





*Probiotic Chitinase
Producing Bacteria
Engineered Against
Crohn's Disease*

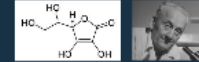


Where? What?

Where Are We From?



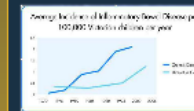
Hungary



Szeged

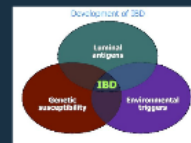


Crohn's Disease



Etiology of Crohn's

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



Treating Methods of Crohn's



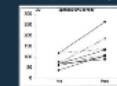
- Fecal transplantation
- Medications
- Surgery
- Probiotic bacteria
- NAG



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

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



Where Are We From?



CZECH REPUBLIC

SLOVAKIA

AUSTRIA

HUNGARY

Szeged

SVENIA

ROMANIA

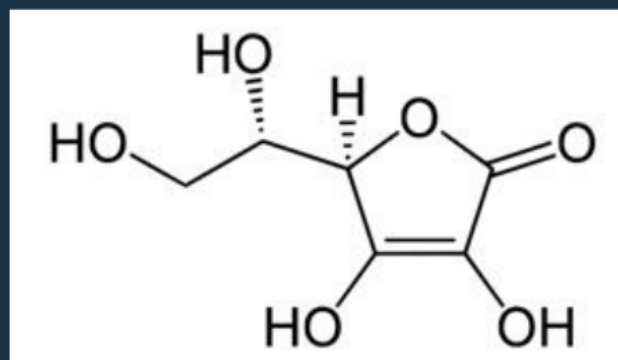
CROATIA

BOSNIA

SERBIA



Hungary

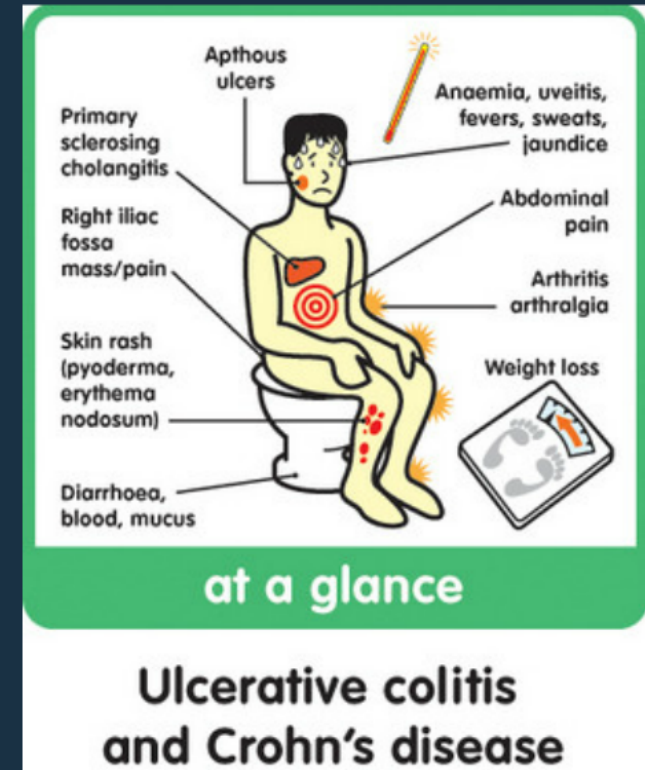
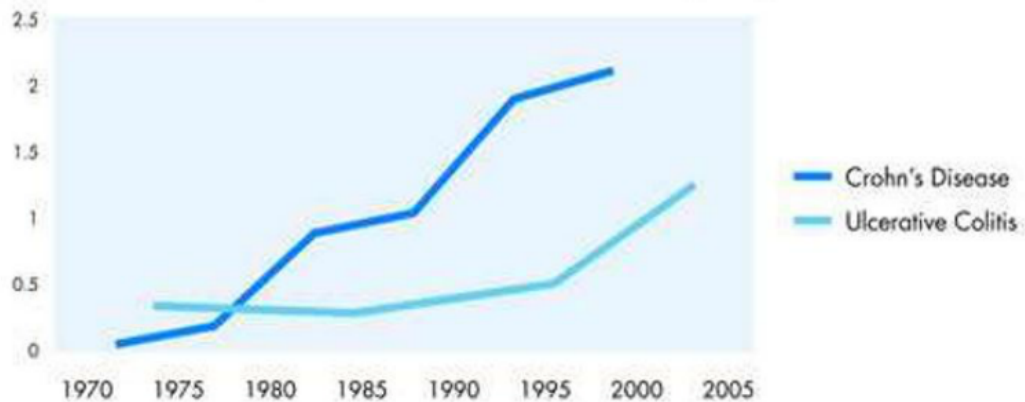


