Construction of bacterial biosensor of interferon gamma based on *Escherichia coli* cells

Tomasz Buko[1], Daniel Grygorowicz[2], Jakub Jóźwik[2], Olga Kowalska[2], Igor Lipiński[2], Adrian Macion[3], Dorota Malicka[4], Weronika Pyter[5], Aleksandra Radziszewska[2], Wiktoria Szymanek[2], Tola Tame[6], Nella Tuczko[5]

1. Department of Molecular Biology, Faculty of Biology, University of Warsaw, Poland
2. Synthetic Biology "Genesis" Students' Society, Faculty of Biology, University of Warsaw, Poland
3. Medical University of Warsaw, Poland
4. Centre of New Technologies, University of Warsaw, Poland
5. Department of Environmental Microbiology and Biotechnology, Faculty of Biology, University of Warsaw, Poland
6. Institute of Genetics and Biotechnology, Faculty of Biology, University of Warsaw, Poland

The authors declare that they have no conflict of interest.

List of abbreviations

BFP – blue fluorescence protein
IFN-γ - interferon gamma
IGRA - interferon gamma release assays
IPTG - Isopropyl β-d-1-thiogalactopyranoside
LTBI - latent tuberculosis infection
OD600 – optical density at 600nm
psp - phage shock protein
RBS - ribosome binding site
TB - tuberculosis

Introduction

"Genesis" is a Polish team competing in the Global Open Genetic Engineering Competition 2023. The team consists of 12 students, 9 of whom were undergraduate students when the project started. All team members belong to the Synthetic Biology "Genesis" Students' Society affiliated with the Department of Biology, University of Warsaw. Genesis's project aims to find a cost-effective, accessible, and simple alternative to present latent tuberculosis infection tests.

Even though the cause of tuberculosis was identified for the first time in 1882 [1], some studies indicate that the *Mycobacterium tuberculosis* complex existed by the human side for at least 15,000 years [1, 2]. TB usually affects the lungs but can spread to other weakened

parts of the human body and has a very high mortality rate when untreated. In fact, before the COVID-19 pandemic, TB was the main reason for death from a single infectious agent [1]. Although the peak of infections falls in Europe and North America in the 17th and 18th centuries, the disease remains a major issue in low-income populations. The newest rapid molecular TB tests detect a particular sequence of nucleic acids and can simultaneously identify antibiotic resistance of the infecting strain [1, 3].

However, the standard evidence of TB is not always manifested even though the organism is infected by *M. tuberculosis*. This state is called latent tuberculosis infection, and despite the asymptomatic form, it can potentially lead to the development of an active disease later in life [4]. The World Health Organization still advises using tuberculin skin tests or interferon gamma release assays to screen risk groups for LTBI [5]. However, the use of both of these methods is associated with limitations such as the low efficiency of distinguishing between LTBI and active disease and the reproducibility of these methods. That being the case, population-wide examinations for LTBI are currently unavailable. Our work aims to address this issue by proposing a way of constructing a bacterial biosensor of IFN-γ based on *Escherichia coli* cells.

Biosensors are analytical instruments that use biological components to detect chemical compounds or physical phenomena. The biological element distinguishes biosensors from classic measuring equipment, characterized by lower sensitivity, specificity, longer reading generation time, and higher production cost than biosensors. The chassis organism, i.e., the basis of the biosensor, can be any type of cell, but the most popular are bacterial cells, primarily *E. coli*.

E. coli is a model organism that represents gram-negative bacteria belonging to *Enterobacteriales*. Its advantages include a small genome, a multitude and availability of non-pathogenic laboratory strains, and easy cultivation. *E. coli* is also one of synthetic biology's most important chassis organisms. Extensive libraries of genetic parts and mathematical models have been designed for this bacterium, including the Register of Standard Biological Parts [6, 7].

