Insulin-Producing Therapeutic Probiotics For Type 1 Diabetics

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Type I diabetics in many parts of the world do not have access to medical grade insulin due to its high cost and limited production in some countries. A synthetic biology approach to regulated insulin production by probiotic bacteria is described that responds to the presence of both nutritional elements such as cellulose and glucose as well as the growth phase properties of the probiotic bacteria themselves. These inducer signals are designed to ensure that insulin is produced when food from a meal enters the small intestines and continues into the colon. Insulin production from proinsulin is facilitated by the presence of membrane-bound basic carboxypeptidase and membrane-bound trypsin found on the surface of the probiotic bacteria themselves. Proinsulin secretion is facilitated by the use of a Type 2 Tat Secretion system composed of both inner and outer membrane transport protein complexes from Gram Negative bacteria. Three cellulose inducible devices are constructed to ensure that the *Escherichia coli* chassis produces insulin, membrane-bound proteases for proinsulin processing and secretion system proteins for production, processing, and release of insulin from bacterial chassis in response to environmental cues from food when entering the intestines.

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Background

What is Diabetes Mellitus?

Diabetes Mellitus (DM) is a chronic disease that can be caused by inherited or acquired deficiency in the production of insulin by β cells in the pancreas. The main problem with treating DM type 1 is that treatments can help but there is no cure. The malfunction of β cells is often caused by an autoimmune response where the patient's immune system attacks the β cells, thus preventing the production of insulin.

What is the Problem?

For diabetic patients, insulin is a necessity. Diabetic patients without insulin will only be able to survive for 7 to 10 days maximum, eventually dying from severe pain. Often, people think that a hormone this essential is probably inexpensive and easily available. However, the reality in some countries is far from what we think. The average price for one vial of insulin in the U.S is around USD 285. One vial of insulin contains 10 mL (1000 units) of insulin. Diabetic patients need 2–4 vials per month. Although the patent for insulin has been expired for a long time the price of this medication has kept increasing even when there is no development or change in Insulin.

About Probiotics

Probiotics refer to live microorganisms such as bacteria and yeasts that are beneficial to the host, specifi-

cally the digestive system, when consumed in healthy amounts. The most common ones include *Lactobacillus* and Bifidobacteria, found in functional foods and dietary supplements. Not only do they demonstrate significant potential for therapeutic purposes but because their mechanisms have yet to be fully explored, the possibilities of using these mechanisms to promote appropriate probiotic strain selections under specific circumstances can lead to the uncovering of new functions.

Examples of important mechanisms underlying the antagonistic effects of probiotics on various microorganisms include the following: modification of the gut microbiota, competitive adherence to the mucosa and epithelium, strengthening of the gut epithelial barrier and modulation of the immune system to convey an advantage to the host. Furthermore, accumulating evidence demonstrates the ability of probiotics to communicate with the host by pattern recognition receptors. This includes Toll-like receptors, nucleotide-binding oligomerization domain-containing protein-like receptors, which modulate key signaling pathways, such as nuclear factor-κB and mitogen-activated protein kinase which enhance or suppress activation and affect downstream pathways. Such recognition is vital to elicit antimicrobial responses while minimizing inflammatory tissue damage. There is potential for suppression of diarrhea, alleviation of lactose intolerance and postoperative complications, prevention of colorectal and microbial cancer activities and reduction of irritable bowels.

When selecting the appropriate probiotics, bacteria must be able to survive under gastrointestinal conditions, with exposure to bile and gastric acid, having to adhere to the mucosa and competitive exclusion of pathogens.

Mechanism-wise, probiotics play a huge role in the enhancement of the epithelial barrier. The intestinal barrier, composed of the mucous layer, antimicrobial peptides, and secretion of IgA as well as epithelial junction adhesion complex, is a primary defense mechanism used for maintaining epithelial integrity and protection. However, once this barrier function is distorted, antigens can penetrate the submucosa and induce inflammatory responses, resulting in intestinal disorders, such as inflammatory bowel disease.

How does insulin work?

Role of insulin

Insulin is a hormone produced by the β cells in the pancreas which regulates blood sugar levels. Cells need glucose for cellular respiration, without it, it won't be able to make energy. Insulin binds to the insulin receptor of the cells, then a vesicle containing Glucose transporter type 4 (GLUT4) is released, then these membrane proteins are placed in the cell membrane allowing glucose to enter the cells and be used in cellular respiration. Simply, insulin is a key used to open a channel for glucose to enter. It is one of the essential hormones used to regulate the blood sugar level to maintain homeostasis.

