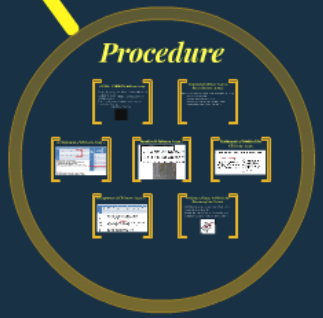
Probiotic Chitinase Producing Bacteria Engineered Against Crohn's Disease

iGEM HUNGENIOUS

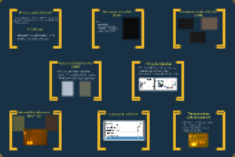
Where? What?

How?

Procedure



Working with DNA



Environment

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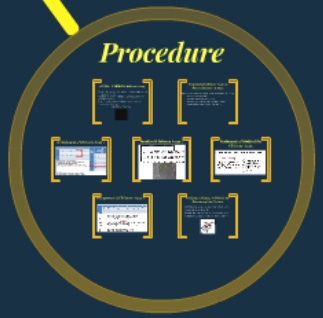

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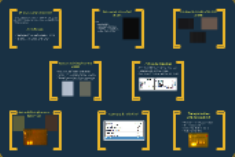


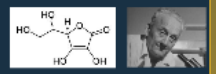
Environment

Probiotic Chitinase Producing Bacteria Engineered Against Crohn's Disease

Where? What?

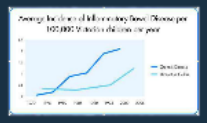
Where Are We From?

Hungary



Szeged

Crohn's Disease



Etiology of Crohn's

- Still unknown
- Foul ways of living
- Smoking
- Genetics

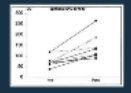
Treating Methods of Crohn's

- Fecal transplantation
- Medications
- Surgery
- Probiotic bacteria
- NAG

Role of NAG in Crohn's Treatment

- Effects of N-acetyl glucosamine (NAG) on patients with inflammatory bowel disease (IBD)
- 8 per 12 cases: NAG had a tissue repairing effect.
- NAG: inexpensive, natural and nontoxic treatment in the future

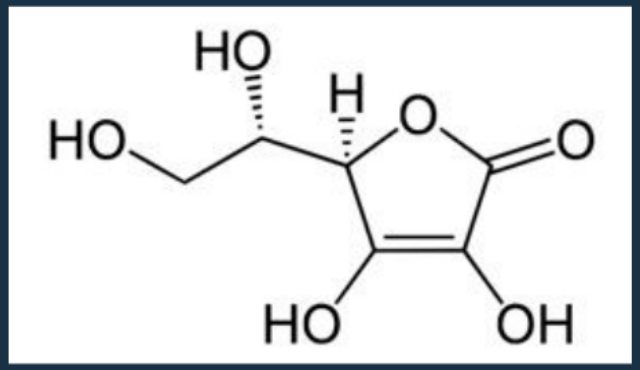
Source: *A pilot study of N-acetyl glucosamine, a nutritional substrate for glycosaminoglycan synthesis, in paediatric chronic inflammatory bowel disease*



Where Are We From?


ECH REPUBLIC
SLOVAKIA
USTRIA
HUNGARY
Szeged
OVENIA
ROM
CROATIA
BOSNIA
SERBIA

Hungary



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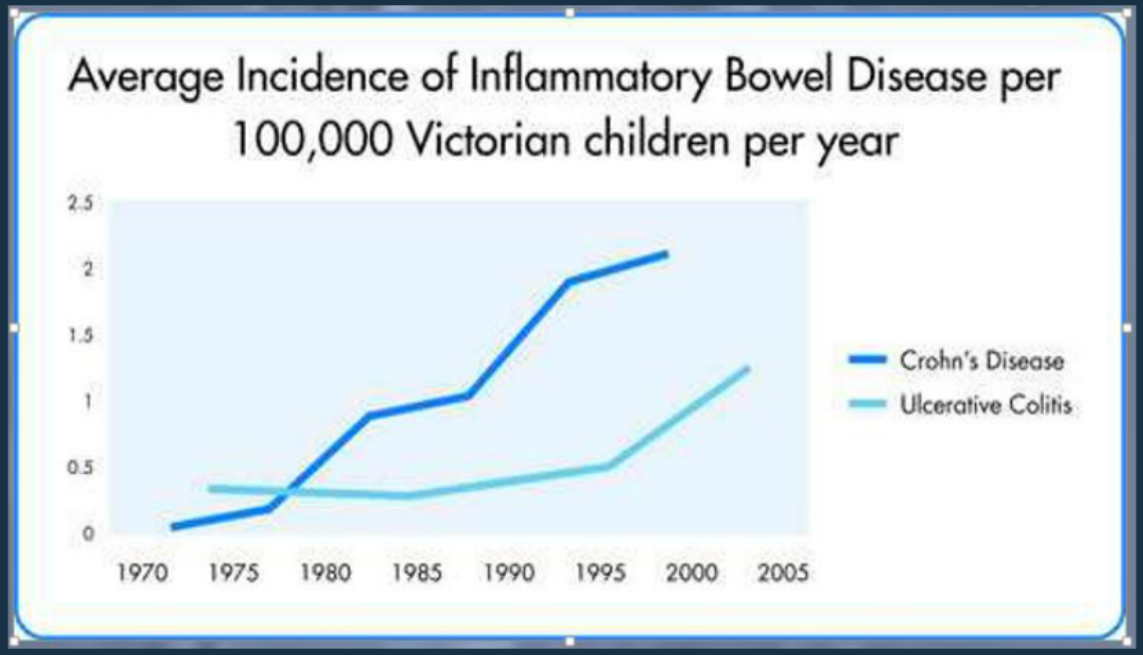

