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Research Article

Successful Treatment of Diabetes II in Adult Patient and New Prospects of Recombinant Vaccine and Recombinant Proteins Engineering *In Situ*

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ABSTRACT

Our aim was to find the efficient cure of Diabetes II in vivo.The selective medium was constructed to ensure, that only bifid bacteria would be isolated from the feces of the volunteer. The additonal work was needed to find the conditions of maintenance of the isolated cultures to ensure the adhesion of said cultures back to the intestinal epithelium of the host organism. The isolated strains of bifid bacteria were tested on their belonging to the species Bifidobacteriumbreve, which has been used prior to us as the probiotic. The recombinant strain of B. breve was constructed as we did that before using the genome tailoring technology. We reached the success with the complete cure of Diabetes II in the volunteer. We have proposed our technology for expression of the recombinant proteins for the future use, for instance, for the long term outer space missions, where the crew would be vaccinated by the same technology, if they feel they would need to do so during the discovery of the new planets, suitable for the relocation of the overcrowded Earth population there. We also propose our technology for use for any recombinant protein expression in the human body when that would be needed for a variety of clinical applications: use of the human intestine as the gate as the entry to the human body for delivery of the recombinant proteins. The application of our described technology economic value would be around $327 billion or above that these days.

Keywords: Normal intestinal microflora; Intestinal bifidobacteria; Recombinant human insulin; Long range outer space flights

INTRODUCTION

An adult individual, working for our businesses, has approached us, when the work has been done, and kindly asked to help him. He is in his 50-s and suffered from Diabetes II, daily taking metformin. With the time, he became resistant to the metforming taken and after his visit to the doctor has received the prescription to add insulin injections to his daily routine along with still taking the metformin. He was very much upset, since now he had to monitor his blood glucose level multiple times over the day, adjusting the insulin and the metformin taken, while having the administered insulin and metformin still taken after he had become resistant to it. So, he approached us and said, that he already saw multiple genetically engineered biocatalysts, that our businesses have created, and he stated, he believes in us and asked us to cure him from his Diabetes II, using the new recombinant engineered strain, he wanted us to construct. We said him, that he would be the volonteer, and we will help him.Our decision was based on our previous knowledge, that the volunteer most likely has *Bifidoabcteriumbreve* in his intestine, and we can make this organism expressing recombinant human insulin.

Bifidobacteriumbreve was originally isolated from the intestinal tract of the infant [1]. A main component of the human gut microbiome, Bifidobacteria species are the part of many probiotic supplements, widely used to treat gastrointestinal and other ailments, associated with the anti-inflammatory and immune-modulatory effects, that are generally thought to promote health, but are not understood in detail [2,3]. *B. breve* is the dominant species in the gut of breast-fed infants and it has also been isolated from human milk of breast-feeding mothers of the newlyborns [2,4,5]. Due to strong beta-gactosidase activity in strains of *B. breve*, *B. breve* is capable of growing on various types of milk [6].

Therefore, we have decided to use the intestinal bifidobacteria of the volunteer to achieve the intestinal expression of the human

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insulin (this report). Specifically, our choice came to isolation of *B. breve***,** as it was shown before, it is a widely used as the probiotic [2,7].

The details of the procedure to cure Diabetes II of the adult individual and its future prospects and developments are the objective of this manuscript. The estimated national cost of diabetes in 2017 was $327 billion [8]. More than 34 million Americans have diabetes (about 1 in 10), and approximately 90-95% of them have type IIdiabetes [8]. Therefore, the evidence of the cost of our way of treating Diabetes II is obvious. The estimated national cost of the future intestinal recombinant proteins proposed has to be evaluated upon the availability of said data.

MATERIAL AND METHODS

Isolation of the *Bifidobacteriumbreve*, the component of normal intestinal microflora, suitable for construction of the insulin-producing strain to be returned to the patient to cure him from Diabetes II.