Szeged



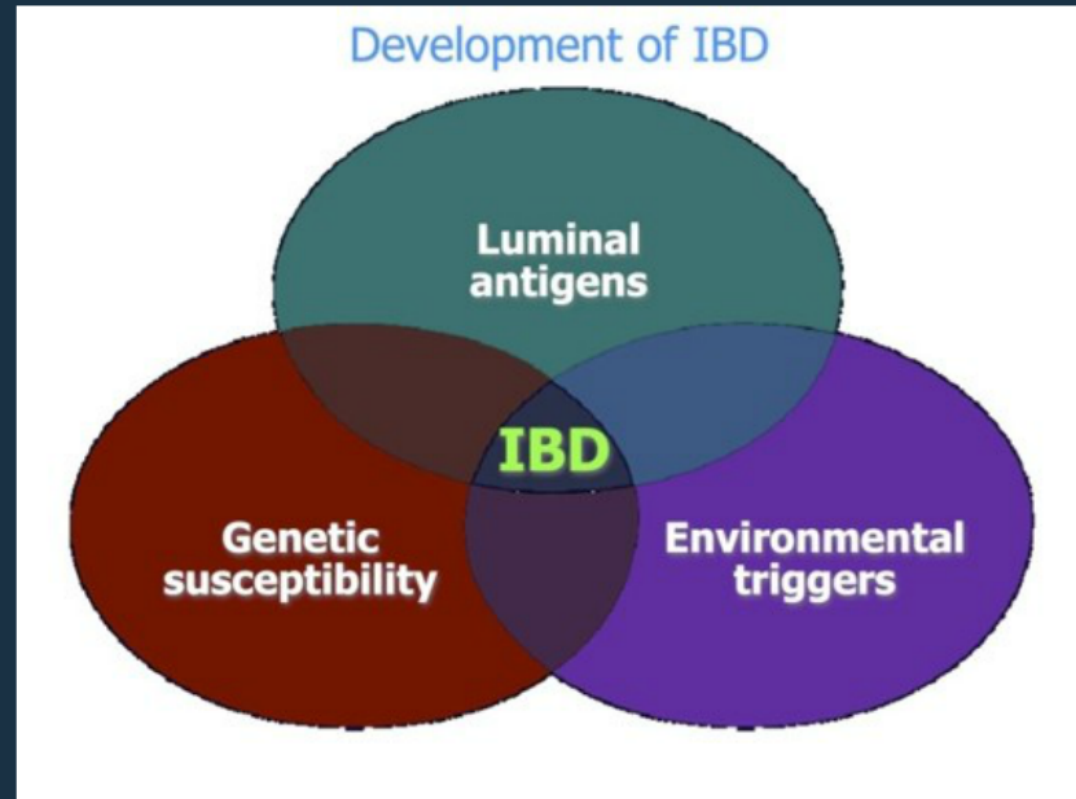
Crohn's Disease

Average Incidence of Inflammatory Bowel Disease per 100,000 Victorian children per year

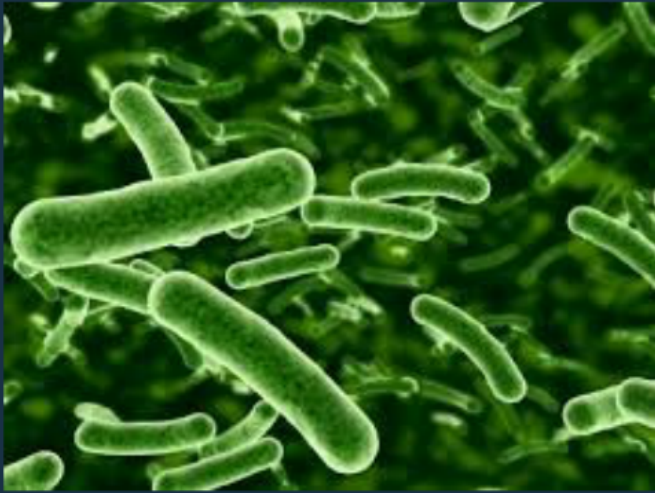


Etiology of Crohn's

- Still unknown
- Foul ways of living
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Treating Methods of Crohn's



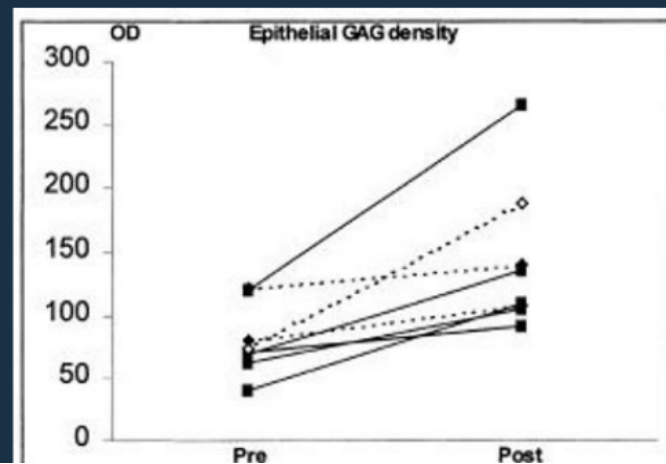
- Fecal transplantation
 - Medications
 - Surgery
 - Probiotic bacteria
 - NAG



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*



How?