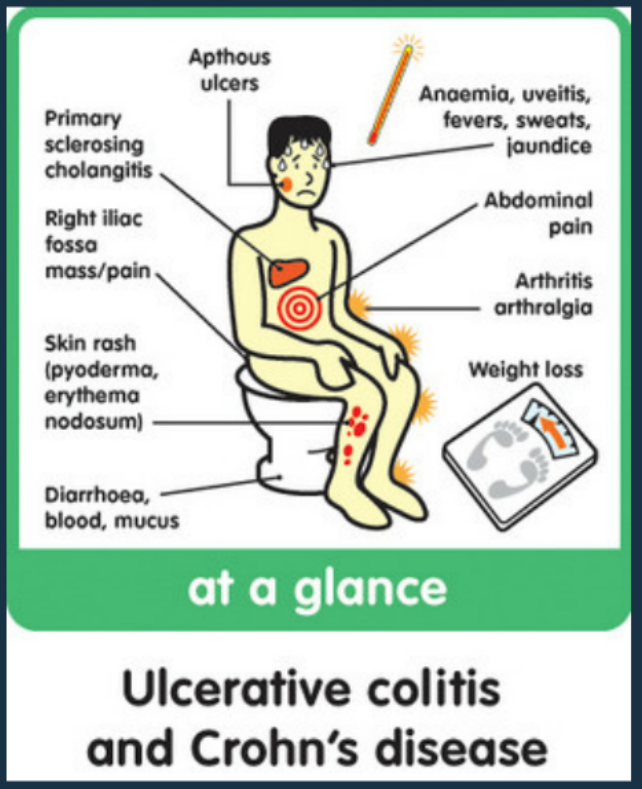

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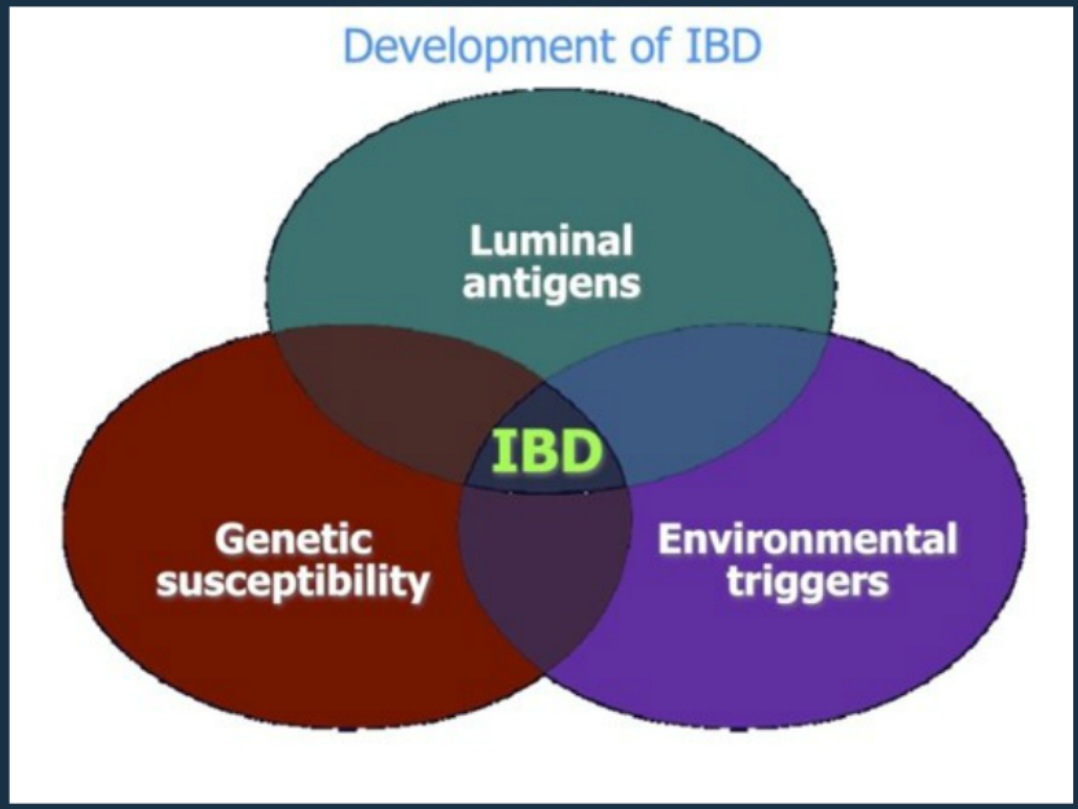

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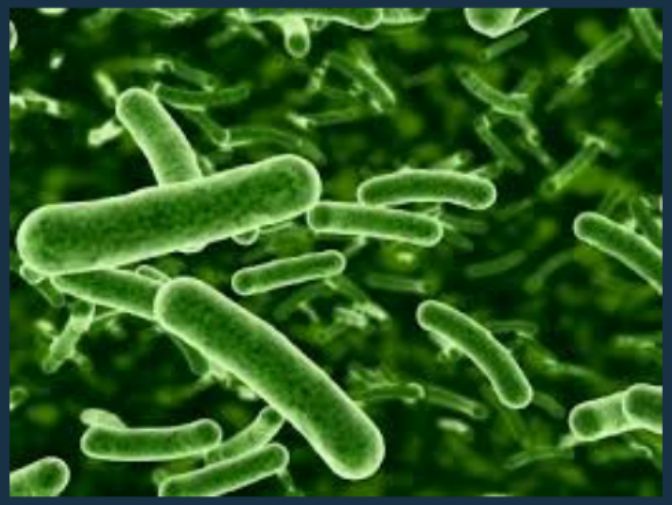

