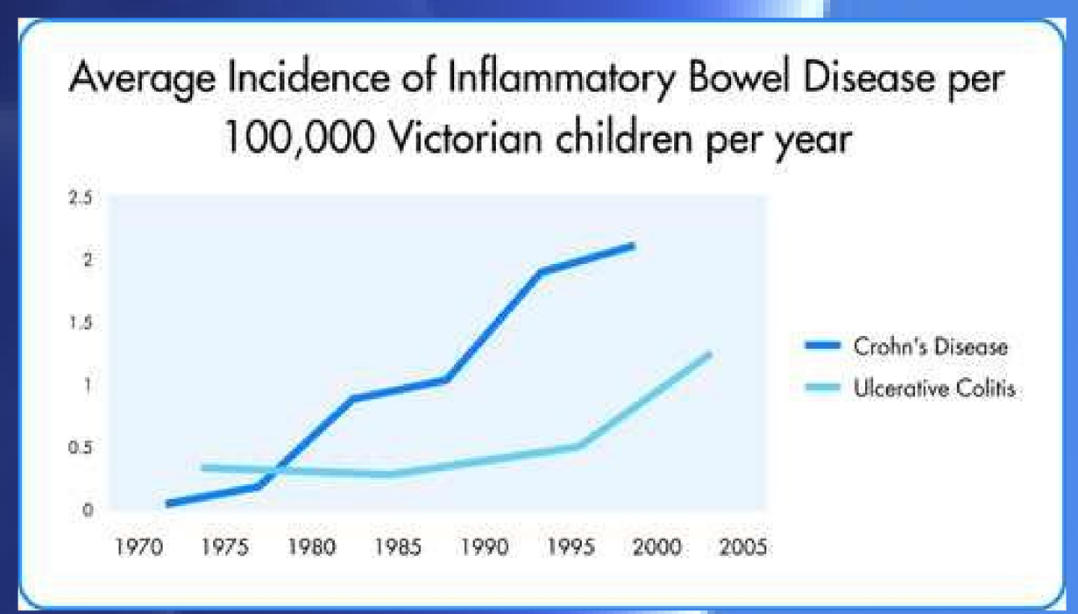
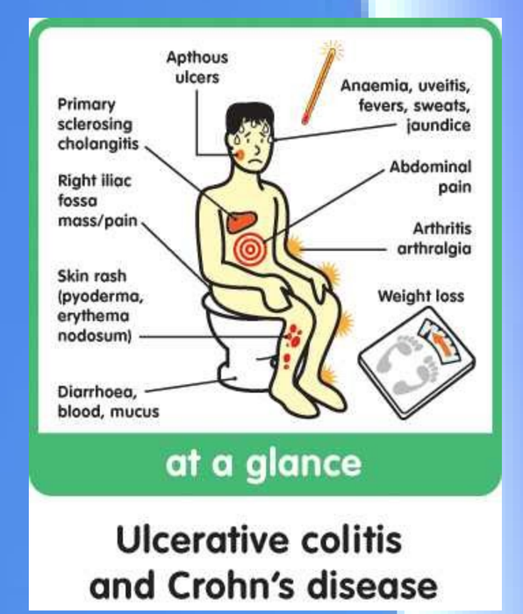
Why Crohn's Disease?

Crohn's disease is a type of inflammatory bowel disease that has symptoms which are not local. Nowadays it spreads more efficiently mostly among the youth (see the graph below). A part of the gastro-intestinal tract is in constant inflammation and apart from that the disease can cause ulcers, vomiting, diarrhea and anemia (see the figure below). Over 600 000 people in the US suffer from this disease.