Interferons are a group of signaling proteins belonging to cytokines that naturally occur in humans. They play an essential role in regulating the immune response against bacteria, cancer cells, and viruses. Interferon gamma has a pleiotropic effect on the human body, including participation in macrophage activation, antibacterial response, antigen presentation, and apoptosis regulation. As stated above, the level of IFN-γ is the basis of IGRA immunological tests, supplementing the tuberculin skin test method. The biosensor of IFN-γ based on *E.coli* may be a method potentially facilitating the diagnosis of LTBI.

The proposed biosensor is based on the chimeric protein OmpA/OprF, i.e., IfnS, which has the ability to bind IFN-γ. OmpA is one of the best-known and evolutionarily conserved proteins of the outer cell membrane of gram-negative bacteria [8, 9]. Its structure is relatively plastic, and its fragments can retain their conformation even after deleting some loops, which enables a simple creation of chimeric proteins [10]. OprF is an orthologous molecule to OmpA that can bind human IFN-γ. Due to the high similarity of the secondary structure of OmpA and OprF, the chimeric protein formed from them can be anchored in the *E. coli* membrane. By swapping selected OmpA loops outside the bacteria to analogous fragments

of OprF, responsible for binding IFN-γ, the chimeric IfnS protein can be obtained. The signal transduction generated by the binding of the IFN-γ molecule to the IfnS protein is mediated by the phage shock protein system [10], and the transcription of genes encoding PpspABCDE is activated. In the case of the described biosensor, a reporter gene has been placed under the pspA promoter, generating an easy-to-read signal. The ifnS gene in *E. coli* cells is linked to the Ptet-rbs system - originally the promoter and RBS of the tetracycline resistance gene. Such a system ensures a sufficiently high protein expression, which determines the efficiency of the biosensor. The transcription termination element is the double terminator BBa_B0015, which is widely used in synthetic biology. The reporter gene is bfp with RBS BBa_B0034 and a terminator. BFP synthesis is regulated by the pspA promoter.

While designing our project, it came to our attention that a very similar biosensor had already been constructed in 2016 [10]. However, it is not used in medical practice and has not been prepared using standard synthetic biology parts. Therefore we present our proposition of constructing a bacterial biosensor of IFN-γ based on *E. coli* cells using standard synthetic biology parts, which can improve design, development, and testing processes. Moreover, design based on standard synthetic biology parts is easier to replicate by other scientists and to scale-up for mass production.

Project goals

1. Assembly of the receptor system
    a. Obtaining a sufficient amount of material in the form of biobrick
        - Will it be necessary to isolate material from more than 3 ml of culture?
    b. The specific occurrence of the PCR reaction leading to the formation of the backbone
        - Will optimization based on changes in template concentration and polymerase operating temperature be necessary?
    c. High transformation efficiency
        - Will more transformation material be required?

2. Assessing promoters' strength
    a. High activity of the pspA and tetracycline promoters
        - Will it be necessary to consider changing the promoters of the reporter and receiver circuits?
    b. The standard deviation of the measurements will not be too high
        - Was the procedure followed correctly by adding the accurate amounts of reagents?
3. Assembly of the receptor system
    a. Obtaining a sufficient amount of material in the form of biobrick
        - Will it be necessary to isolate material from more than 3 ml of culture?
    b. The specific occurrence of the PCR reaction leading to the formation of the backbone
        - Will optimization based on changes in template concentration and polymerase operating temperature be necessary?
    c. High transformation efficiency
        - Will more transformation material be required?

4. Evaluation of biosensor operation
    a. No errors in the construct sequence
    b. Will the applied doses of the inducer enable drawing sound conclusions about the activity?
        - Will reduced or increased doses of the inducer be required?
        - The system will not detect sufficiently low concentrations of IFN-γ

Materials and methods

Organisms

Escherichia coli:

- TOP10F'
  F'[lacIq Tn10 (TetR)] mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL(StrR) endA1 nupG λ-
- K12
  Wild type

Antibiotics:

- ampicillin - 100 µg/ml (Sigma-Aldrich)
- chloramphenicol - 25 µg/ml (Sigma-Aldrich)
- kanamycin - 30 µg/ml (Sigma-Aldrich)
- tetracycline - 10 µg/ml (Sigma-Aldrich)

Cultivation of bacterial strains

Strains that are the source of plasmids containing the appropriate biobricks will be cultured in 25 ml of liquid LB medium supplemented with an antibiotic according to the plasmid resistance at 37°C with overnight shaking at 120 rpm. Recipient strains and strains used to amplify the tetracycline and lactose promoters will be grown under the same conditions.