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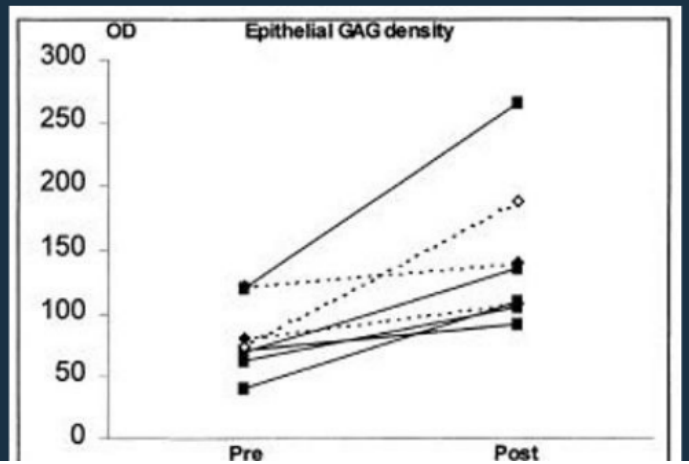



How?
NAG in the Intestinal System
Transferring Probiotic Bacterium Into the Intestines as Therapy
Producing NAG from Chitin in the Intestines, Locally
Combination of the mentioned researches and synthetic biology:
MAKING A CHITINASE-PRODUCING PROBIOTIC BACTERIA AND TRANSFERRING IT INTO THE INTESTINES
What Do We Need for This?
• Chitinase
• Bacteria, which:
- Are easily transformable
- Are probiotic
- Die if they get out into nature
Natural Chitinases
• Chitin is the basic material of fungi, insects, worms and many other organisms
• Due to the presence of chitin in soil, some organism also produce chitinases
• Soil samples were collected
• We have made colloidal chitin medium to examine them
The Problems with Chitinases
• There are more than 30,000 different chitinases in nature
• Chitinases are usually large : 2,500–2,800 bp therefore it is difficult to transform them
Chitinase of Chromobacterium violaceum
Our BioBricks

NAG in the Intestinal System

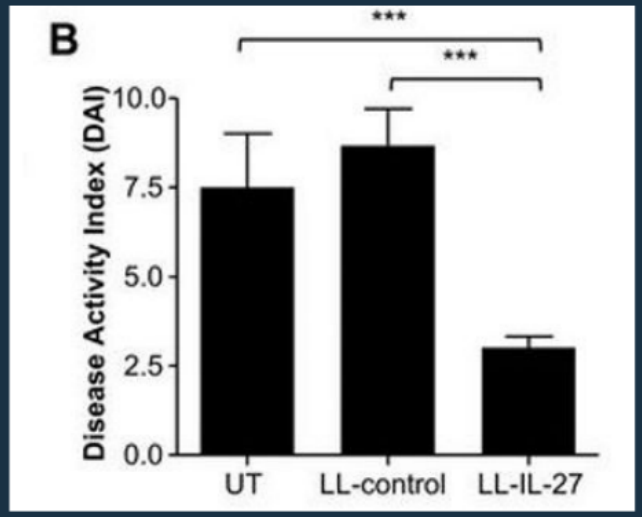
- **Chitinases** have been found in human tissues
- **Their role**: defense against parasite infections, some allergic conditions
- **This works only**
  - In the stomach
  - In the lung
  - In macrophages

Source: *Role of Chitinases in Human Stomach for Chitin Digestion: AMCase in the Gastric Digestion of Chitin and Chit in Gastric Pathologies*

Transferring Probiotic Bacterium Into the Intestines as Therapy

- Transferring genetically modified bacteria into mice's intestines
- IL-27 protein – possible treatment for IBDs
- Treatment of IBD: specific targeting of therapeutics – in the intestines
- Successful: the mice, treated with the modified bacteria showed improvement

Source: *Oral delivery of IL-27 recombinant bacteria attenuates immune colitis in mice*



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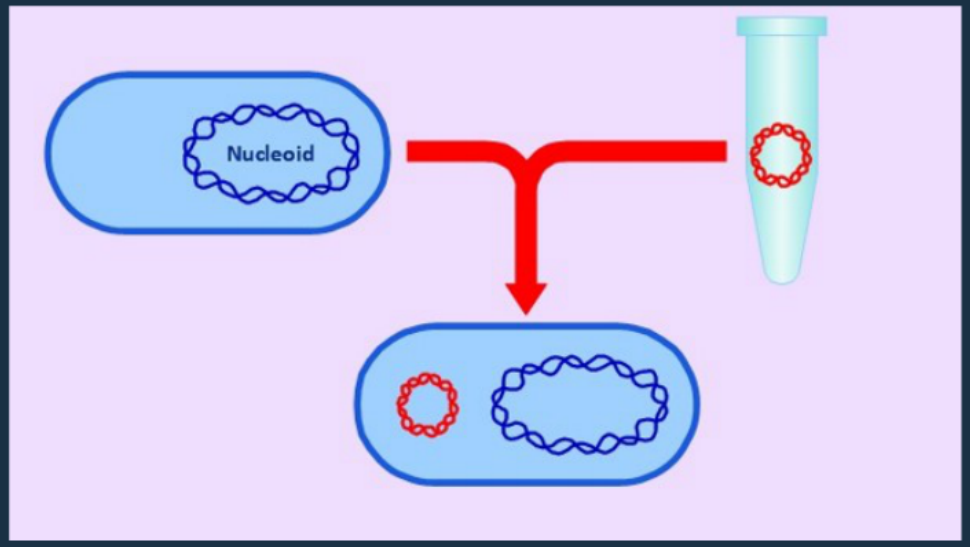