http://www.thegutsygroup.com.au/crohns-colitis/incidenCce/



http://www.crohnsdiseaselifeexpectancy.com/

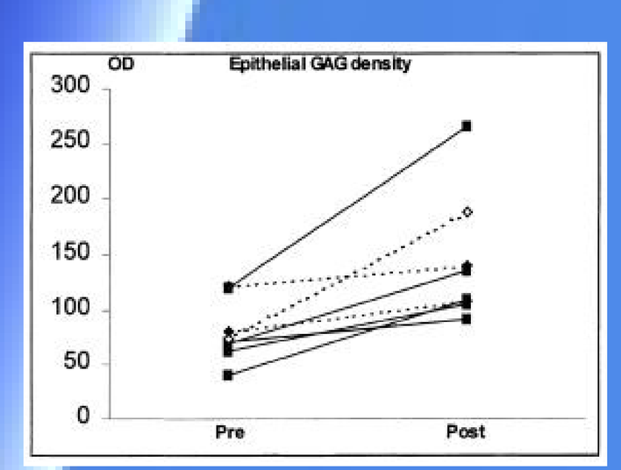
Why NAG and Why Probiotic Bacteria?

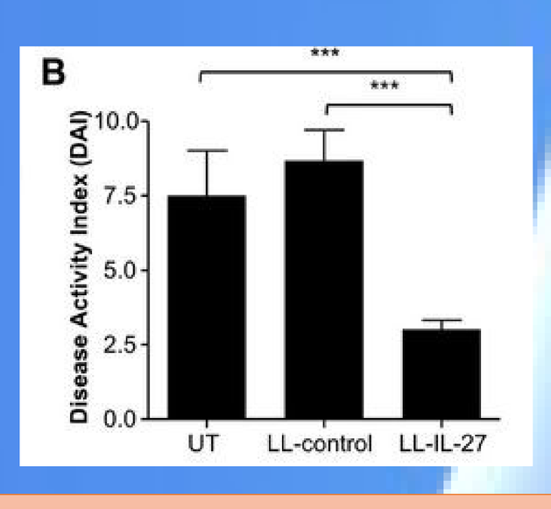
Researchers from England treated their patients (age up to 16), with NAG (N-acetyl glucosamine) orally and rectally and observed that 8 out of 12 of them showed improvement. They verified it by biopsy and other means and published their results.

(A pilot study of N-acetyl glucosamine, a nutritional substrate for glycosaminoglycan synthesis, in paediatric chronic inflammatory bowel disease S. Salvatore et al.: Alimentary Pharmacology & Therapeutics; Volume 14, Issue 12, pages 1567–1579, December 2000)

American researchers published an article on making and transferring genetically modified IL-27 citokyn producing bacteria into the intestines of mice with severe symptoms of bowel disease. The researchers have found that the rate of the symptoms decreased.

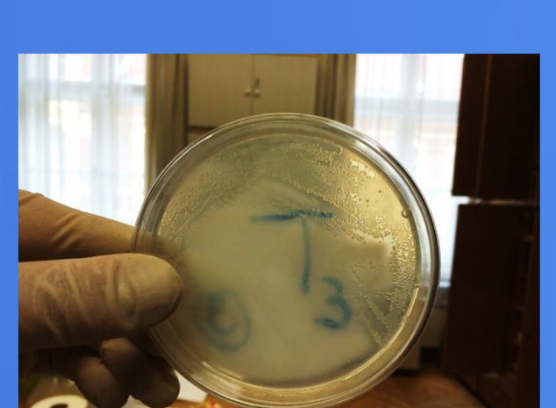
(Hanson, M.L. et al.: Oral Delivery of IL-27 Recombinant Bacteria Attenuates Immune Colitis in Mice. *Gastroenterology*, 146(1), 210-221.)

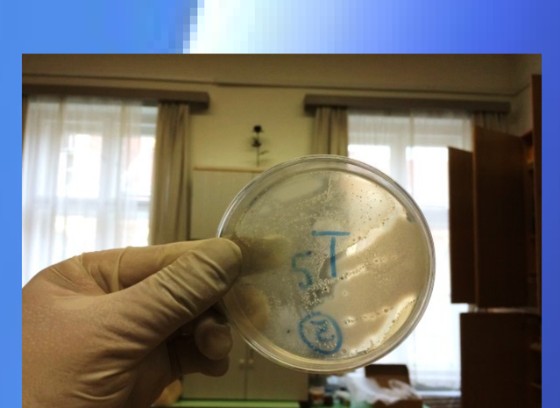




The Role of Natural Chitinases in Our Project

Chitin is the basic material of many organisms such as fungi, insects and worms. Due to the presence of chitin in soil, some soil decomposers produce chitinases.
We collected soil samples from different locations and in order to examine them, we have made colloidal chitin agar medium. If they break down chitin, we can see clearing zones around the colonies on the colloidal chitin agar as visible on the figures below.