NAG in the Intestinal System

- Chitinases (also known as NAGase) are found in all vertebrates
- They are the primary enzymes responsible for the breakdown of chitin in the digestive tract
- They are also found in the gut of many invertebrates
- They are also found in the gut of many plants

Source: *W. G. "NAGase" in *Encyclopedia of Food and Nutrition*, 2nd ed., pp. 100-101, © 2004, John Wiley & Sons, Inc.*

Transferring Probiotic Bacterium Into the Intestines as Therapy

- Probiotic bacteria are used to treat various conditions
- They are used to treat the gut and the immune system
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Source: *Probiotic Bacteria: A Practical Approach*, pp. 100-101, © 2004, John Wiley & Sons, Inc.



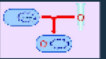
Producing NAG from Chitin in the Intestines, Locally

Combination of the mentioned receptors 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
 - Live if they get out from culture



Natural Chitinases

- Chitin is the basic material of fungi, insects, worms and many other organisms
- Due to the presence of chitin in soil, some organisms also produce chitinases
- Soil samples were collected
- We have made natural chitin medium to examine them



The Problems with Chitinases

- There are more than 100,000 different chitinases in nature
- Chitinases are usually large: 2,500 - 2,800 bp, therefore it is difficult to transform them

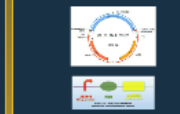


Chitinase of *Chromobacterium violaceum*

- This chitinase gene is 1,100 bp
- Gene is located in the *Chromobacterium violaceum* genome
- We have transformed the gene into *E. coli*

Source: *Expression and optimal secretion of a bacterial chitinase from *Chromobacterium violaceum* in *Escherichia coli**

Our BioBricks



NAG in the Intestinal System

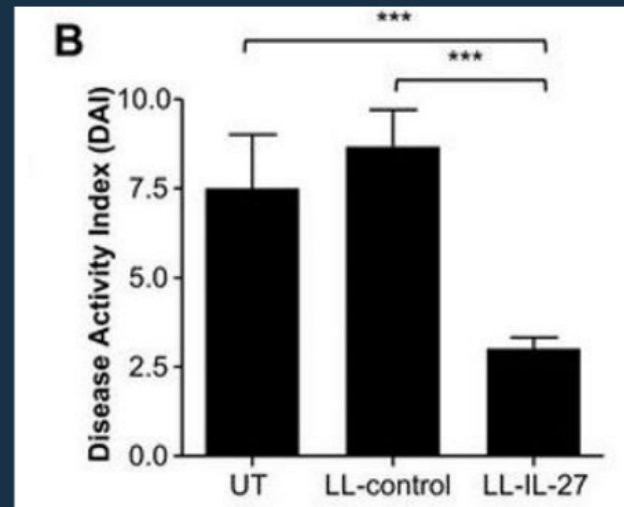
- **Chitinases** have been found in human tissues
- **Their role:** defense against parasite infections, some allergic conditions
- **This works only**
 - In the stomach
 - In the lung
 - In macrophages

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*



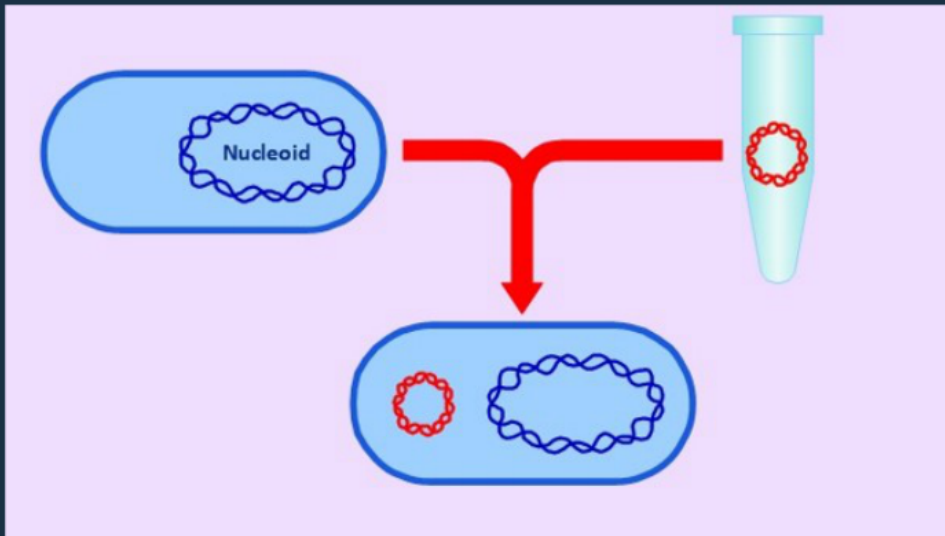
Producing NAG from Chitin in the Intestines, Locally