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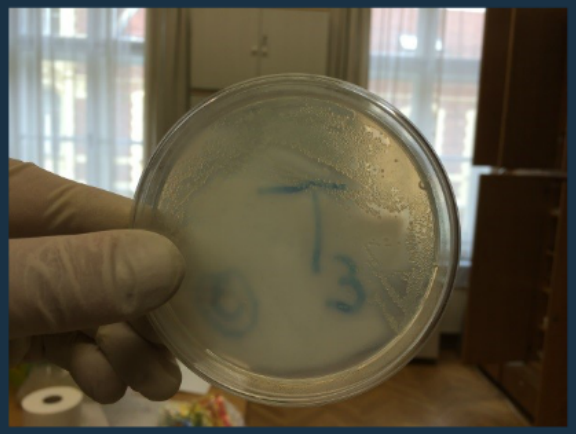
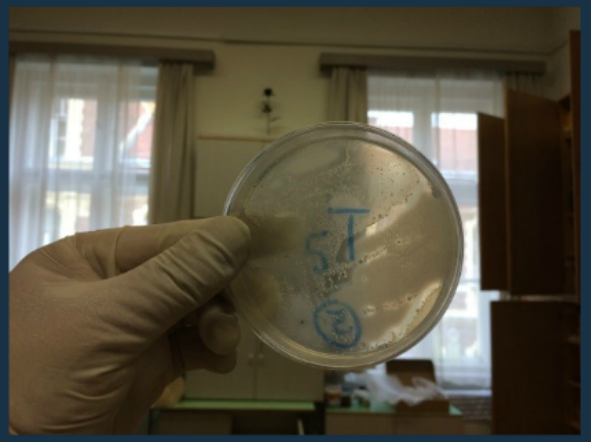




The Problems with Chitinases

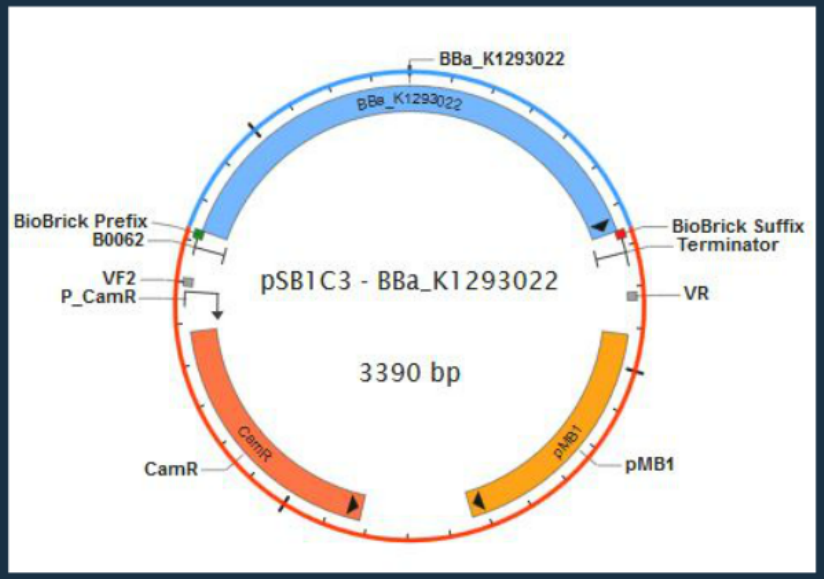
- There are **more than 30,000** different chitinases in nature
- Chitinases are **usually large** : 2,500-2,800 bp therefore it is difficult to transform them

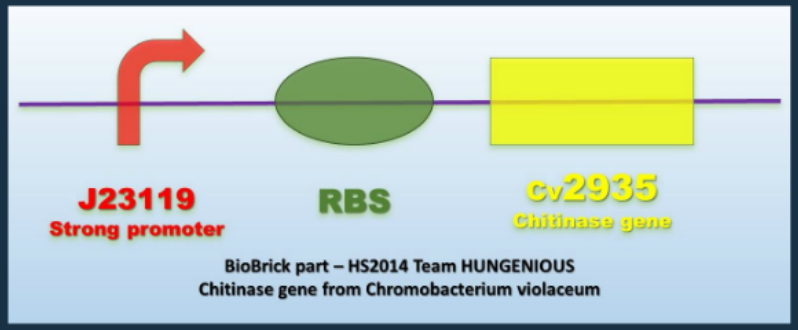
Chitinase of Chromobacterium violaceum

- This chitinase gene isn't too large: 1320 bp
- Goal: making a BioBrick containing the gene CV2935
- Our plan: transform the gene from C. violaceum into E. coli

Source: *Expression and efficient secretion of a functional chitinase from Chromobacterium violaceum in Escherichia coli*

Our BioBricks





Procedure

SIGMA-ALDRICH Chitinase Assay

- Based on the enzymatic hydrolysis of chitinase substrates, it releases p-nitrophenol
- Detection of chitinase activity in bacterial growth media containing chitinase
- Product can be measured colorimetrically at 405 nm
- Beer-Lambert Equation:

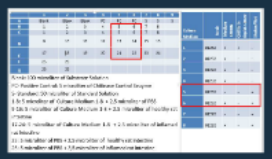
  Absorbance = $\log_{10}(I_0/I)$

Performing Chitinase Assay to Prove Chitinase Activity

We measured different kinds of chitinases with this assay:

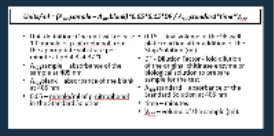
- Bacillus thuringiensis
- Chromobacterium violaceum
- Trichoderma viride (assay control enzyme)
- Natural chitinases from soil samples

Arrangement of Chitinase Assay 1.