After transformation by the rubidium-calcium method, bacteria will be cultured on a solid LB medium (1,5% agar) supplemented with an antibiotic according to backbone resistance for 24 hours at 37°C. After this time, a streaking of 4 selected colonies will be performed to confirm the positive results of the transformation and purity of the culture.

Plasmid isolation

Plasmids will be isolated from a 3 ml overnight bacterial culture using a commercially available EurX kit according to the manufacturer's instructions. The isolation efficiency will be checked by measuring the DNA concentration.

Biobricks isolation

In order to isolate individual biobricks, the NEB Biobrick Assembly Kit will be used, and the procedure will be carried out according to the manufacturer's instructions. The procedure will be assessed by 1-2% gel electrophoresis, which will enable assessing the digestion product length.
Reagent volumes according to the protocol:

Upstream Part Plasmid:	500 ng
EcoRI-HF:	1 µl
SpeI:	1 µl
10X NEBuffer 2.1:	5 µl
H2O:	to 50 µl

Downstream Part Plasmid:	500 ng
XbaI:	1 µl
PstI:	1 µl
10X NEBuffer 2.1:	5 µl
H2O:	to 50 µl

Destination Plasmid DNA:	500 ng
EcoRI-HF:	1 µl
PstI:	1 µl
10X NEBuffer 2.1:	5 µl
H2O:	to 50 µl

Preparation of backbones

A plasmid backbone with a resistance different from that of the plasmids carrying the specified biobricks will be amplified by PCR with Taq A&A Biotechnology polymerase and reaction mix with primers complementary for the suffix and prefix (orientation 5' ->3'):

Lprefix GAATTCGCGGCCGCTTCTAG
Psuffix CTGCAGCGGCCGCTACTAGTA

Ingredients:

- Template DNA - 10 ng
- Primers - 0.5 µM each
- PCR MIX 25 µl
- Water up to 50 µl

Reaction conditions:

Temperature	Time [min]	Repeats
95°C	2:00	x1
95°C	**0:30**	**X 37**
55°C	**0:30**	
68°C	**3:00***	
68°C	10:00	x1
10°C	inf	

*can be extended or shortened if needed.

After the reaction, the template will be digested with the DpnI enzyme for 90 minutes at 30°C, after which the DNA concentration will be measured. The procedure will be assessed by 1-2% gel electrophoresis, which will enable assessment of the reaction specificity.

Ligation – 3A assembly

The ligation mixture will be assembled according to the manufacturer's instructions using the NEB Biobrick Assembly Kit. After ligation, 1-2% gel electrophoresis will be done to assess product length.

The sequence of procedures for ligation biological parts:

1. BBA_B1006 (terminator - T), bfp
2. BBa_B0034 (rbs), promoter PJ23
3. BBa_B0034, promoter pspA
4. BBa_B0034, lactose promoter
5. BBa_B0034, tetracycline promoter
6. Each promoter-rbs, bfp-T
7. ifnS-TT (double terminator), tetracycline promoter-rbs
8. Tetracycline promoter-rbs-ifnS-TT, PpspA-bfp-T

Competence and transformation

The strain of *E. coli* TOP10F' or K12, in the case of compatibility of genomic resistance and the introduced plasmid, will be qualified by the rubidium-calcium method according to the following procedure [11]:

1. Rejuvenate the overnight *E. coli* culture and then incubate with shaking at 37°C until it reaches the exponential growth phase (approximately 1.5 hours).
2. Centrifuge 1.5 ml of the culture in an Eppendorf tube at 4°C, 12 thousand. rpm, 10 min.
3. Remove the medium, add 1.5 ml of the culture back to the tube, and centrifuge as above.
4. Remove the supernatant thoroughly, suspend the bacterial pellet in 1 ml of Solution I, and then centrifuge as above.
5. Carefully remove the supernatant and suspend the resulting pellet in 0.2 ml of Solution II.
6. Incubate the suspended bacteria for 10-15 minutes on ice.
7. Add 3 µl of plasmid DNA.
8. Incubate the bacterial mixture for 50 seconds at 43°C (heat shock).
9. Quickly add 1 ml of LB medium and incubate for about 30 min at 37°C without shaking.
10. The mixture of cells should be concentrated by centrifugation - the obtained pellet should be suspended in 100 µl LB and plated on plates with a selection medium.
11. Incubate for 24 hours at 37°C.

Solution I 10mM MOPS pH 7.0, 10mM RbCl
Solution II 100 mM MOPS pH 6.5, 50 mMCaCl-2, 10 mM RbCl

As a positive control, a whole isolated plasmid will be used. A negative control will be performed using water instead of plasmid material.

Multiplication of the lactose and tetracycline promoter

The template for the PCR reaction allowing the multiplication of the lactose promoter will be a small amount of bacterial colony collected from the solid medium. The PCR reaction will be performed with primers allowing specific amplification of the lactose promoter. In addition, thanks to the 5' overhangs, suffix and prefix sequences will be added to the promoter.

Primer sequences (orientation 5'->3'):

- Pplac1004 GGAATTCGCGGCCGCTTCTAGACAATACGCAAACCGCCTCTC
- Lplac1004 GGCTGCAGCGGCCGCTACTAGTAATCATGGTCATAGCTGTTTC

An analogous reaction will be performed to obtain the tetracycline promoter. The template will be plasmid PSB1T3. Primer sequences will also have an overhanging suffix and prefix (orientation 5' ->3'):

- BB_Ptet_F GTTTCTTCGAATTCGCGGCCGCTTCTAGAGAGATTCTCATGTTTGACAGCTTA
- BB_Ptet_R GTTTCTTCCTGCAGCGGCCGCTACTAGTAACACGGTGCCTGACTGCG

The reaction template will be digested using DpnI (90 minutes, 37°C). After digestion, the concentration of DNA will be measured. The specificity of the PCR reaction will be assessed by 1-2% gel electrophoresis.

Evaluation of the strength of promoters

The bacterial culture will be inoculated in an LB liquid medium and grown overnight. Overnight cultures will be rejuvenated and brought to OD600 = 0.4. Inducible promoters will be induced by the addition of appropriate compounds:

- Plac – IPTG 0,3 mM
- PpspA – ethanol 5 and 10%

PJ23 and tetracycline promoters as constitutive ones will not be induced. The pspA promoter will be induced by the addition of ethanol. After the incubation period, the level of BFP fluorescence will be measured.

Fluorescence measurement

A 96-well plate reader will be used for cell fluorescence measurements. 200 µl of the culture after induction will be transferred to the well. A sterile, liquid LB medium will be used as a control. Fluorescence measurements will be carried out at the excitation wavelength λ=381 and the emission wavelength λ=445. There will be 16 readings per well taken from the bottom of the clear plate, and there will be 3 technical replicates for each sample. Then, the average will be calculated from the obtained results.

Biosensor activity assessment

Cultures will be diluted to an OD600 of 0.05 with fresh LB medium and incubated at 30°C until OD600 of 0.2 prior to adding IFN-γ [10].