We had to develop the selective medium to isolate *Bifidobacteriumbreve* from the intestine of the volunteer. Bifidobacteria are the naturally occurring intestinal microorganisms, obligate anaerobes, which are transferred from the mother's intestine to the mouth of the newly born child during the natural birth process. Mother's Bifidobacteria protect the newly borns from a variety of potential food-borne pathogens to up to one year of the newly born's life [1]. Bifidobacteria were found in the breast milk of the brest-feeding mothers [1]. Bifidobacteria produce organic acids, and hydrogen peroxide along with potentially beneficial naturally occuring proteins in the host intestine. The most important, the produced by bifidobacteria formic acid has strong antibacterial activity [5]. To make sure, that the isolate of bifidobacteria will again adhere to the intestine of the volunteer, we made sure, that the isolation of the intestinal bifidobacteria will be on the special selective medium to make sure, that it would take only 48 hours to grow up the intestinal bifidobacteria of the volunteer and only 192 hours to get the bifidobacterium, capable of adhesion back to the volunteer's intestine. Said recombinant bifidobacterium would be capable of expressing the recombinant insulin at the gut of the patient, our volunteer, specifically. Therefore, the total cell duplication time did not exceed 200 cell duplications, as it is mandatory to store intact the capability to adhere back to the intestine of the host [9].

Therefore, we have constructed the Selective Bifidobacterium Medium to isolate the intestinal bifidobacteria from the feces of the volunteer (Table 1).

Table 1: The Selective Bifidobacterium Medium to isolate *B. breve* from the volunteer's feces, which we have constructed, contained, g/l.

Caseine Triptone	10,0
Meat Extract	10,0
Yeast Extract	5.0
Pepton from soymeal	6.0
Sodium chloride	3.0
di-Potassium hydrogen phosphate	3.0
X-gal	0.5
Ox gal powder	3.0
Agar powder	15
Norfloxacin	0.3
Amphotericin B	0.2

The significance of the nutrient and selective components of the Selective Bifidobacterium Medium: The rich Selective Bifidobacterium Medium composition, presented by Caseine Triptone, Meat Extract, Yeast Extract and espesially Pepton from Soymeal ensures, that Bifidobacteria grow on the selective medium for 48 hours anaerobically at 37°C. X-gal (5-Bromo-4-Chloro-3-Indolyl β-D-Galactopyranoside) is the selective agent, showing the presence of beta-galactosidase activity in *B. breve* isolates [10]. X-gal in the Selective Bifidobacterium Medium will ensure, that the colonies of *B. breve* will be dark blue, when they grow on the Selective Bifidobacterium Medium as described [5]. Ox gal is the selective agent, originating from bovine bile, serving as the bile substitute in the Selective Medium to isolate bifidobacteria. Norfloxacin comes in tablets, 400 mg. Norfloxacin is the selective agent, blocking growth of intestinal lactobacilli [9]. Strains of *B. breve* are naturally resistant to norfloxacin [9]. Norfloxacin powder was obtained from norfloxacin tablets.Amphorericin B is the anti fungal agent [9]. It is added to the *Selective Bifidobacterium Medium* to inhibit growth of molds and candida, should they happen to be in the feces, used to isolate bifidobacteria [10].

The Selective Bifidobacterium Medium without norfloxacin and amphotericin B was autoclaved at 0.5 atm for 30 min. The selective agents Norfloxacin powder and Amphotericin B powder were addedto the Selective Bifidobacterium Medium after autoclaving of the medium, when it was cooled down to 45°C for distribution into Petri Dishes [11]. Selective Bifidobacterium Medium was cooled down to 45°C and the norfloxacin powder and amphotericin powder were added. The medium was poured by 20 ml in the 100 mm Petri dishes, which were dried in the Laminar Flow Cabinet [12]. Dried medium in the Petri dishes was marked with thepermanent marker [10], making five sectors of approximately equal size. The 20 microliter aliquotes of the decimal 3rd, 4th, 5th, 6th and 7th dilutions of the volunteer faeces in the dilution buffer (DB) were plated on each Petrydish by P200 pipetman [13]. Each pipetman aliquote was then spreaded on the whole surface of the medium sector (not to cross the neighboring sector), using the plastic microbiological loop [14]. The plates then were incubated in microanaerostates [15] at 37°C for 48 hours. Colonies from the last feces dilutions were stricked once on the Selective Bifidobacterium Medium to make sure the pure cultures were isolated. Purified bifidobacterial strains were stored at -80°C, each in 300 microliters of the liquid Selective Bifidobacterium Medium, added to the sterile 1.5 ml Eppendorf tubes [16] before inoculation.