Results of Chitinase Assay 1.

Mathematical Model of the Chitinase Assay



Arrangement of Chitinase Assay 2.

Verifying Chitinase Activity in the Presence of Gut Extract

- Rat large intestine extract was added to the bacterial growth media
- Result: the extract has no obstructive, nor stimulating effect on the chitinase activity

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Arrangement of Chitinase Assay 1.

	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

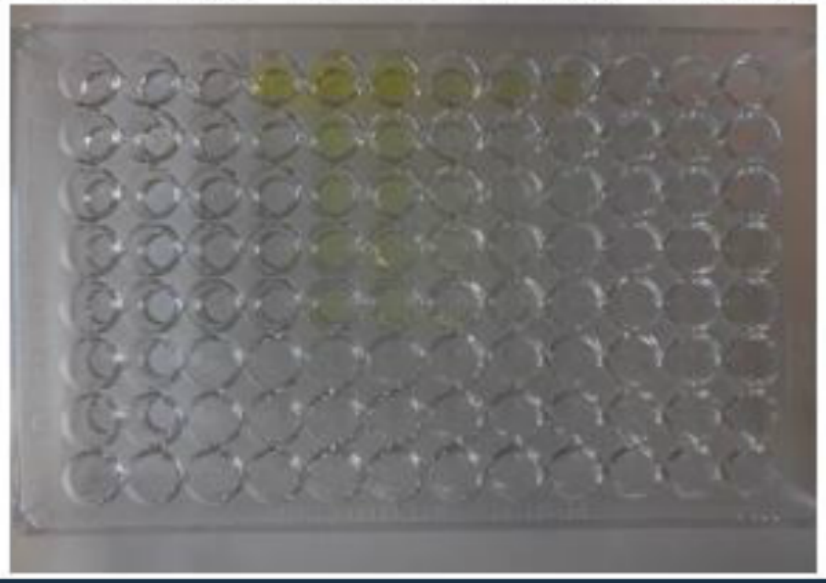
25: 5 microliter of PBS + 2,5 microliter of healthy rat intestine

26: 5 microliter of PBS + 2,5 microliter of inflamed rat intestine

Culture Medium	Strain	Medium CHITIN	CHITIN in liquid culture	Shaken/Not
1	01262	+	+	-
2	01262	+	-	-
3	01292	+	+	-
4	01292	+	-	-
5	01262	-	-	+
6	01262	+	-	+
7	01292	-	-	+
8	01292	+	-	+

Results of Chitinase Assay 1.

	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



Mathematical Model of the Chitinase Assay

$$Units/ml = (A_{405}sample - A_{405}blank)*0.05*0.15*DF / A_{405}standard*time*V_{enz}$$

- Unit definition: One unit will release 1.0 mmole of p-nitrophenol from the appropriate substrate per minute at pH 4.8 at 37 °C.
- A_{405}sample – absorbance of the sample at 405 nm
- A_{405}blank – absorbance of the blank at 405 nm
- 0.05 – mmole/ml of p-nitrophenol in the Standard Solution
- 0.15 – final volume of the 96 well plate reaction after addition of the Stop Solution (ml)
- DF - Dilution Factor - fold dilution of the original chitinase enzyme or biological solution to prepare sample for the test
- A_{405}standard – absorbance of the Standard Solution at 405 nm
- time – minutes
- V_{enz} – volume of the sample (ml).

Arrangement of Chitinase Assay 2.

	1	2	3	4	5	6	7	8	9
A	Blank	Blank	Blank	PC	PC	PC	S	S	S
B	L1.S1	L2.S1	Tr1.S1	Tr1.S1	Tr2.S1	Tr2.S1	Soil1.S1	Soil2.S1	
C	L1.S2	L2.S2	Tr1.S2	Tr1.S2	Tr2.S2	Tr2.S2	Soil1.S2	Soil2.S2	
D	5.S1	5.S2	6.S1	6.S2					

L1: liquid culture of assumed transformed bacteria Nissle
L2: liquid culture of assumed transformed bacteria Nissle
Tr1: transformed Bacillus thuringiensis 01262 sample
Tr2: transformed Bacillus thuringiensis 01292 sample
Soil1: bacteria sample from the bank of river Tisza shown chitinase activity on colloidal medium
Soil2: bacteria sample from flower bed shown chitinase activity on colloidal medium
S1: substrate for measuring exochitinase activity
S2: substrate for measuring endochitinase activity

Verifying Chitinase Activity in the Presence of Gut Extract

- Rat large intestine extract was added to the bacterial growth media
- Result: the extract has no obstructive, nor stimulating effect on the chitinase activity

Working with DNA

The Source of the Target DNA

- We have ordered synthetic DNA from IDT – unsuccessful
- New plan: genomic DNA of Chromobacterium violaceum (DSMZ-Germany)

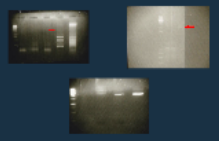
Two Subgroups

- Biological Research Center - BRC
- RMG & University of Szeged

Experiments Carried Out at BRC

PCR
- Gradient PCR
- Annealing temperature
- Amplification of backbone
- Polymerase problems

Checking the Results of the PCR at BRC



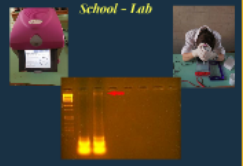
Ligation and Transformation at BRC

- Ligation and transformation
- p7A31+CV2935 into Nissle (E.coli)
- Transformation: electroporation