Culture response to IFN-γ will be examined at a concentration of 0,1 to 1 nM and, after preliminary tests, at higher concentrations up to 2.5 nM. All cultures will be incubated for 90

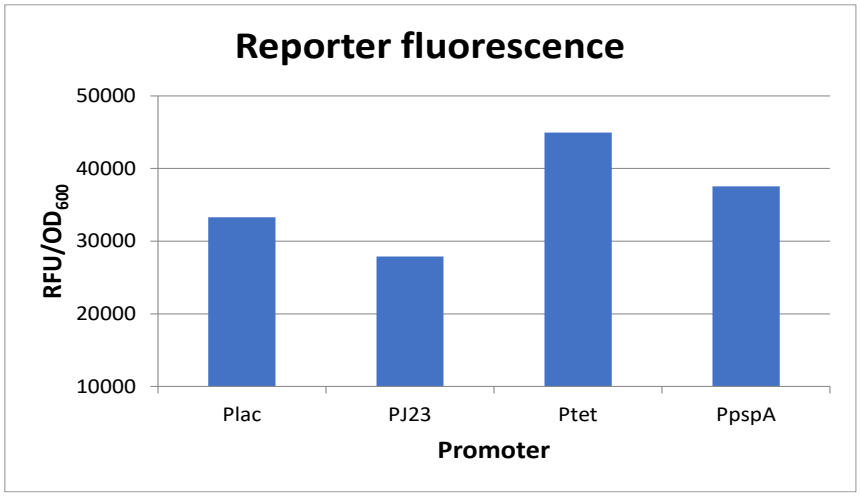
min at 30°C post-induction. After that time, the fluorescence of BFP will be measured as described in the *Fluorescence measurement* section.

Expected results

Cultivation of bacterial strains

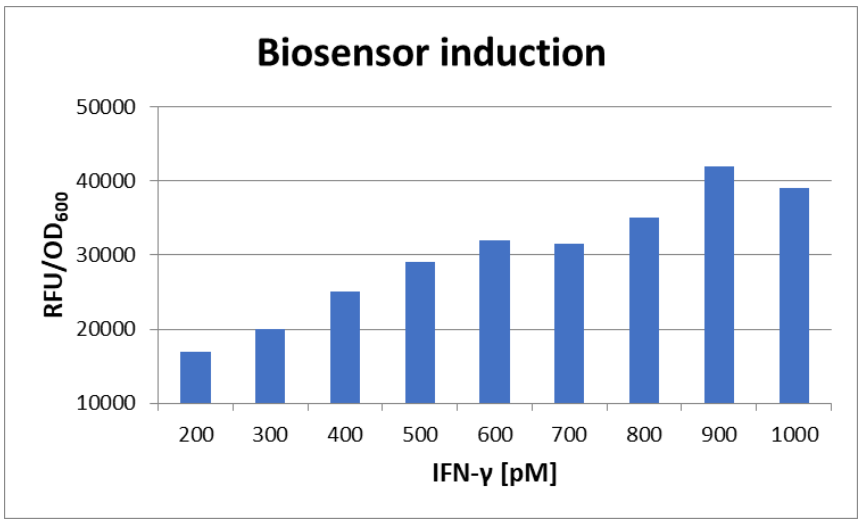
After all transformation procedures, we expect at least 15 colonies per plate. After streaking, we expect that all colonies will grow.

Evaluation of the strength of promoters



After performing BFP fluorescence measurements to assess promoter strength, we expect the highest fluorescence levels to be noted for the pspA and tetracycline promoters. Lower fluorescence levels may be noted for the lactose and PJ23 promoters since they are not as strong.

Biosensor activity



We expect a steady rise in the level of fluorescence as the inducing agent increases. The concentration of 1nM should not cause saturation of the ifnS receptor, so in later assays, an increase in the level of fluorescence should be observed.

Biosafety and biosecurity

The constructed biosensor will have to be tested and optimized using IFN-γ protein, preferably human. Working with factors that regulate the human immune system always generates potential risks, like bacteria developing new, previously unknown methods of responding to those factors or accidentally leaking them to the environment. Therefore all wet-lab experiments should be performed in the spirit of the biorisk management culture and by researchers who underwent safety training, for example, the one offered by the University of Warsaw at the very beginning of Bachelor's studies. It is also possible to use mice's interferon gamma, which may, unfortunately, lead to a slight decrease in the effectiveness of optimization.