When the INS gene is transcribed and translated, it produces preproinsulin. Preproinsulin has a signal sequence used in the maturing of the protein in our cells. However, our project does not need this signal sequence, therefore, the 24 base pair was removed. This means that when our INS gene without the signal sequence is transcribed, Proinsulin is made directly. Proinsulin just has a C-peptide chain that is used in protein folding, which is later removed by trypsin and carboxypeptidase B to be turned into insulin.

Systems Level

Our main design idea was to create a therapeutic gut bacteria that diabetic patients would regularly (once per week) take which produces insulin in the patients' gut. Our main design goals were to produce and regulate insulin. Production of insulin is simple, we just have to express the INS gene, which makes 51 amino acids arranged in two chains, the A chain (21 amino acids) and B chain (30 amino acids) that are linked by two disulfide bonds. Some of these amino acids can be changed to produce different types of insulin that react at a different speed. We plan to implement an insulin expression system so that the bacteria do not need to die to expose the insulin in our gut—they can just secrete it. For this, we plan to use either the Type Two Secreation System (T2SS) or flagellin fliC.

Regulating insulin production is crucial since too much or too little can affect the patient. If too little insulin is produced it wouldn't be effective at helping the diabetic patient, but if too much insulin is produced then it would cause hyperinsulinemia which may lead to hypoglycemia. We didn't want to create harmful bacteria for the diabetic patients so we needed a complex regulatory system. We plan to use the osmY promoter and cellulose induced promoter. This means that insulin will only be produced when the bacteria is going through the log phase and with the presence of cellulose. This way we can make sure that insulin is only produced after the patient's meal.

Regulation of Insulin production is also crucial since under or overproduction could be fatal for the patient. Since rapid-acting insulin was used, insulin had to be quickly made then secreted out of the bacteria.

To prevent insulin from being produced continuously, the osmY promoter and the glucose-induced cellulose promoter were chosen. This allows the bacteria to only start producing insulin when it is going through a lag phase and with the presence of both glucose and cellulose in the gut.

This is purposely made to target the diet of the patient, the insulin will only be produced when the patients start to eat their meal. This allows a regular production of rapid-acting insulin which will, in turn, help the patient's cells get the glucose they need to survive.

Device Levels

Device 1

The osmY promoter only allows the gene to be expressed during the log phase. Then proinsulin is produced with the T2SS signal sequence. If glucose and cellulose are present, the rest of the gene turns on producing more insulin. When the bacteria go on to the stationary phase, the system will shut down preventing more insulin from being produced.

Device 2

This device is used to produce the necessary enzymes and membrane protein used for insulin secretion and turning proinsulin into insulin. Trypsin and Carboxypeptidase B are produced to turn proinsulin into insulin.

Parts Level

The osmY promoter

The osmY promoter is used for the regulation of insulin production. When the cells are in log phase there is a promoter that will 'turn on' transcription and there is another for the stationary phase. When the bacteria enters the stationary phase 'inverter' can be used with the osmY promoter to turn off production- hopefully resulting in a 'burst' of insulin production.

BBa_K1819009 can be used to produce only during the 'log' phase but BBa_j45992 can replace it if the production of insulin in the stationary phase is more desirable. As the bacteria moves from each phase the insulin production will switch on or off. When the bacteria die, more should be added through consumption.

INS gene

The INS gene codes for the proinsulin. By removing the signal sequence that preproinsulin has, we are able to directly produce the INS gene. The INS gene can be easily altered to suit patients preference. Although the gene sequence is used for the initial design code for rapid-acting insulin aspart, it is possible to change the gene sequence of the INS gene to produce a different type of insulin. The INS gene still produces proinsulin because it is nearly impossible to make insulin directly since insulin is made up of two chains. Proinsulin basically just has a C_peptide chain that joins the a and b chains that makes insulin. The proinsulin, however, cannot work like insulin so eventually, the proinsulin must be changed into insulin with the help of some enzymes.