PCR in the School lab

- PCR for iGEM BioBrick BBa_K293022
- Amplification of CV2935 gene
- Digestion of the gene with PstI & EcoRI

PCR and Electrophoresis in School – Lab

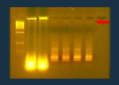


Ligation in the School Lab

Linear DNA	100 ng/ml
Phosphorylated linker	1 µl
10X T4 DNA Ligase buffer	2 µl
50% PEG 4000 solution	2 µl
T4 DNA Ligase	2 µl
Water, nuclease-free	to 20 µl
Total volume	to 20 µl

Transformation with the BioBrick

- We used E. coli DH 5 alpha strain
- Heat – shock method
- out in School Lab (biosafety)
- check: colonial PCR



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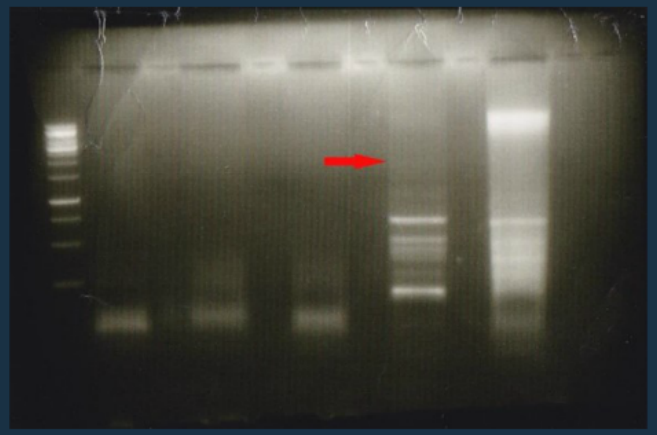

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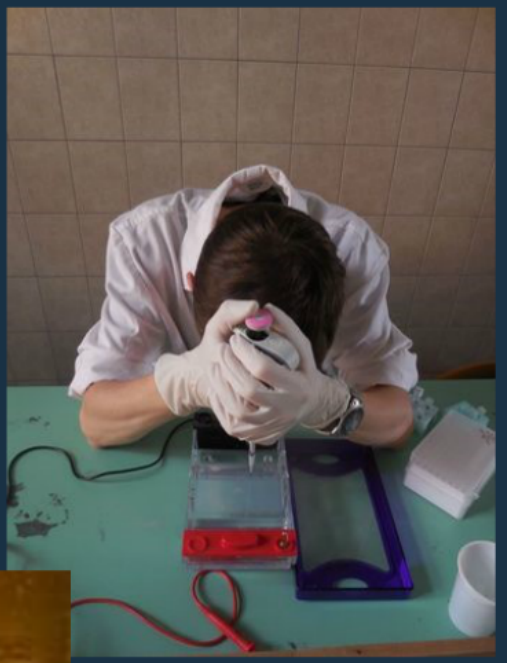
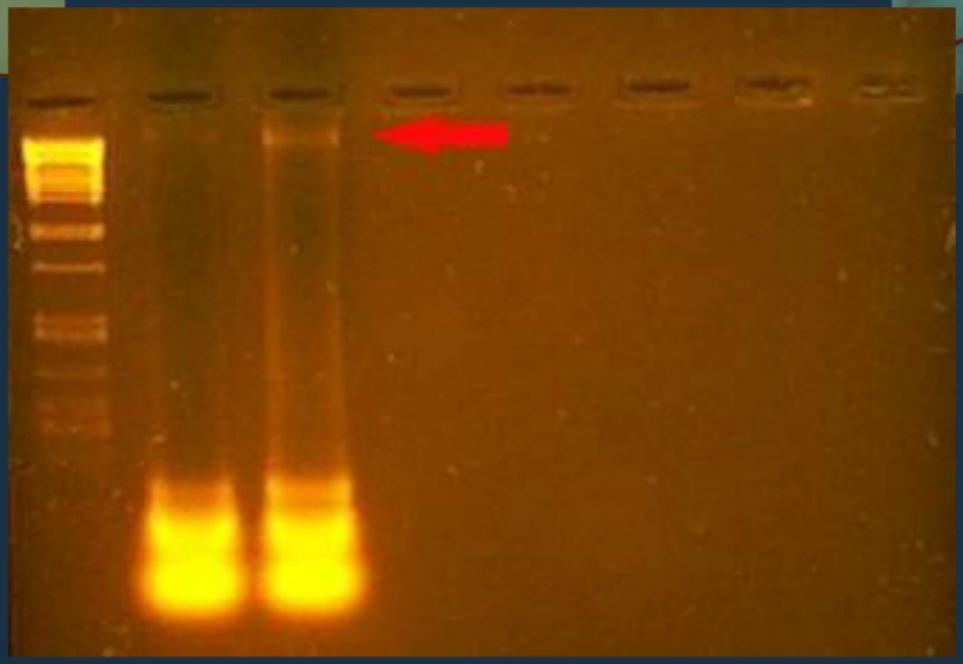
- PCR for iGEM BioBrick BBa_K1293022
- Amplification of CV2935 gene
- Digestion of the gene with PstI & EcoRI

Step	Temperature, °C	Time	Number of cycles
Initial denaturation / enzyme activation	95	4 min	1
Denaturation	95	30 s	25-40
Annealing	Tm-5	30 s	
Extension	72	1 min/kb	
Final Extension	72	5-15 min	1

Maxima Hot Start Green PCR Master Mix (2X)	25 µl
Forward primer	0.1-1.0 µM
Reverse primer	0.1-1.0 µM
Template DNA	10 pg - 1 µg
Water, nuclease-free (#R0581)	to 50 µl
Total volume	50 µl