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



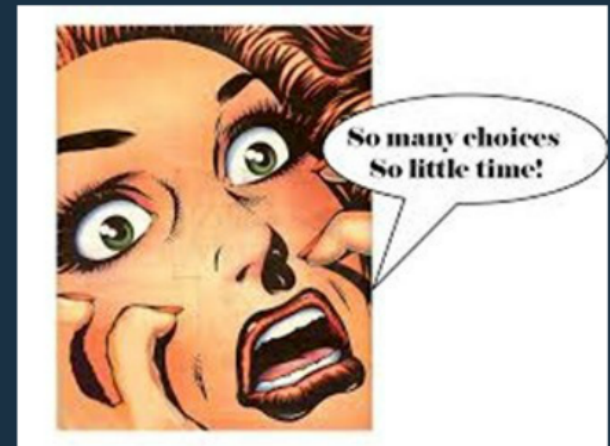
Natural Chitinases

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The Problems with Chitinases

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- Chitinases are **usually large** : 2,500–2,800 bp therefore it is difficult to transform them

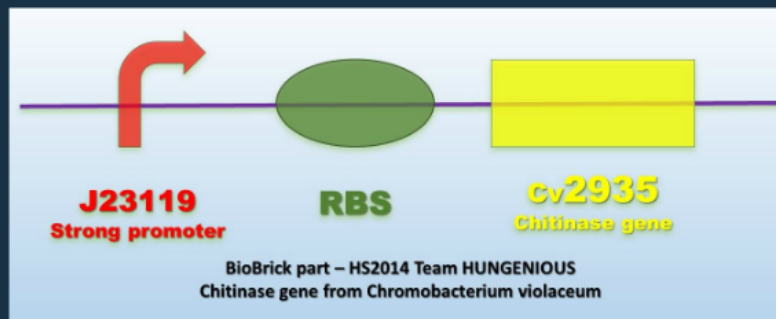
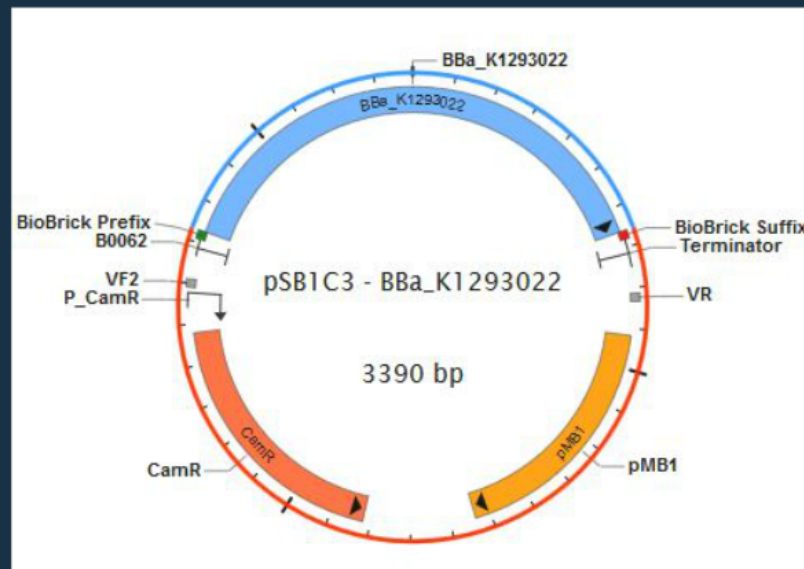


Chitinase of Chromobacterium violaceum

- 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.

Well	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	100 µl of 10% chitinase	100 µl of 10% chitinase	100 µl of 10% chitinase	100 µl of 10% chitinase	100 µl of 10% chitinase	100 µl of 10% chitinase	100 µl of 10% chitinase	100 µl of 10% chitinase	100 µl of 10% chitinase	100 µl of 10% chitinase	100 µl of 10% chitinase	100 µl of 10% chitinase	100 µl of 10% chitinase	100 µl of 10% chitinase	100 µl of 10% chitinase	100 µl of 10% chitinase	100 µl of 10% chitinase	100 µl of 10% chitinase	100 µl of 10% chitinase	100 µl of 10% chitinase

Results of Chitinase Assay 1.