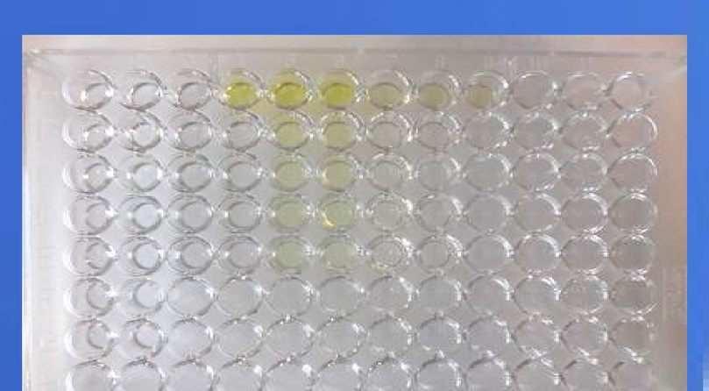




In order to determine how chitinases are able to work in the intestine, we ordered Sigma Aldrich Chitinase Assay Kit (SACAK) to study the activity of natural chitinase enzymes under different environmental conditions. The assay is based on the enzymatic hydrolysis of chitinase substrates and it releases p-nitrophenol which can be measured colorimetrically at 405 nm. We used BioRad Benchmark microplate reader to carry out these measurements.

Our key findings were as follows,

1. The **SACAK was appropriate for measuring natural chitinase activity**. We could see strong color changes at the kit's own chitinase enzyme.
2. The measurment of the **natural chitinase of Bacillus thuringiensis** liquid media showed **2.08 U/ml activity** according to the given formule by the SACAK provider.
3. The chitinase activity of Bacillus thuringiensis bacteria was the highest if it was cultured in **shaking plate on 37°C, at pH≈4.8-5.8**. These findings strengthen the idea of GMO-based chitinase activity in a normal large intestine environment conditions.
4. The **large intestine extract of the rat didn't show any chitinase activity** itself.
5. The **large intestine extract didn't have any enhancing or inhibiting effect** on natural chitinase activity.
6. We found strong exochitinase activity, but **no endochitinase activity was measured**.



Microplate Manager Bio-Rad Laboratories, Inc.
Raw Data Report

Reader Type : Benchmark   Plate File : Plate5
Date : 2014.19.06 16:57

Measurement Wavelength: 405nm
Incubator Temperature: 29.0 °C
Reading Type: Endpoint
Mix Time: 0 sec