PCR and Electrophoresis in School - Lab





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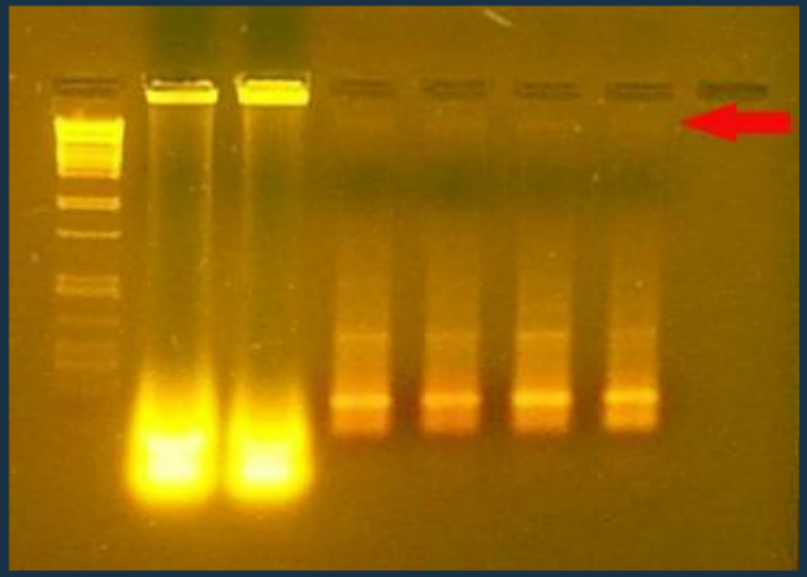


Environment

Biosafety - Experiments

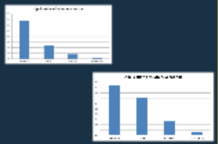
Biosafety - bacteria

- BSL1 level
- Non-living Chromobacterium violaceum
- The product of the gene is not connected with the pathogenic effect

Human practices 1.

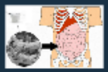
Meeting with medical doctors (e.g.: dr. Klaudia Farkas)
Meeting with other pupils in the school in a reunion with Aaron Chiecanower Nobel Prize winner

Human Practices 2.

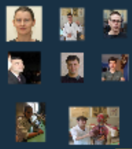


Our Future Plans

- Histological studies proving the effect of NAG
- Transplantation into intestines
- Yoghurt
- Prevention of Environmental risks



Our Team and Mentors



Where Did We Do the Experiments?

- RMG
- BRC
- The Department of Physiology, Anatomy and Neuroscience

Our Sponsors

Thank you for your attention!
Do you have any questions?

Biosafety - Experiments

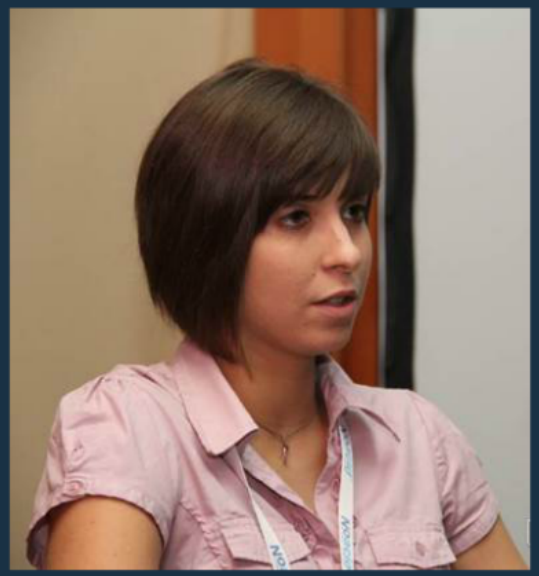
Biosafety - bacteria

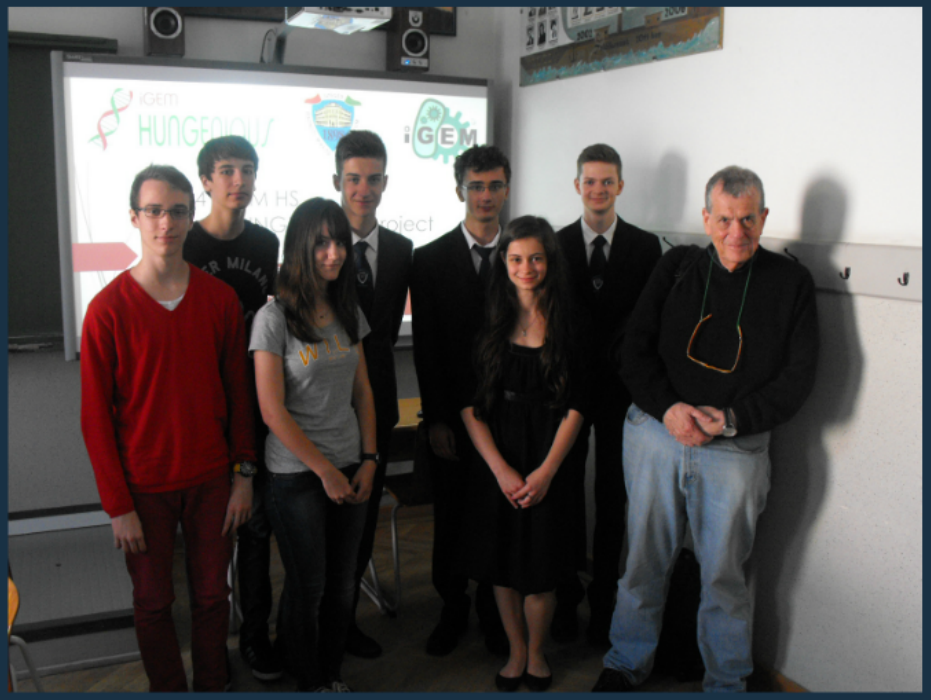
- BSL1 level
- Non-living Chromobacterium violaceum
- The product of the gene is not connected with the pathogenic effect