Well	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Mathematical Model of the Chitinase Assay

- Mathematical model of the chitinase assay is based on the Beer-Lambert law, which relates the absorbance of a solution to its concentration and the path length of the cuvette.
- The Beer-Lambert law is expressed as: $A = \epsilon \cdot c \cdot l$, where A is the absorbance, ϵ is the molar absorptivity, c is the concentration, and l is the path length.
- In this assay, the absorbance is measured at 405 nm, and the path length is 1 cm.
- The concentration of the chitinase is determined by comparing the absorbance of the sample to that of a standard solution.

Arrangement of Chitinase Assay 2.

Well	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	100 µl of 10% chitinase	100 µl of 10% chitinase	100 µl of 10% chitinase	100 µl of 10% chitinase	100 µl of 10% chitinase	100 µl of 10% chitinase	100 µl of 10% chitinase	100 µl of 10% chitinase	100 µl of 10% chitinase	100 µl of 10% chitinase	100 µl of 10% chitinase	100 µl of 10% chitinase	100 µl of 10% chitinase	100 µl of 10% chitinase	100 µl of 10% chitinase	100 µl of 10% chitinase	100 µl of 10% chitinase	100 µl of 10% chitinase	100 µl of 10% chitinase	100 µl of 10% chitinase

Verifying Chitinase Activity in the Presence of Gut Extract

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



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:

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



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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.

	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

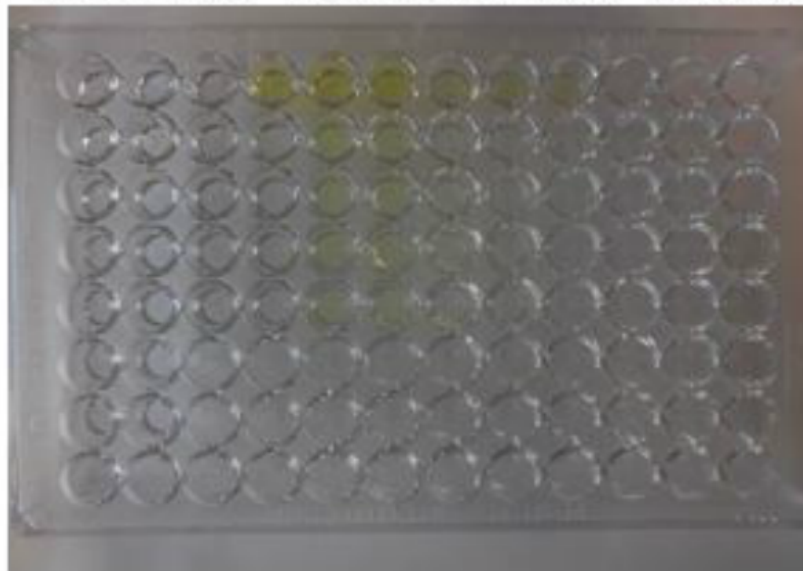
25: 5 microliter of PBS + 2,5 microliter of healthy rat intestine

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

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

- 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.
- $A_{405\text{sample}}$ – absorbance of the sample at 405 nm
- $A_{405\text{blank}}$ – 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
- $A_{405\text{standard}}$ – 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

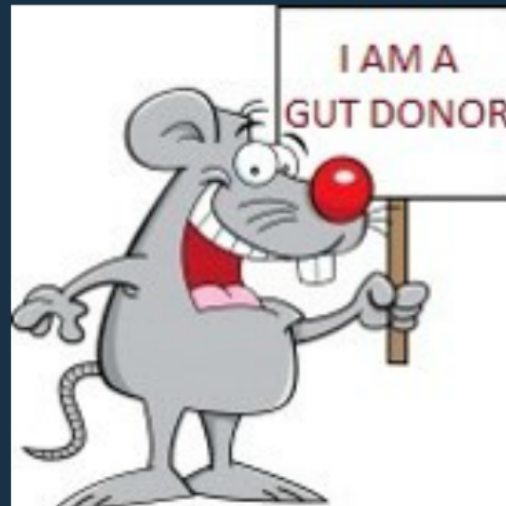
Soil2: bacteria sample from flower bed shown chitinase activity on colloidal medium

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 BCC – unsuccessful
- New plant genome DNA of *Chenopodium album* (DNA2-1-acromy)

Two Subgroups

- Biological Research Center – BRC
- RMG @ University of Szeged

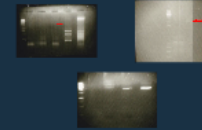
Experiments Carried Out at BRC

PCR

- Gradient PCR
- Annealing temperature
- Amplification of trehalose
- Polymerase proofing

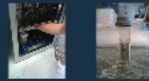


Checking the Results of the PCR at BRC



Ligation and Transformation at BRC

- Ligation and transformation
- pZAp1-CV-3975 into Nissle (*E. coli*)
- Transformation: electroporation