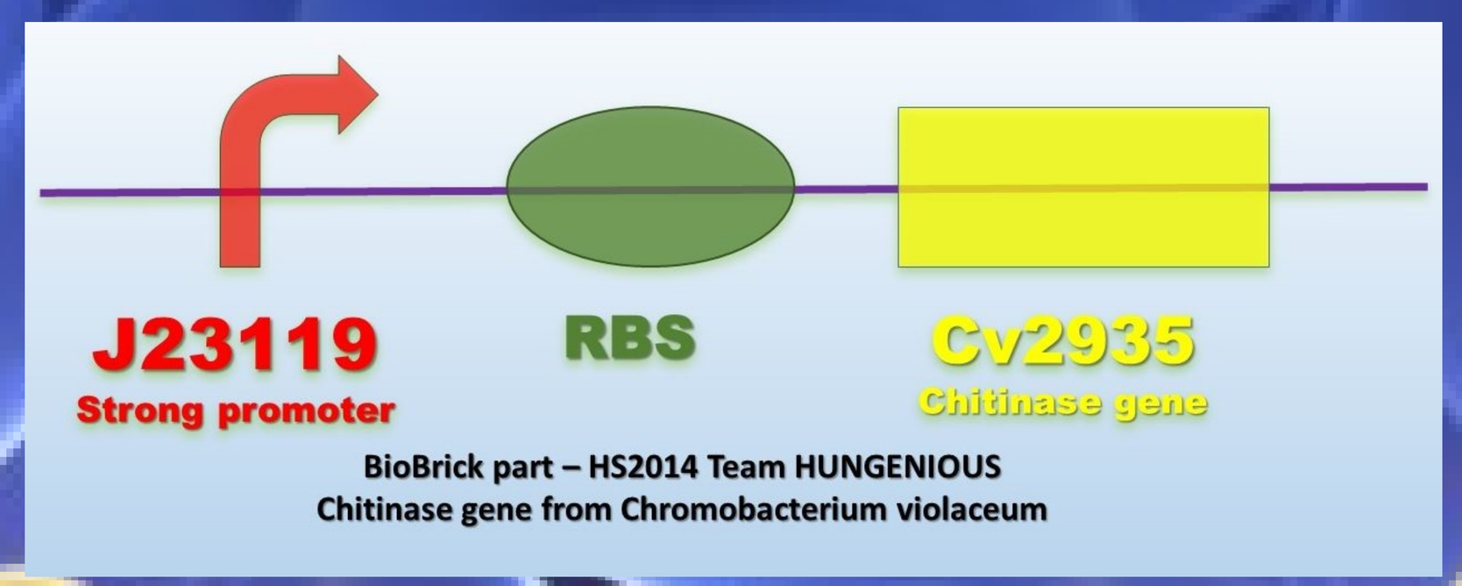
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.009	0.009	0.007	2.896	2.766	2.650	0.445	0.454	0.450	0.071	0.067	0.060
B	-0.013	-0.045	-0.013	0.004	0.638	0.430	-0.011	-0.018	0.082	0.077	0.079	0.078
C	0.018	-0.001	0.020	0.034	0.681	0.469	0.023	0.014	0.085	0.074	0.063	0.060
D	0.039	0.041	0.049	0.035	0.684	0.457	0.051	0.056	0.072	0.087	0.078	0.073
E	0.070	0.017	0.026	0.022	0.650	0.421	0.045	0.061	0.094	0.089	0.099	0.096
F	0.035	0.024	0.092	0.075	0.067	0.075	0.090	0.070	0.077	0.075	0.055	0.072
G	0.023	0.011	0.076	0.085	0.084	0.065	0.077	0.072	0.078	0.078	0.071	0.069
H	0.094	0.091	0.091	0.081	0.069	0.082	0.104	0.094	0.083	0.074	0.077	0.068

	1	2	3	4	5	6	7	8	9
A	Blank	Blank	Blank	PC	PC	PC	S	S	S
B	1	2	3	4	5	6	7	8	
C	1	2	3	4	5	6	7	8	
D	9	10	11	12	13	14	15	16	
E	17	18	19	20	21	22	23	24	
F	25	26							
G	25	26							

Blank: 100 microliter of Substrate Solution
PC- Positive Control: 5 microliter of Chitinase Control Enzyme
S- Standard: 50 microliter of Standard Solution
1-8: 5 microliter of Culture Medium 1-8 + 2,5 microliter of PBS
9-16: 5 microliter of Culture Medium 1-8 + 2,5 microliter of healthy rat intestine
17-24: 5 microliter of Culture Medium 1-8 + 2,5 microliter of inflamed rat intestine
25: 5 microliter of PBS + 2,5 microliter of healthy rat intestine
26: 5 microliter of PBS + 2,5 microliter of inflamed rat intestine