The DB composition: 0.1 M Tris, 0.15 M NaCl, 1.0 M urea, 10 mM $CaCl_2$, 0.1 M citric acid monohydrate, 5 g/l BSA and 1.0 g/l cysteine-HCL (pH 6.00). The DB was distributed into the sterile 1.5 ml Eppendorf tubes [16] by 900 microliters.

Dilution of the volunteer feces.0.1 g of the freshly collected volunteer's feces was dissolved in the first 1.5 ml Endorphin tube, containing 900 micro liters of DB (the 1st dilution of the feces). The O.1 ml of the resulting the first dilution of the volunteer feces were transferred to the second Eppendorf tube with 900 microliters of DB (the 2nd dilution of faeces), and so on, to make the necessary for the plating decimal dilutions of the volunteer's feces. The procedure was, as follows. The 100 microliter aliquiotes of the first dilution of the volunteer feces were added [13] to the second 1.5 ml Eppendorf tube with 900 microliters of DB, etc,to make the second, 3rd, 4th, 5th, 6th and 7th, etc. dilutions of the feces of the volunteer.

Hybridization to distinguish *Bifidobacterium breve* from other

bifidobacteria. It was important to make sure, that the isolated on the Selective Bifidobacterium Medium cultures were indeed *Bifidobacteriumbreve*. To distinguish *B. breve* from other bifidobacterial species, we have used the described procedure [1,5].

The 1.8 kb fragment of the *B. breve* chromosomal DNA, FERM P-11932, was used as the hybridization probe. It was obtained from the Fermentation Research Institute (Japan) [1].

It is very important, that the isolated strains of bifidobacteria were isolated and purified in 96 hours, then stored at -80°C in the same medium, as used for the isolation and purification of the intestinal bifidobacteria, only without agar-agar. The time to obtain the recombinant strain of *B. breve* should not exceed 96 hours. Therefore, the total time, which the isolated bifidobacteria were outside of the volunteer's intestine did not exceed 192 hours. As that was sted before [9], only in such case the ingested recombinant strain, which was manteined outside of the volunteer's intestine, regains the capability to adhere back to the host intestinal epitheliumand therefore, express the sub cloned recombinant human insulin *in vivo* (this report). The total time to isolate, purify and metabolically engineer *B. breve* took only about 192 hours (1.6 hour is the bifidobacterium cell duplication time). As stated before [9], any elongation of the time for being outside the volunteer's intestine removes the capability of the isolated culture to adhere backto the the volunteer's organism intestinal epithelium, and, therefore, makes the attempts to get the constant recombinant human insulin expression *in vivo* futile (this report).

Construction of the recombinant *Bifidobacteriumbreve*, excreting recombinant human insulin. We have used Bifidobacteriumbreve bb387 strain, isolated from the volunteers intestine, to do the metabolic engineering and make sure, that said strain would be capable of excreting human recombinant insulin. Using genome tailoring methodology [17], in *Bifidobacteriumbreve* bb387 strain we have removed [17] the unnecessary for strain maintenance genes at their positions 4346…4816 bp,10023…10574 bp, 16239…17477 bp, 19324…20316 bp, 20927…21592 bp,22486…23799 bp,237007…238836 bp, 24676…26007 bp, 31295…33502 bp,34410…36290 bp, 37707…39254 bp, 538583…541012 bp,643441…645516 bp,817368…820625 bp,1476554…1481404 bp,and 2258202…2261981 bp [3,17,18]. The recombinant strain of *Bifidobacteriumbreve* was produced as described [17,19,20]. The recombinant human insulin, expressed by the intestinal isolate *Bifidobacteriumbreve* bb387insulin, has been deposited to NCBI (Submission ID is 2442803).