PCR in the School Lab

- PCR for iGEM BioBrick BBa_K109022
- Amplification of CV2975 gene
- Digestion of the gene with PstI & EcoRI

Run	Temp	Time	Volume	Final Volume	Final Conc.
1	95	1:00	20	20	1.00
2	55	0:30	20	20	1.00
3	72	1:00	20	20	1.00
4	72	5:00	20	20	1.00
5	72	1:00	20	20	1.00
6	72	1:00	20	20	1.00
7	72	1:00	20	20	1.00
8	72	1:00	20	20	1.00
9	72	1:00	20	20	1.00
10	72	1:00	20	20	1.00
11	72	1:00	20	20	1.00
12	72	1:00	20	20	1.00
13	72	1:00	20	20	1.00
14	72	1:00	20	20	1.00
15	72	1:00	20	20	1.00
16	72	1:00	20	20	1.00
17	72	1:00	20	20	1.00
18	72	1:00	20	20	1.00
19	72	1:00	20	20	1.00
20	72	1:00	20	20	1.00

PCR and Electrophoresis in School - Lab

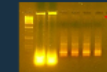


Ligation in the School Lab

Volume	Final Volume
100 µl DNA Ligase	100 µl
100 µl DNA Ligase Buffer	200 µl
50 µl Pfu 4000 units	250 µl
10 µl DNA	260 µl
Water, nuclease-free	300 µl
Total volume	300 µl

Transformation with the BioBrick

- We used *E. coli* DH5 alpha strain
- First – shreck method
- not in School Lab (Biosafety)
- check: colonial PCR



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

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- RMG & University of Szeged

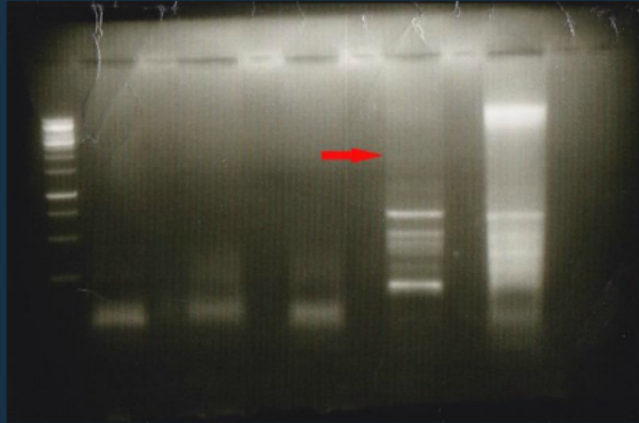
Experiments Carried Out at BRC

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
- pZA31+CV2935 into Nissle (E.coli)
- Transformation: electroporation



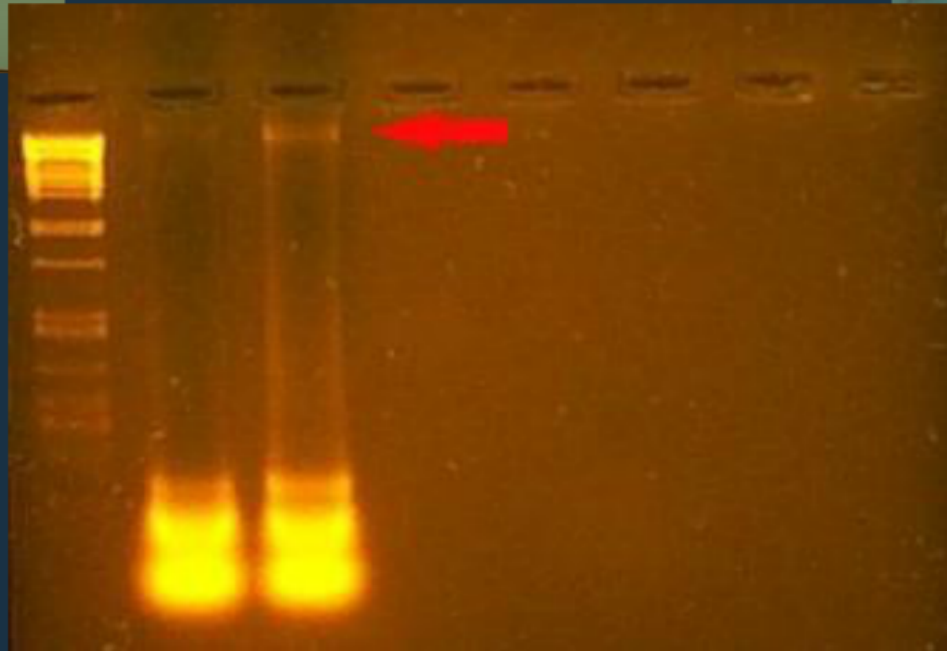
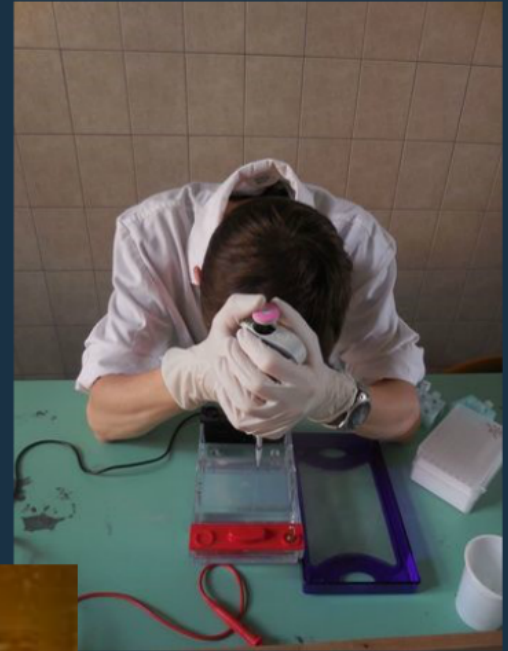
PCR in the School lab