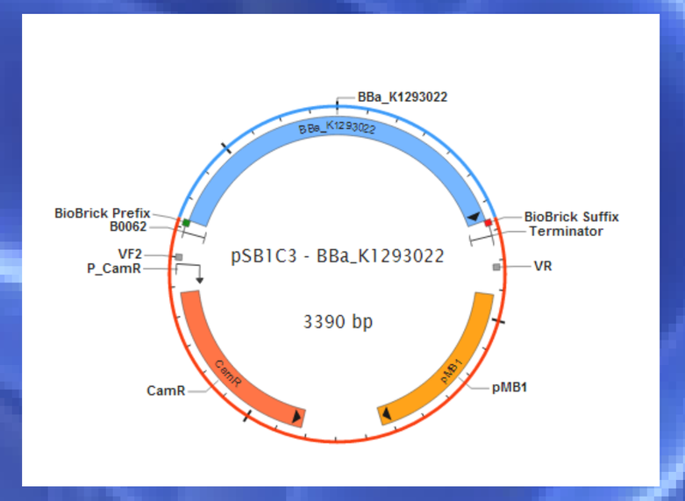
Our New BioBricks

We have constructed two BioBricks based on our experimental design. One of them is **BBa_K1293022** which contains the Cv2935 gene of Chromobacterium violaceum. The other one is **BBa_K1293023** which is constructed from a strong promoter BBa_23119 and our aforementioned basic part. The article in which we found our target gene is:
Expression and efficient secretion of a functional chitinase from Chromobacterium violaceum in Escherichia coli.
Lobo et al. *BMC Biotechnology* 2013, **13**:46 doi:10.1186/1472-6750-13-46



PROBIOTIC CHITINASE PRODUCING BACTERIA ENGINEERED AGAINST CROHN'S DISEASE

iGEM HUNGENIOUS



Biosafety

Name	DNA /living cells	Biosafety Level	ATCC No.	Source	Used for
Chromobacterium violaceum	DNA-part	BSL2	12472	DSMZ	amplification template of chitinase gene
Escherichia coli DH5 alpha http://ecoliwiki.net/colipedia/index.php/DH5_alpha	living cells	BSL1	67878	BRC	transformation
Escherichia coli Nissle 1917	living cells	BSL1	8739	BRC	transformation
Bacillus thuringiensis NCAIM - 01262	living cells / freeze dried	BSL1	33679	NCAIM	chitinase assay
Bacillus thuringiensis NCAIM - 01292	living cells / freeze dried	BSL1	10792	NCAIM	chitinase assay

We always wore labcoats and gloves. We kept the safety rules which were given by our experienced teachers. All disposable materials, (pipette tips, Eppendorf- and PCR-tubes, inoculating loops, etc.) connecting with microorganisms were put into a biohazard container, filled with strong oxidative disinfectant.
We have used microorganisms belonging only to BSL1 level. The only exception is *Chromobacterium violaceum* which is itself a BSL2 bacterium; however we have used only parts of its DNA (chitinase gene). The product of this gene is not connected with the *Chromobacterium violaceum*'s pathogenic effect.

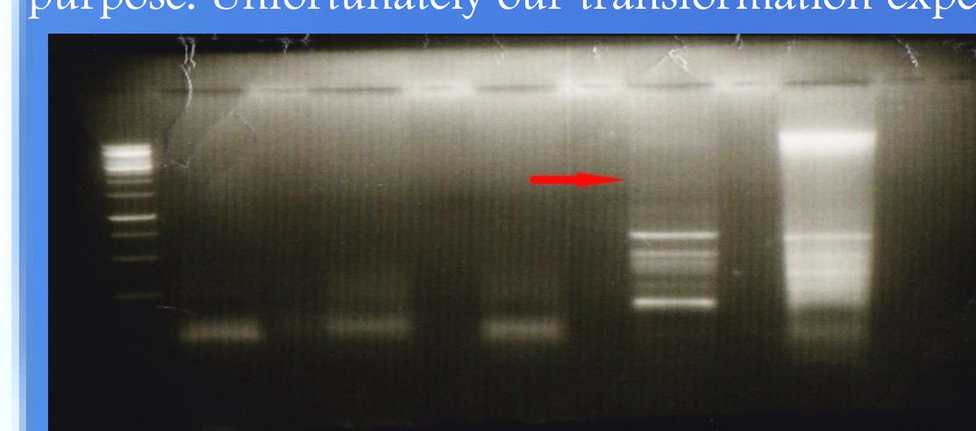
Our Future Plans

Research quetsions:
- We would like to prove the tissue repairing effect of NAG coming from our GMO bacteria
- We would like to find an appropriate mean of transfer of GMO bacteria into mice/humans

Biosafety questions:
- Does our GMO bacteria cause any changes in the natural microflora of the colon?
- Can we ensure that Gmo bacteria will die after leaving the patients' digestive system?