RESULTS

The recombinant human insulin producing strain of *Bifidobacteriumbreve*, *Bifidobacteriumbreve* bb387Insulin, was then developed as described [16]. The primers for the PCR, to check out the presence of the recombinant insulin gene in the recombinant strain of intestinal bifidobacteria *Bifidobacteriumbreve* bb387Insulin, were designed, using the publically availalable tool at the NCIB web site [20]. The PCR with the primers (the forward primer AGCATCTGCTCCCTCTACCA and the reverse primer TCCATCTCTCTCGGTGCAGA), showed the production of human recombinant insulin by *Bifidobacteriumbreve* bb387Insulin, as that was confirmed clinically.

The strain *Bifidobacteriumbreve* bb387Insulin was originally tested as sensitive to the therapeutic doses of amplicillin, achievable in the intestine of the volunteer. This testing has been done to immediately stop all the biological effects of the ingestion of *Bifidobacteriumbreve* bb387Insulin by the volunteer.

Clinical effect of ingestion of the *Bifidobacteriumbreve* bb387Insulin by the volunteer.Shortly after ingestion of the 25 ml of the Selective Bifidobacterium Medium, containing frozen stock of *Bifidobacteriumbreve* bb387Insulin, the volunteer has achieved its adhesion to the intestinal content, as he noted the absence of his Diabetes mellitus II clinical symptoms after regular meals, taken three hours after the *Bifidobacteriumbreve* bb387Insulin intake, with no further insulin injections and additional metformin intake. There was no glucose in the urine of the volunteer (tested in my lab), and the blood glucose levels exceeded 100 mMols/d only one hour after the meals, or corresponded to the normal blood glucose levels of the normal individual. With my sincere interest, I have ordered the volunteer, who has continued to report for regular work, to do the three days continuous blood glucose testing, especially one hour after having meals with even the sugar content. The data, obtained during said testing, have convinced me, that there was no the need for the blood glucose monitoring daily, only once a week, since the volunteer seemed to be free from Diabetes II.Three weeks passed. No blood glucose level spikes were ever observed, even after sugar-containing meals. At my request, the volunteer has interrupted the actions of *Bifidobacteriumbreve* bb387Insulin in his intestine by the intake of a single dose of 2.0 grams of ampicillin (capsules). Shortly after the ampicillin intake, the volunteer started experiencing again all the clinical symptoms of his existing Diabetes II. The blood glucose levels started exceeding 200 mMol/dL, if no insulin injections were made.After three days of said Diabetes II clinical manifestation, the volunteer was ordered to ingestagain the frozen at -80°C and dissolved in the 25 ml of liquid Bifidobacterium Selective Medium with no norfloxacin and amphotericin B added *Bifidobacteriumbreve* bb387Insulin.The volunteer was again cured fromhis Diabetes II, and he has continued his life, living as the healthy individual.

DISCUSSION

We have discussed the prospects of our planet in the future at our program web site, and noted the coming in 20-50 years from now the shortage of the fresh water.Indeed, accumulated in the air CO_2 is one of the heaviest gasses in the air blend, reaching its density 1.97 g/cubic meter [3]. The CO_2 in the air gas mixture under no wind conditions spreads on the ground surface and selectively absorbs all the infrared energy of the Sun, thus heating the ground significantly. That causes the extra evaporation of the fresh water from soil to the air. As you know, Global Warming presents itself in various forms, specifically with increased frequency of tornadoes, rains, etc.But the Earth gravity has been stable for the last few million years from now.Therefore, under the constant gravity force applied, more fresh water vapors are in the air.The space, surrounding Earth, as any Space anywhere, has vacuum. That vacuum sucks fresh water vapors from Earth air, and such fresh water vapors travel in the Space in the unknown direction way for the Earth. In 2010 NASA has bombarded the Moon and found plenty of ice on its dark and very cold surface.They were guessing, where said ice came from?Moon worked as the cold trap for the fresh water vapors coming from Earth in the Space vacuum [http;//syngasbiofuelsenergy.com]. What will happen next and the most important, when?It is impossible to anticipate, that the fresh water loss to the outer Space may be stopped at any time even if the Earth population is suddenly decreased in its amount. The