- 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

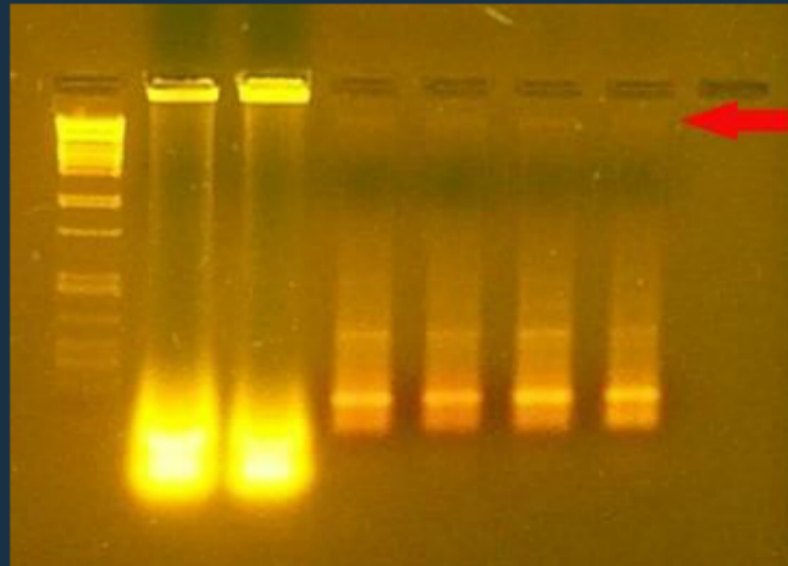


Ligation in the School Lab

Linear DNA	100-500 ng
Phosphorylated linkers	1-2 μg
10X T4 DNA Ligase buffer	2 μl
50% PEG 4000 solution	2 μl
T4 DNA Ligase	2 u
Water, nuclease-free	to 20 μl
Total volume	to 20 μl

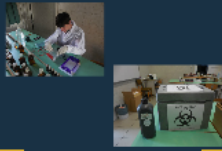
Transformation with the BioBrick

- We used E. coli DH 5 alpha strain
- Heat - shock method
- not in School Lab (biosafety)
- check: colonial PCR



Environment

Biosafety - Experiments



Biosafety - bacteria

- BSL-1 level
- Non-pathogenic *Chromobacterium violaceum*
- The product of the gene is not connected with the pathogenic effect

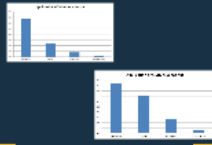
Gene	Product	Function	Location	Reference
...
...
...
...

Human practices 1.

Meeting with medical doctors (e.g. dr. Almuta Parkas)
Meeting with other people in the school (discussion with Aureus Christa and Václav Petrášek)

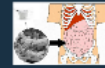


Human Practices 2.



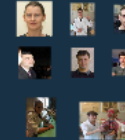
Our Future Plans

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



Our Team and Mentors

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Where Did We Do the Experiments?