Genetic Experiments

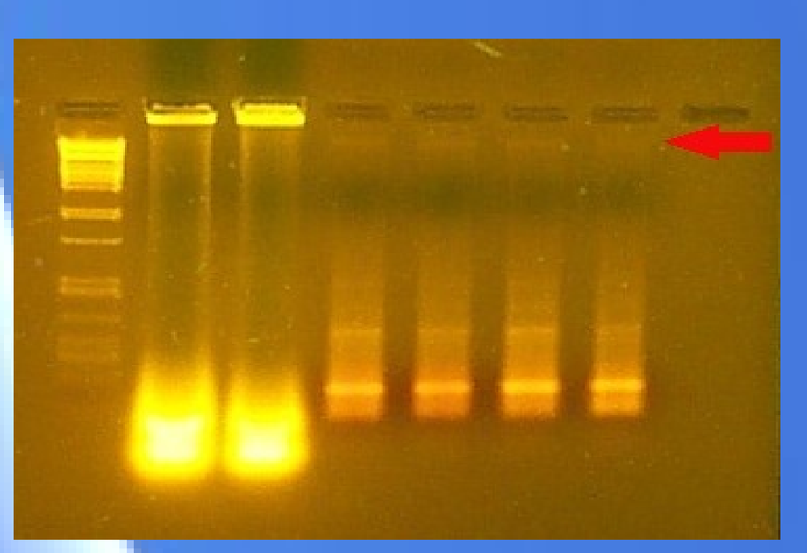
At first, we were going to get DNA from IDT (Integrated DNA Technologies), but unfortunately, after many attempts, the company was **unable to synthesize** the chitinase gene. In the lack of time our plan was the following: we ordered genomic DNA of Chromobacterium violaceum from DSMZ.
As we had plenty of things to do, our team split up into 2 smaller groups: someone was working at the Biological Research Centre (BRC) with local researchers, whilst the others were working at the School Lab.
In the BRC we have used gradient PCR technique instead of standard, and annealing temperatures also had to be set to an appropriate value. Simultaneously, we also amplified the **pZA31 plasmid, into which the Cv2935 gene was going to be inserted**. To avoid 3' overhangs different polymerases and buffers had to be used in both reactions. To decide whether the PCR reactions were successful, we ran the products on gel. The results showed that, the 'Phusion polymerase' was the most successful with the annealing temperature of 58°C, as it can be seen in these pictures.
We prepared the electrocompetent cells by suspending, chilling and centrifuging 3 times, then we subjected the cells to electroporation in order to transform our plasmid, containing the chitinase gene, into Nissle bacteria (E.coli strain). Special cuvettes and an electroporator was used for this purpose. Unfortunately our transformation experiment was unsuccessful.