extra air CO_2 comes from the intensified petroleum use, and the use of its products for combustion, producing CO_2. People breath and produce CO_2 as well. It is anticipated 14 billion people on Earth by 2050. That increases more the air CO_2 content, leading to the exelerated fresh water loss as discussed. We have no any idea, what will happen soon, if no new planets, similar to Earth, will be discovered and the overcrowded Earth population will not start to move there.We do anticipate, that by that time a glass of water will be available for significant money, and there would not be no washing of our bodies and clothes, no crops production. No livestock and crops production are anticipated with the reasonable justification above. The solution would be to restructure the current economy for inclusion as wide as possible of our private technologies of the manufacture of seven commodity chemicals and fuels, made solely from air CO_2 and N_2 and the hydrogen [3], projected to be produced by electorlysis of the petroleum production waters and other heavily contaminated waters, while the byproduct oxygen adds to the air, and the proposed hydrogen would be produced at $0.20 per 1 kg or 500 moles.

The prospects of the future use of this recombinant techonlogy and its potential applications as related to the coming soon long range outer space flights of the manned crews, hoping to find the appropriate place for humans to reside in the Universe due to the enormously increasing Earth population, going to reach 14 billion people by 2050. Private investors started investing much into the prospective coming soon long range outer space missions of the manned crews. The US model of the economic development has been proven to be immaculate and directed only to moving forward. We are here, in the US, we are ready to do anything possible to make the life of said manned crews as simple as possible. We offer 1) the crew vaccination *in situ* via the described herein way of Diabetes II treatment in adults and 2) foods for the manned crews, which they can prepare themselves during said long range space flights.Both our offers are intended to decrease the lift-off weight of the spacecrafts. The meals, that we offer to use, are in the next our possible manuscript in this magnificent peer-reviewed Journal. The procedure of making the recombinant strain(s) from the intestinal content is carefully described herein, as that pertains to the expression *in vivo* of the recombinant protein recombinant human insulin.

Imagine, the long term outer space mission has reached something, which they consider to further explore for the proposed relocation of the crowded Earth population to the outer space. Imagine, that the samplers have taken the biological samples to be detail examined for the potential microbial dangers for the crew of said long range spacecraft.Certain objects may be determined as causing damage to the crew, which has never been in contact with said potentially dangerous organisms in the outer Space.Now, we offered herein the technology to produce recombinant therapeutic proteins right in the intestine of the particular individual chosen.In general, the expression of any new antibody, new vaccine and other forms of therapeutic proteins will work.It is extremely important, that the intestine, where the recombinant proteins are produced, is in the tight connection with the whole human organism and, therefore, will ensure the random blood distribution of said recombinant protein (s).Therefore, the intestine will work as the internal gate for the therapeutic proteins. As such, the said manned crew members may get the essential vaccines in their own bodies in just less than 200 hours.This approach may have crucial importance for the manned crews life during said long term outer space missions proposed.

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DECLARATIONS

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CONFLICT OF INTERESTS

The author declares his personal conflict of interests with the law firm in Houston, TX Hirsch and Westheimer, major petroleum and gasoline/diesel fuel companies, with the Houston Police (City of Houston) and with the Houston FBI.

ETHICAL APPROVAL

This article does not contain any section, requiring Ethical Approval.

CONSENT TO PARTICIPATE

This article is the opening gate for the wide use of the recombinant proteins *in situ*.

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