Cellulose promoter

The fungus,*Trichoderma reesei,* has a special promoter activated by the presence of several saccharides. The cbh1 promoter is an inducible promoter which is induced by cellulose, lactose, sophorose, etc and regulated by catabolic repression. When the cbh1 promoter is used for protein expression, an inducer (or inducers) has to be added to trigger the expression of the target genes.

This promoter is especially useful to regulate the expression of the gene. As the production of insulin has to be easily and heavily regulated. The cbh1 promoter was added together with the osmY promoter so that insulin would not be able to be produced without the right conditions. For insulin to be produced, it has to be in the log phase and any of the cellulose or other saccharides must be present. These conditions are only met when the diabetic patient eats. As these patients will have regulated diets with different types of nutrients, cellulose will be usually present as it exists in plants. This way the patients will be encouraged to consistently consume a healthier diet in order to maintain their blood sugar level.

Trypsin-1 gene Carboxypeptidase B2

Both Trypsin and Carboxypeptidase are used for transforming the proinsulin into insulin. Trypsin is a serine protease that is formed in the small intestine. It is used to hydrolyze proteins. Trypsin alone can still change proinsulin to insulin. However, studies show that when Carboxypeptidase B2 is added, the transformation becomes much faster. It was observed that the Arg-Gly and Arg-Glu bonds in the carboxyl and amino-terminal regions were split very rapidly by trypsin, whereas further cleavage of insulin between Lys-Als and at other sites, proceeded much more slowly.

Proinsulin must be turned into insulin in order to have any effect. Since both of these enzymes can speed up the process and it is essential to transform proinsulin into insulin, they must be present inside the bacteria. This is why it is present in the second device that is triggered by the presence of proinsulin. So when proinsulin

is created in the first device, it then triggers the second device to start producing trypsin and carboxypeptidase.

The CPB2 gene was used for the production of carboxypeptidase and the PRSS1 gene was used to produce Trypsin-1.

One issue with this method is that if trypsin is produced in the cytoplasm of the cell, it might react with other molecules resulting in the death of the bacteria. That is why our team is still trying to find a better way to transform proinsulin to insulin. For example, using the periplasmic space or having a membrane-bound protein which can do the same job.

System level

When insulin is harvested from the recombinant bacterium. It often requires the bacteria to be broken apart to expose the insulin in the cytoplasm of the bacteria. The main reason for this is that *E. coli* doesn't have a naturally built-in insulin secretion system.

Needing to burst open the bacteria is not ideal for the diabetic patient because it can be difficult to control how much or when the insulin is produced. Also once the bacteria bursts it will die.

When insulin is produced in our β cells, it is secreted through the glucose-stimulated insulin secretion (GSIS) system. This process has multiple steps with multiple proteins. This process requires multiple proteins to be present to work which is difficult to stimulate in a bacteria

The type T2SS was used to secrete insulin into the gut. There are two main ways to transport a protein using a T2SS. Using the hemolysin transport system (Hly) or using a twin-arginine translocation (Tat) to transport the polypeptide to the periplasm, then using the general secretory pathway (GSP) to secrete the protein out of the bacteria.

For both Hly and Tat transport system, it requires a signal sequence to be attached in the end so that the membrane protein can recognize it. This signal sequence is attached right after the insulin gene as shown in Device 1. This sequence is then removed from the INS gene as it makes its way into the periplasm. When the INS gene is first translated in the cytoplasm it will be sent to the periplasm without it maturing. This means that protein folding must take place outside the bacterium or in the periplasmic space. chaperone proteins exist in the periplasmic space to help with protein folding.

The General secretory pathway is used to secrete the proteins out of the periplasm. The GSP consists of 4 distinguished subassemblies: inner membrane platform, outer membrane complex, pseudopilus, and secretion ATPase. Small proteins such as insulin are able leak from the periplasmic space into the environment, the GSP was added to make sure that the secretion occurs.

Although some gram-negative bacteria have the T2SS on their membrane, the *E. coli* strand living in our gut does not have this. That is why all the protein used to make this system must be made. This will be added in the second device that is triggered by the production of insulin. The secretion method might be changed in the future into a much simpler version which does not require multiple proteins. However, currently, this might be the safest method to make sure that the secretion of insulin occurs when needed. The secretion system is not controlled however the production of insulin is. Thus there is no need to have a regulatory system for secretion of insulin.

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