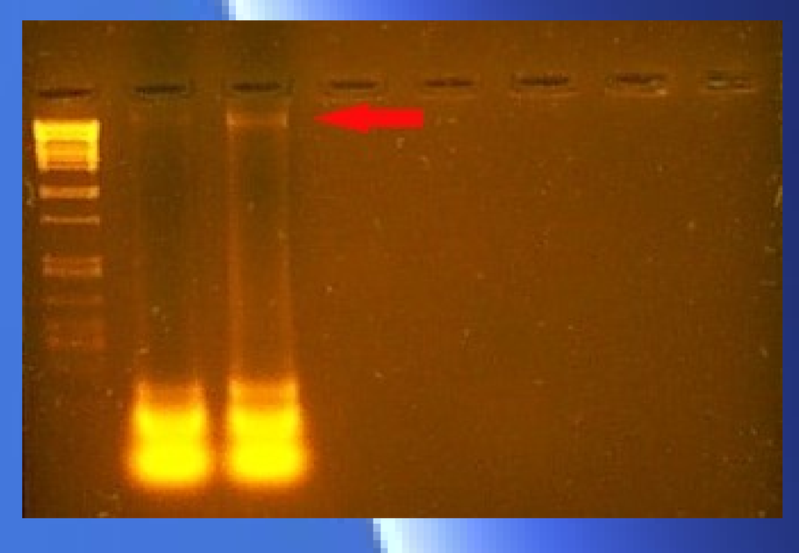


Results of the first PCR of gene CV2935
1st well: size ladder
2nd-6th well: Samples treated at different annealing temperatures. The red arrow indicates the slightly visible line of DNA under the 5th well. During the PCR of the DNA of the 5th well, the annealing temperature was set to 58C.

1st well: size ladder
2nd-4th well: Samples treated with different polymerases: we treated the DNA in the second and third well with the standard Taq polymerase. We added Phusion High-Fidelity polymerase to the DNA, that can be found in the 4th well. The red arrow shows the line of chitinase gene at 2kbps.

While the procedures at BRC were going on, similars were carried out at our school and at the University of Szeged. Though, here we wanted to construct a **BioBrick in a pSB1C3 backbone**, not just to transform a plasmid into bacteria. We amplified the chitinase gene using colonial PCR and iGEM prefix and suffix primers. PstI & EcoRI were used for digestion. After we have purified the enzyme, a ligation was performed according to the table above. At last, the product of the ligation was transformed into **DH5-alpha strain of E. coli** with heat shock method. All the experiments was checked by gel-electrophoresis, even from the transformed colonies we have made colonial PCR. The results can be seen on the figures below.