- IMG
- 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

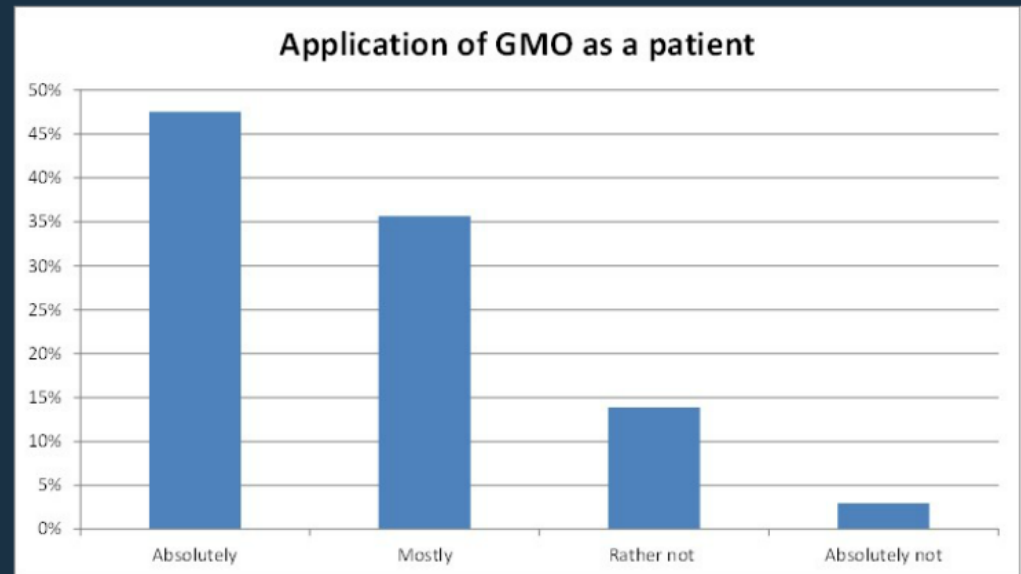
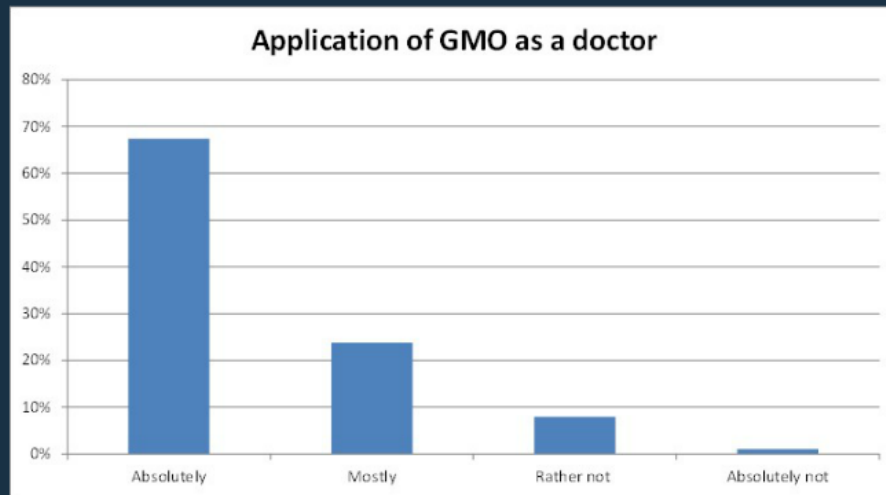
Name	DNA /living cells	Biosafety Level	ATCC No.	Source	Used for
<i>Chromobacterium violaceum</i>	DNA-part	BSL2	12472	DSMZ	amplification template of chitinase gene
<i>Escherichia coli</i> DH5 alpha http://ecoliwiki.net/colipedia/index.php/DH5_alpha	living cells	BSL1	67878	BRC	transformation
<i>Escherichia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

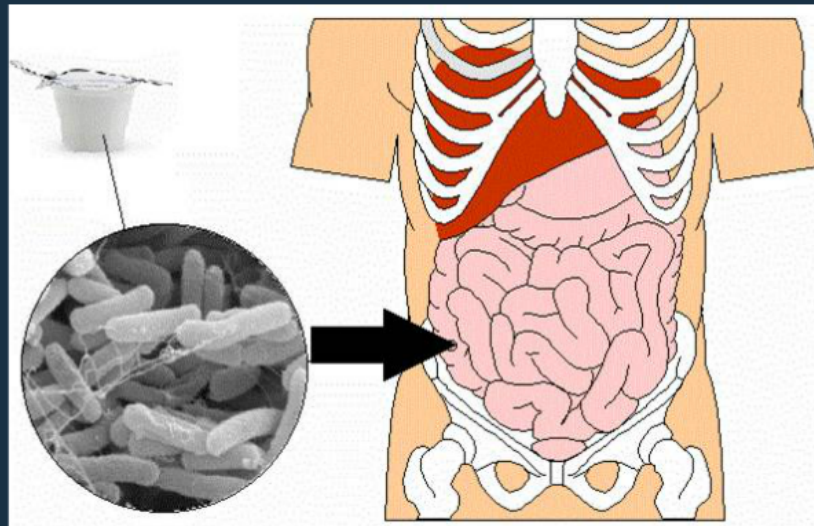


Human Practices 2.



Our Future Plans

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



Our Team and Mentors

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Where Did We Do the Experiments?



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

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EMBERI ERŐFORRÁSOK
MINISZTERISÉGE



*Thank you for your
attention!*

*Do you have any
questions?*