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

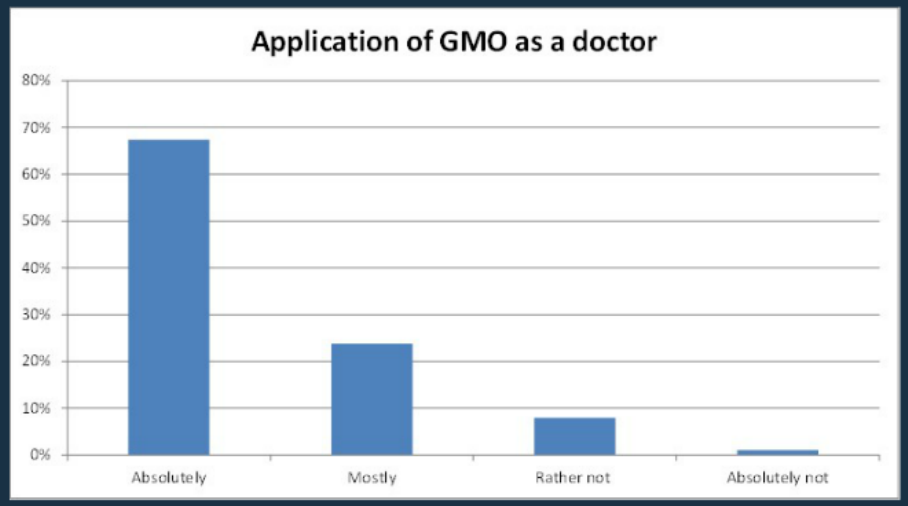
Human practices 1.

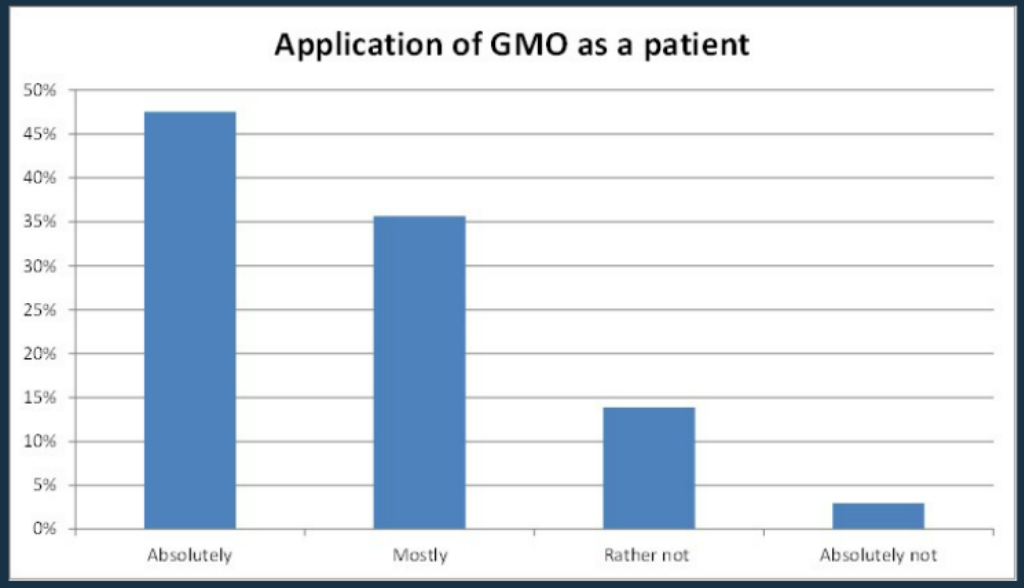
Meeting with medical doctors (e.g.: dr. Klaudia Farkas)
Meeting with other pupils in the school in a reunion with Aaron Chiecanover Nobel Prize winner





Human Practices 2.





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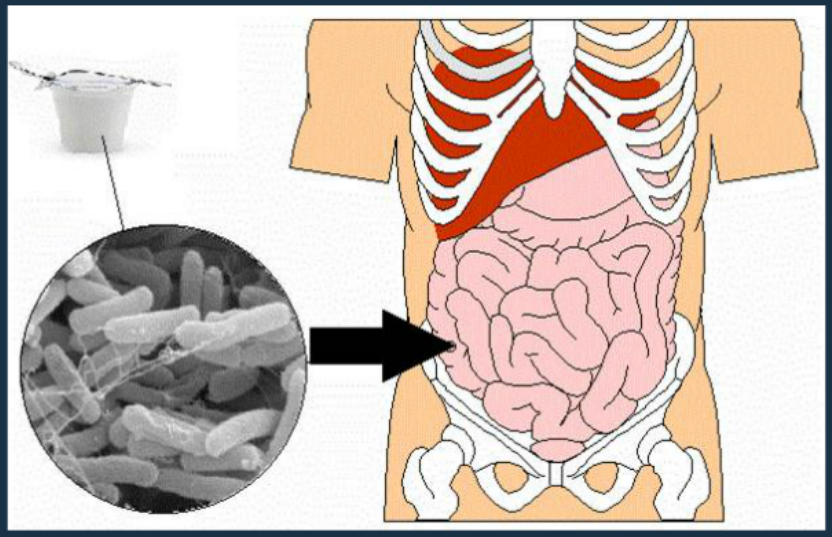

Our Team and Mentors

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