. Hydrogenotrophic bacteria are found in the gut normally and these would reduce the amount of hydrogen gas; however, this is probably counteracted by bacteria producing hydrogen gas found in the gut (Strocchi and Levitt 1992).

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