Another potential issue is that the OprF protein, one of the elements that IfnS consists of, is a virulence factor in *Pseudomonas aeruginosa*. However, only small fragments of the OprF responsible for binding IFN-γ are used to assemble IfnS. Therefore, properly isolating those fragments without transferring *P. aeruginosa* cells should eliminate this issue altogether.

In principle, the proposed biosensor will be used among low-income communities. That being the case, the risk of environmental contamination with modified *E. coli* cells is relatively high when the device is not handled correctly. Tests using this biosensor should be performed by trained medical practitioners who understand the risks of leaking modified bacteria into the environment.

Conclusions

Although there are many promising, theoretical indications that the bacterial biosensor of interferon gamma based on *E. coli* cells could become a suitable alternative for standard LTBI testing, experimental studies are crucial to obtain a definitive answer. Naturally, the biosensor constructed in the described way should be extensively tested and optimized to evaluate its sensitivity, reliability, and, therefore, the value of its practical use. Preparing and implementing this technology will be a long, demanding, but exciting process that may contribute to the quality of latent tuberculosis infection testing.

References

[1] Global tuberculosis report 2022. Geneva: World Health Organization; 2022. License: CC BY-NC-SA 3.0 IGO

[2] Hershkovitz, I., Donoghue, H. D., Minnikin, D. E., May, H., Lee, O. Y., Feldman, M., Galili, E., Spigelman, M., Rothschild, B. M., & Bar-Gal, G. K. (2015). Tuberculosis origin: The Neolithic scenario. Tuberculosis (Edinburgh, Scotland), 95 Suppl 1, S122–S126. https://doi.org/10.1016/j.tube.2015.02.021

[3] WHO consolidated guidelines on tuberculosis. Module 3: diagnosis - rapid diagnostics for tuberculosis detection, 2021 update. Geneva: World Health Organization; 2021. License: CC BY-NC-SA 3.0 IGO.

[4] Narasimhan, P., Wood, J., Macintyre, C. R., & Mathai, D. (2013). Risk factors for tuberculosis. Pulmonary medicine, 2013, 828939. https://doi.org/10.1155/2013/828939

[5] Latent tuberculosis infection: updated and consolidated guidelines for programmatic management. Geneva: World Health Organization; 2018. License: CC BY-NC-SA 3.0 IGO.

[6] Guo, M., Wang, J., Du, R., Liu, Y., Chi, J., He, X., Huang, K., Luo, Y., & Xu, W. (2020). A test strip platform based on a whole-cell microbial biosensor for simultaneous on-site detection of total inorganic mercury pollutants in cosmetics without the need for predigestion. Biosensors & bioelectronics, 150, 111899. https://doi.org/10.1016/j.bios.2019.111899

[7] Na, D., Lee, S., & Lee, D. (2010). Mathematical modeling of translation initiation for the estimation of its efficiency to computationally design mRNA sequences with desired expression levels in prokaryotes. BMC systems biology, 4, 71. https://doi.org/10.1186/1752-0509-4-71

[8] Krishnan, S., & Prasadarao, N. V. (2012). Outer membrane protein A and OprF: versatile roles in Gram-negative bacterial infections. The FEBS journal, 279(6), 919–931. https://doi.org/10.1111/j.1742-4658.2012.08482.x

[9] Wang Y. (2002). The function of OmpA in *Escherichia coli*. Biochemical and biophysical research communications, 292(2), 396–401. https://doi.org/10.1006/bbrc.2002.6657

[10] Aurand, T. C., & March, J. C. (2016). Development of a synthetic receptor protein for sensing inflammatory mediators interferon-γ and tumor necrosis factor-α. Biotechnology and bioengineering, 113(3), 492–500. https://doi.org/10.1002/bit.25832

[11] Kushner, S. R. (1978). An improved method for transformation of *Escherichia coli* with ColE1-derived plasmids (Vol. 173). Elsevier Amsterdam.