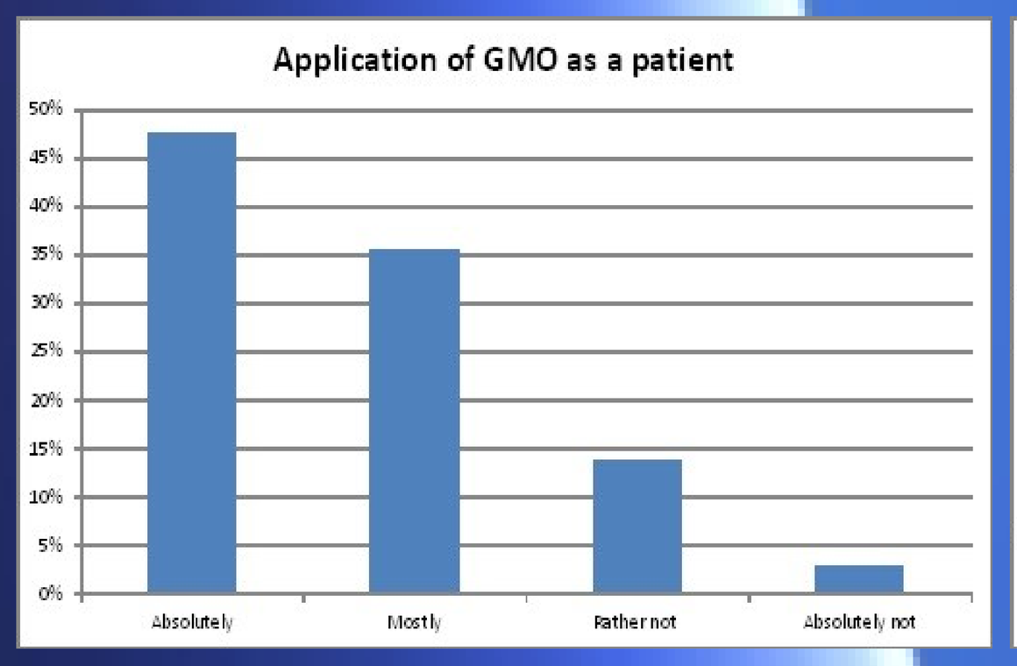


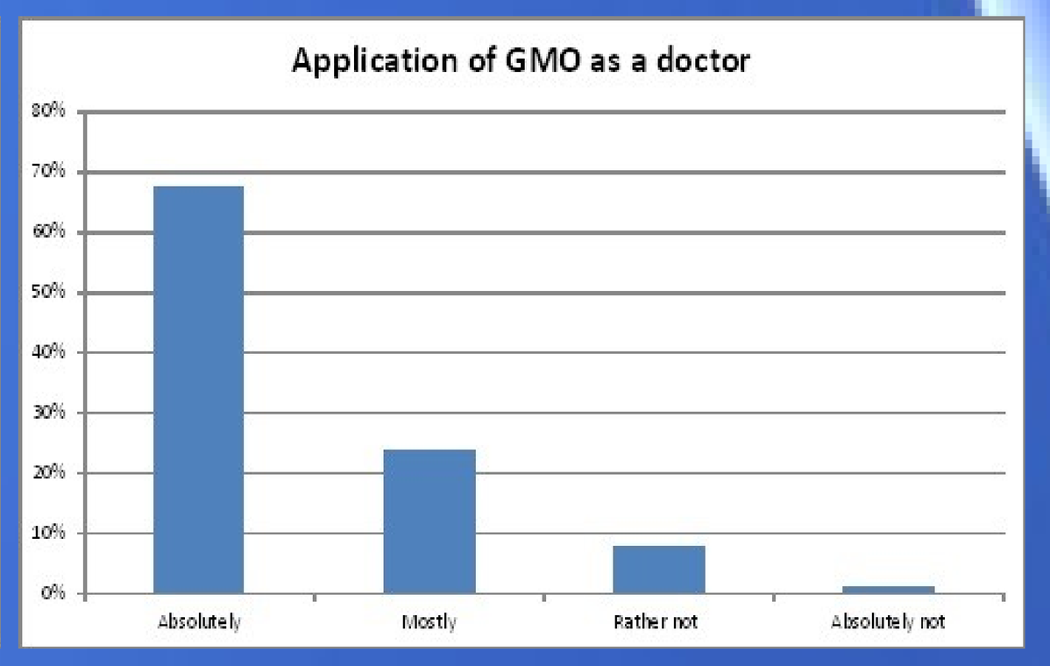


Human Practices

In order to propagate our project we had several meetings with people who can be affected the problem we worked on. We met for example:
- A medical doctor (Klaudia Farkas M.D.), who works with patients affected by Crohn's disease.
- We had an opportunity to talk to medical students.
- We had the privilege to talk about our project to Nobel prize winner Aaron Chiecanover, who visited our school.

Moreover we have decided to make a survey to find out people's opinion about our idea and other topic in which it may be involved. We created this survey using SuveyMonkey. It has been filled 101 times mostly by people from Hungary.
Our key findings were as follows:
1. Considering these responses, this disease is believed to be a moderately spread disorder.
2. In accordance to the results of the survey more than 35% approves non-medical application of a genetically modified organism.
3. We could also conclude the rate of those, who would take part in a treatment which involves GMO: 45% would not refuse to be cured with GMO.
4. Interestingly, 70% claimed that if they were a doctor they would absolutely use a treatment involving genetically modified probiotic bacteria.





Team and Acknowledgments

Our team consists of 8 pupils all from RMG: Miklos Boldogkoi, Reka Fabian, Mark Harangozo, Akos Marton, Gergo Nikolenyi, Martin Papos, Petra Varga, Andras Volford.
Our mentors were: Sandor Ban (RMG-Szeged), Balint Csorgo (BRC-Szeged), Akos Nyerges (BRC-Szeged)
Main designer of our website: Csongor Kiss (RMG-Szeged)
Institutions involved:
- BRC: Biological Research Centre of Hungarian Academy of Sciences,
- RMG: Radnoti Miklos Experimental Grammar School, Szeged
- Department of Physiology, Anatomy and Neuroscience at the University of Szeged

The sources of bacteria and genom:
- Soil samples
- NCAIM: National Collection of Agricultural and Industrial Microorganisms – Hungary
- DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen