

Synthetic Biology – Friend or Foe? What Kind of Threats Should We Expect?

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Synthetic biology is a newly emerging branch of dual-use technology. It is a combination of biology and different branches of engineering. *The aim of this article* is to show the main technological methods of synthetic biology and to give specific examples of its use to create new types of biological agents and methods of biological warfare, previously unthinkable and presented only in science fiction. Basic tools and techniques of synthetic biology are: DNA synthesis and DNA sequencing; «chassis», i.e. host system harboring the genetic toolbox for expression of the desired genes, delivered by suitable vectors, of the engineered biological pathway; engineering of transcription systems that do not deplete the resources of the cell (synthetic promoters and transcription factors); genome modification tools (CRISPR/Cas9 nuclease, zinc finger nucleases, TALE nucleases, meganucleases); computer-aided tools (involved in basic structural design and synthesis; in network design; in prediction of behavior/function/response). Synthetic biology has already demonstrated its capabilities in re-creating known pathogenic viruses and pathogenic bacteria; in making existing pathogenic bacteria and viruses more dangerous for humans; in creating new pathogens; in manufacturing toxic chemicals or biochemicals by exploiting natural and artificial metabolic pathways; in making toxic chemicals and biochemicals via in situ synthesis; in modifying the human microbiome; in modifying the human immune system; in modifying the human genome (through addition, deletion, or modification of genes or through epigenetic changes that modify gene expression and can pass from parent to child during reproduction and thus spread a genetic change through the population over time). The article discusses in detail the possibilities of synthetic biology for the development of new means of biological warfare. The author believes that it is necessary not only to constantly monitor these new dual-use biotechnologies, but also to improve traditional and scientific methods of their monitoring.

Keywords: synthetic biology; threat; pathogen; bacteria; virus; human.

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Synthetic biology is a combination of biology and different branches of engineering, such as electrical, mathematical, mechanical, and computer science. It provides a greater ability of understanding and manipulation of the biological systems or creation of novel life forms [1]. Synthetic biology intends to (re)construct novel artificial biological systems, which can maintain the integrative complexity of central dogma of molecular biology¹ in

a rational and translational manner for the efficient production of desired biomolecules beneficial to the society. Synthetic biology as a scientific direction is based on certain mechanistic ideas about the nature of living organisms. According to these ideas, living organisms can be assembled from blocks of nucleotide sequences encoding certain functions, just like toys are assembled from LEGO bricks. The foundation of synthetic biology was

¹ Central dogma of molecular biology. URL: https://en.wikipedia.org/wiki/Central_dogma_of_molecular_biology (date: 20.01.2021).

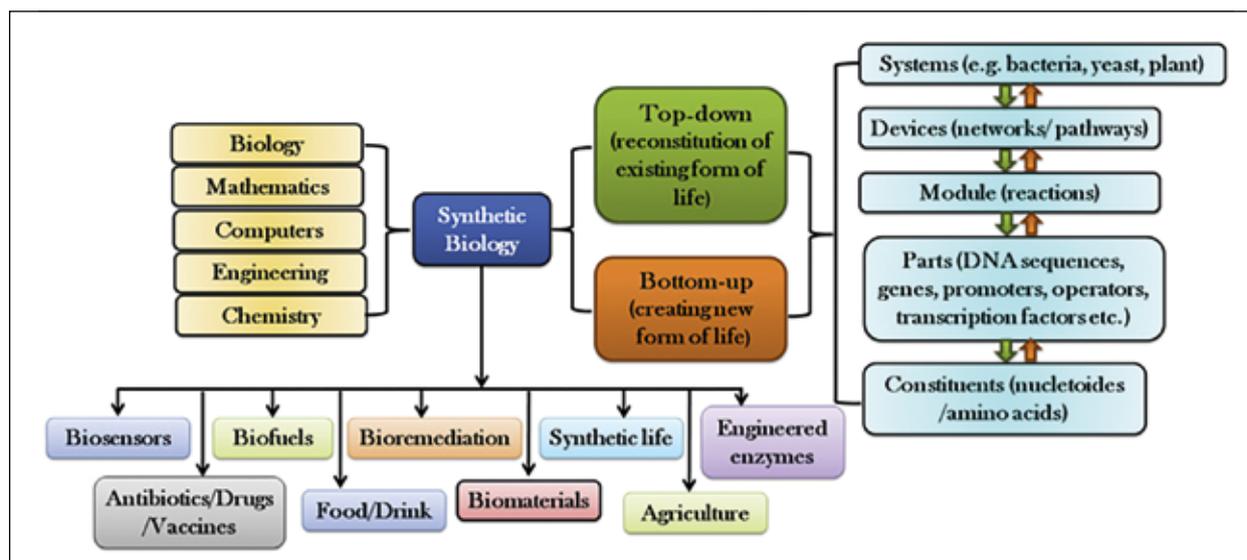


Figure 1 – An overview of synthetic biology showing the combination of different associated fields (copied from [2])

laid by medieval works, dedicated to the attempts to create the homunculus, by Van Helmont's (1580–1644)² recipe for spontaneous generation of mice from dirty laundry, by the O.B. Lepeshinskaya's (1871–1963) theory of «living matter» and by other teachings about the «spontaneous generation of the living»³. Attempts to mechanize («synthesize») life ended always with a fiasco and up to date did more harm than good. The aim of this article is to show the main technological methods of synthetic biology and to give specific examples of its use to create new types of biological agents and methods of biological warfare, previously unthinkable and presented only in science fiction.

According to S.P. Singh et al. [2], synthetic biology integrates «the expertise from interdisciplinary fields, synthetic biology approaches are capable of addressing the unpredictable challenges associated with the intricate complexity of cellular systems. Synthetic biology has inspired researches to bioengineer biological systems to perform specific tasks in the area of therapeutics, diagnostics, and biomanufacturing of high-value biomolecules» (Figure 1)

- Basic tools and techniques:
- DNA synthesis and DNA sequencing.
- Organism («chassis»).
- Engineering of transcription:
- Synthetic promoters;
- Transcription factors.
- Genome modification tools:
- CRISPR/Cas9 nuclease;

- Zinc finger nucleases;
- TALE nucleases;
- Meganucleases.
- Computer-aided tools.

Basic tools and techniques of synthetic biology [2]:

DNA synthesis and DNA sequencing

The recent developments in DNA synthesis and DNA sequencing have opened new horizons in genetic modification technologies. Previously this manipulation was difficult because of template-based DNA synthesis; however, with the advent of de novo DNA synthesis, construction of new genes, control elements, basic building blocks, and even whole genome are possible. Construction of synthetic genome has led to creation of a synthetic version of life in yeast. Since the establishment of the first synthesis by phosphoramidite method⁴, numerous advancements have been made in this field, which ensured a high rate of oligo-synthesis, gene assembly, less error rate, and cost. Mere oligo-synthesis and gene-assembly do not ensure the synthesis of the desired sequence as these are prone to errors. Thus, to attain an error-free and verified DNA sequence (gene, promoter, genome, etc.), the assembled sequences are cloned in plasmids and subjected to sequencing. In one method, fluorescent selection marker GFP is fused with the gene sequences in such a way that the addition of the correct sequence will lead to a fluorescence, while error-containing sequence because of frameshift mutation leads to the loss in activity. Application

² Jan Baptist van Helmont. URL: https://en.wikipedia.org/wiki/Jan_Baptist_van_Helmont (date: 20.01.2021).

³ Spontaneous generation. URL: https://en.wikipedia.org/wiki/Spontaneous_generation (date: 20.01.2021).

⁴ Phosphoramidite Ligands // Phosphorus(III) Ligands in Homogeneous Catalysis: Design and Synthesis / Paul C. J. Kamer and Piet W. N. M. van Leeuwen. John Wiley and Sons. 2012. P. 133–157. <https://doi.org/10.1002/9781118299715.ch4>

of various polymerases harboring exonucleases and endonuclease activities has also been used to cut heteroduplexes, which on re-amplification can make the sequence error-free. Next-generation sequencing (NGS) approaches promise reduction in error rate by a factor of 500, when compared with the initial oligo pool. NGS uses different platforms for reading nucleotide sequences which are based on different principles of sequencing but ensure low cost, high speed, and accuracy in sequencing. Another area of DNA synthesis includes the addition of novel synthetic base pairs which would alter the regular codon set and will introduce novel amino acids, and therefore production of novel compounds. Two new bases, one the analog of pyrimidine 6-amino-5-nitro-3-(10-b-D-20-deoxyribofuranosyl)-2(1H)-pyridone (dZ) and other its purine analog complement 2-amino-8-(10-b-D-20-deoxyribofuranosyl)-imidazo [1,2-a]-1,3,5-triazin-4(8H)-one (dP), exhibited pyDDA:puAAD hydrogen bonding pattern, where «py» indicates a pyrimidine analog and «pu» indicates a purine analog. Here A and D represent acceptor and donor groups in hydrogen bonding. These developments in conglomeration of artificial genetic information systems have enhanced the wide applicability of synthetic biology.

Organism («chassis»)

The host system harboring the genetic toolbox for expression of the desired genes, delivered by suitable vectors, of the engineered biological pathway is termed as chassis. The prerequisite of an efficient chassis is its ability to grow on minimal media so as to lower the production cost, robust growth, and stability in response to the environment or the toxins released by the intermediates during the biosynthesis of macromolecules. Moreover, it should have a strong cell envelope which can tolerate the harsh conditions and at the same time allow secretion and attachment of molecules. The commonly used chassis are *Escherichia coli*, yeast, and *Bacillus subtilis* as they have been widely studied, and modification in these microbial systems is easier.

Other microbes have also been considered for chassis, such as Cyanobacteria, which has been used for biofuel production, and *Geobacter*, which utilizes electricity for carbon dioxide fixation (i.e., electrosynthesis). It is the chassis that provides the raw material and machinery to the synthetic system for performing various cellular, transcriptional, and translational functions. Therefore, correct selection of chassis is essential to achieve the appropriate effectiveness of the synthetic system. The host system has regulatory elements which may suppress the expression of foreign genes or the endogenous gene circuit may compete with the foreign gene system for resources. Therefore, synthetic circuits should be constructed in such a way that it can

operate independently of the endogenous circuit or mutated chassis needs to be designed that can utilize less of its resources and provide more to the synthetic system. The remedy to this issue lies in the synthesis of engineered chassis which is based on the concept of the minimal genome. A breakthrough in this area was the development of bacterial cell controlled by the genome which was chemically designed. Moreover, numerous engineered chassis have been attempted, which are designed keeping in mind the compatibility of the synthetic system and the flux of cellular resources directed toward the synthetic system.

Engineering of transcription

Overexpressing a gene above a threshold level may deplete the resources, which otherwise may be utilized for metabolic function. In addition, accumulation of intermediates may be toxic to final metabolite such that the enzymes work in a coordinated manner, which does not allow accumulation of intermediates above the required level. Therefore, genetic manipulation or engineering of metabolic pathways should be mediated by regulating transcriptional phenomenon.

i) Synthetic promoters

Transcription can be modulated by controlling the behavior of parts of the synthetic circuit, i.e., promoters, transcriptional activators, and repressors. In the microbial system, several promoters (lac promoter, arabinose-inducible promoter, T7 promoter, etc.) are in use to achieve protein expression of the desired gene. However, use of natural promoters often diminishes their utility in achieving intricate gene regulation of the genetic tool. Inducible promoters also have some concerns related to the variable effects in different host systems and additional cost involved in the «inducers». Therefore, it is desirable to decipher the structure of cis elements so that they can be molded by rearranging the cis motifs into the synthetic promoter for the desired fashion of transcriptional control of synthetic toolkit. The synthetic promoter should be preferably short in length but aggressive in transcription. Furthermore, the synthetic promoter is designed to perform constitutive or tissue or cell-type specific or temporal or inducible expression of the gene system. A deep interrogation of the cis-regulatory architecture, which includes motif sequence, position, copy number, and spacer length, governs the strength, temporal, and spatial expression of the promoter. New motifs can be investigated by screening motif libraries or by bioinformatics-based *de novo* motif discovery tools. Once a suitable motif is isolated, its copy number and spacing also need to be optimized as they link to promoter strength and arrangement of a transcription factor (TF) to access RNA polymerase complex. The transcriptional activity of a promoter should be examined using combinatorial promoter

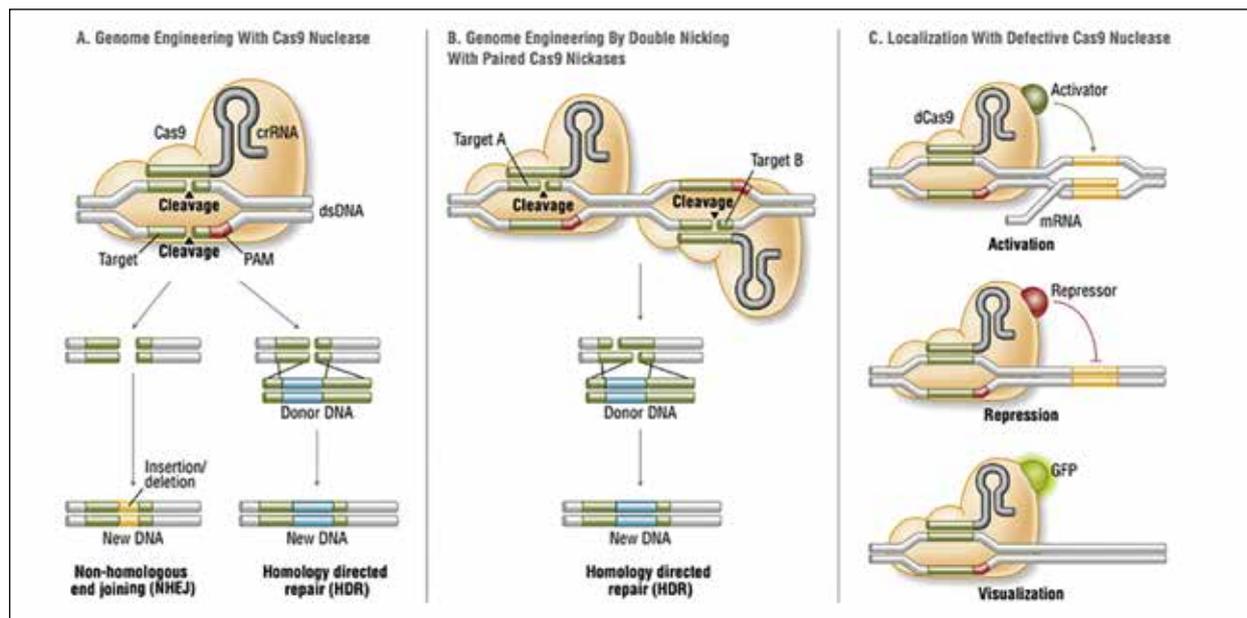


Figure 2 – CRISPR/Cas9 System Applications.

A – Wild-type Cas9 nuclease site specifically cleaves double-stranded DNA activating double-strand break repair machinery. In the absence of a homologous repair template non-homologous end joining can result in indels disrupting the target sequence. Alternatively, precise mutations and knock-ins can be made by providing a homologous repair template and exploiting the homology directed repair pathway. B – Mutated Cas9 makes a site specific single-strand nick. Two sgRNA can be used to introduce a staggered double-stranded break which can then undergo homology directed repair. C – Nuclease-deficient Cas9 can be fused with various effector domains allowing specific localization. For example, transcriptional activators, repressors, and fluorescent proteins (The whole Figure with text has been taken from [3])

libraries and reporter proteins. Such experiments fine-tune the cis-regulatory architectures for construction of efficient synthetic promoters with desirable regulatory ranges.

ii) Transcription factors

The level of transcription of a gene is dependent on the efficiency of promoter transcription factor (TF) interaction, i.e., quick and potent binding of TF to the cis DNA element. TFs provide regulatory links to the gene circuit, which synthetic biologists intend to create in a biological system. Engineering of TF proteins becomes essential when many genes are to be altered simultaneously to develop a «biofactory». Natural TF's DNA-binding domain (DBD) may recognize multiple cis motifs. TF engineering can be done in the DBD in such a way that it can bind to the defined targets in the promoter sequences for controlled gene expression. Fusion of engineered DBD with effector domain and nuclear localization signal leads to the development of synthetic TF with controlled activity. Several examples of synthetic TFs are available which are being used to regulate the genetic network. An example of synthetic TF is the fusion of tetracycline-dependent repressor (from *E. coli*) to transactivation domain (from the herpes simplex virus). The synthetic TF regulates transcription by its ability to bind tetracycline responsive element.

Apart from activation, methylation, acetylation, amination, recombination can also be achieved via synthetic TF. Synthetic promoter elements and synthetic transcriptional factors are foremost utensils in rewiring or reconstructing or novel designing of gene expression regulatory networks in the synthetic biological system.

3. Genome modification tools

Correction of defective genes or introduction of foreign genes requires efficient editing tools for targeted genome modification. The genome editing tool comprises components for identification of target sites and creation of double-stranded breaks (DSB) in DNA, and the breaks are repaired by homologous recombination or nonhomologous end joining. Various new generation nucleases are available, which are more precise in their catalytic action and can be modified as per the genetic requirements.

i) CRISPR/Cas9 nuclease

CRISPR (clustered, regularly interspaced, short palindromic repeats) technology is a universal tool for genome engineering and has revolutionized biotechnology. Only recently identified unique CRISPR/Cas (CRISPR associated) systems, as well as re-engineered Cas proteins, have rapidly expanded the functions and applications of CRISPR/Cas systems. The CRISPR/Cas system (Fig. 2) is the

most popular gene editing tool developed from the indispensable nuclease, Cas9 [3].

Type II CRISPR/Cas system which provides immunity to bacteria against invading viruses, and plasmids led to the foundation of CRISPR/Cas9 system. The Cas9 component acts as the endonuclease which is guided by a 20 nucleotide guide RNA (gRNA) that has RNA sequence complementary to the DNA of the target site. For genome editing, this gRNA needs to be designed according to the requirement that recognizes the site of cleavage by Cas9, thereby introducing a site for deletions and additions. Apart from gRNA, another prerequisite for cleavage is the presence of a protospacer adjacent motif (PAM), which is a short 2–6 bp long DNA sequence present adjacent to the target site. The absence of PAM restricts the cleavage event. Normally the PAM sequence is 50-NGG-30 where N represents any nucleotide base. The aforementioned genome engineering toolboxes have offered several advantages over conventional transgenic approaches by providing opportunities of genetic loci specific gene integration or correction [4].

ii) Zinc finger nucleases

These are chimeric proteins composed of DNA-binding (ZF) and DNA cleavage domains. The cleavage domain was isolated from Fok I, a type IIS restriction enzyme that has different binding and cleavage sites. Fok I activity requires dimerization of cleavage domain; therefore, two sets of zinc finger nucleases (ZFNs) are required, which recognize the target sites on two DNA strands. Binding of the two finger nucleases, using a short linker, to the recognition sequence increases their concentration, thereby facilitating dimerization followed by cleavage.

iii) TALE nucleases

These are similar to ZFN as the cleavage site is derived from Fok I and requires dimerization of nuclease. However, it shows higher target specificity. The target site is greater than 30 bp because of the incorporation of 15e20 repeat variable diresidue in the monomer unit. The only limitation associated with TALE nucleases (TALEN) is its large size that makes its entry difficult in the host cell system. ZFN and TALEN have helped in exploring the novel way of sequence-specific genetic correction opportunities in the organism.

iv) Meganucleases

Meganucleases catalyze cleavage functions at specific loci in the genome. Meganucleases have been reported from a variety of organisms such as archaea, bacteria, phages, fungi, yeast, algae, and some plants. Intron-encoded endonuclease catalyzes DNA cleavage in the intron-lacking alleles. This helps in the movement of introns from intron-containing alleles to intron-less alleles, leading to gene conversion events. Therefore, they are known as homing endonucleases. Based on sequences

and motifs, meganucleases are classified into five families. Contrary to the name, meganucleases, these are the smallest nucleases (165 amino acids) which makes their delivery easy. Meganucleases, with potential to generate DNA DSB at targeted loci of interest, are promising enzymatic tools for genome engineering. However, the number of native meganucleases is limited and insufficient to target a large number of desired loci. Because the meganucleases are nonmodular protein, its redesigning is tough as DNA-binding amino acids overlap with the DNA cleavage amino acids, thereby affecting the catalytic activity. However, methods have been developed for custom designing of meganucleases using machine learning approaches.

Computer-aided tools

As the area of synthetic biology is expanding, novel computational tools need to be developed, aggressively, to understand the current demand of synthetic aspects in biology. Despite the presence of numerous pieces of software, it suffers from certain limitations in terms of their biological counterparts, which sometimes behaves in an unpredictable manner, and due to this, repetition of experiments and analysis is required. Computeraided (CAD) tools are indispensable to synthetic biology as the tools bestowed by it are utilized by biological engineers to understand and ameliorate the properties and functions of synthetic biology parts, devices, networks, etc. CAD tools aid in developing and optimizing parameters based on which synthetic biology devices can be designed and tested. It offers the advantage to judge the feasibility of a model constructed for targeting a specific function. These developments assist in assembling the biological parts to form circuits and networks and simultaneously predict the fate of the assembly. These tools also help in determining the details of the alternates of a design. As the tasks performed by CAD tools are variable, these can be categorized under three sections based on the type of work they are participating in:

- i) tools involved in basic structural design and synthesis;
- ii) tools involved in network design;
- iii) tools involved in prediction of behavior/function/response.

Another progress could be achieved after employing of artificial intelligence (AI). The progress is exciting however AI is not a universal replacement for the investigations of the natural world. Nevertheless, modern AI will (probably) dominate biological data science for its unprecedented learning capabilities to process complex data [5]. Here we would like to focus on one, recent (press release) example:

«Scientists at the Department of Energy's Lawrence Berkeley National Laboratory have developed a new tool that adapts machine learning

algorithms to the needs of synthetic biology to guide development systematically. The innovation means scientists will not have to spend years developing a meticulous understanding of each part of a cell and what it does in order to manipulate it; instead, with a limited set of training data, the algorithms are able to predict how changes in a cell's DNA or biochemistry will affect its behavior, then make recommendations for the next engineering cycle along with probabilistic predictions for attaining the desired goal⁵.

The «desired goal» can be anything... No doubt, that such publications appearing in prestigious journals [6] have a broader reading forum than the civil one. In a study J. Thomsen et al. [7] researchers have developed an AI tool which dramatically speeds up a research of protein dynamics. Interestingly, the used software is freely available and is accessible to all teams in the world rather than be limited to few laboratories with specialist expertise. This are few examples of a great progress in this area.

In the previous article we have put a schema of the division (and the possible (mis)use synthetic biology) as follows [8]:

i) **Binary bioweapons** (these are two-component systems that are relatively safe to handle but become deadly when the two components come together on deployment).

ii) **Designer genes** (where specific unnatural gene sequences are built into viruses or other life forms to incorporate into the genome of the unsuspecting host, which later becomes the victim).

iii) **Gene therapy** (today a medical (partial) reality; the technology that allows medicine to repair or replace defective genes in a diseased individual might be subverted to introduce pathogenic sequences into healthy individuals).

iv) **Stealth viruses** (viruses that could be fashioned by a researcher to infect the host but remain silent until activated by some physiological or environmental trigger).

v) **Host-swapping** diseases (new zoonotic agents which might be developed specifically for bioweapon purposes by modifying existing pathogens to seek human hosts).

vi) **Designer diseases** (where the detailed knowledge of biochemical signaling pathways could conceivably be used to create designer diseases).

However, according to⁶ we will now use a slightly different approach: It should be noted that in the era of synthetic biology, the technologies

themselves pose no inherent harm, and it would generally take a collection of technologies to create a specific capability that warrants concern. Here we can see how the framework applied to assess capabilities (rather than technologies) that potentially pose a concern because of the harm they might enable. A list of potential capabilities to evaluate was identified by gathering a range of possibilities that have been mentioned in various venues as potential concerns associated with synthetic biology and augmenting that list with additional possibilities that had not been previously raised. These potential capabilities were grouped into categories to ensure a consistent approach to their evaluation using the framework.

i) **Re-creating known pathogenic viruses:** Constructing a known, naturally occurring pathogenic virus from the starting point of information about its genetic sequence.

ii) **Re-creating known pathogenic bacteria:** Constructing a known, naturally occurring pathogenic bacterium from the starting point of information about its genetic sequence.

iii) **Making existing viruses more dangerous:** Creating a modified version of a known virus in which one or more traits have been altered to make the virus more dangerous (such as by enhancing its virulence).

iv) **Making existing bacteria more dangerous:** Creating a modified version of a known bacterium in which one or more traits have been altered to make the bacterium more dangerous.

v) **Creating new pathogens:** Constructing a pathogen from the novel combination of multiple parts, which may be derived from various organisms, designed computationally, or created through other strategies.

vi) **Manufacturing chemicals or biochemicals by exploiting natural metabolic pathways:** Producing a naturally occurring product, such as a toxin by engineering an organism (e.g., bacterium, yeast, or alga) to contain the known biosynthetic or metabolic pathway for the desired product.

vii) **Manufacturing chemicals or biochemicals by creating novel metabolic pathways:** Creating a new biosynthetic pathway that enables an engineered organism to produce a chemical that is not normally produced biologically.

viii) **Making biochemicals via in situ synthesis:** Engineering an organism, such as a microorganism that can survive in the human gut, to produce a desired biochemical and delivering this

⁵ Machine learning takes on synthetic biology: algorithms can bioengineer cells for you Scientists develop a tool that could drastically speed up the ability to design new biological systems / Science Daily 2020. September 25. URL: <https://www.sciencedaily.com/releases/2020/09/200925113447.htm> (date: 14.02.2021).

⁶ Biodefense in the Age of Synthetic Biology 2018. ISBN 978-0-309-46518-2. <https://doi.org/10.17226/24890> (Committee on Strategies for Identifying and Addressing Potential Biodefense Vulnerabilities Posed by Synthetic Biology, Board on Chemical Sciences and Technology, Board on Life Sciences).

microorganism in such a way that it can produce and release this product *in situ*.

Modifying the human microbiome: Manipulating microorganisms that form part of the population living on and within humans, for example, to perturb normal microbiome functions or for other purposes.

Modifying the human immune system: Manipulating aspects of the human immune system, for example, to upregulate or downregulate how the immune system responds to a particular pathogen or to stimulate autoimmunity.

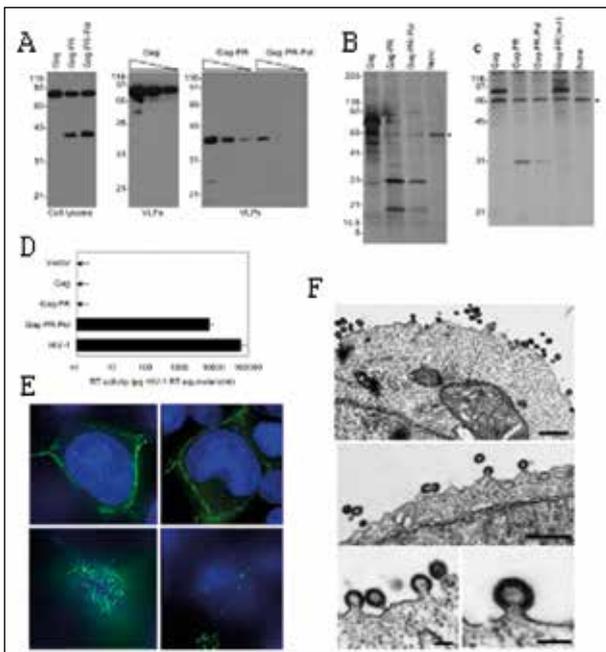


Figure 3 – Assembly, Processing, and Release of HERV-K Virus-Like Particles. (A–D) 293T cells were transfected with Gag-, Gag-PR-, or Gag-PR-Pol-expressing vectors. (A) Western blot analysis of cell lysates (left) and virions (center and right) using a commercially available antibody to HERV-K Gag. Center shows VLPs from 293T cells transfected with a plasmid-expressing Gag, and right shows VLPs from Gag-PR- and Gag-PR-Pol-expressing 293T cells. Decreasing amounts of virion lysate (0.1, 0.05, or 0.025 μ l for Gag; 0.4, 0.2, or 0.1 μ l for Gag-PR and Gag-PR-Pol) were loaded to semiquantitatively estimate relative levels of VLP production. (B) Silver stain analysis of a 4% to 20% gradient SDS-PAGE gel loaded with VLPs harvested from 293T cells transfected with plasmids expressing Gag, Gag-PR, Gag-PR-Pol, or empty plasmid control. An asterisk marks a nonspecific 66-kDa protein band, most probably BSA, that is abundant in the culture medium. (C) Silver stain analysis of VLPs harvested from 293T cells containing Gag, Gag-PR, Gag-PR-Pol, or Gag-PR(mut) encoding an active site mutation (DTG-AAA) in protease. An asterisk marks a nonspecific 66-kDa protein band, most probably BSA, that is abundant in the culture medium. (D) Reverse transcriptase activity in culture supernatants of 293T cells transfected with empty pCRV1 (vector) or vectors expressing HERV-KCON Gag, Gag-PR, or Gag-PR-Pol proteins, as indicated. Enzymatic activity was determined relative to a recombinant HIV-1 reverse transcriptase standard and is representative of three experiments. Supernatants from 293T cells transfected with an HIV-1-based proviral plasmid are included for comparison. (E) Two representative 293T cells transfected with HERV-KCON Gag and Gag-GFP expression plasmids. Cells were fixed 18 h post-transfection, and nuclei were stained with DAPI (blue) prior to visualization by deconvolution microscopy. Top, Images acquired at the mid-section of the cell to show localization of Gag-GFP proteins; bottom, focused on the bottom of the cell to show accumulated VLPs at the cell–coverslip interface. (F) Gallery of electron micrographs of 293T cells transfected with a Gag-PR-expressing plasmid. Black scale bars in the upper and middle panels represent 500 nm, while scale bars in the lower two panels represent 100 nm. <https://doi.org/10.1371/journal.ppat.0030010.g002> [10]

⁷ NCBI (National Center for Biotechnology Information). 2017. GenBank. URL: <https://www.ncbi.nlm.nih.gov/genbank> (date: 20.01.2021).

[9]. In 2007, an infectious form of the ancient endogenous retrovirus HERV-K (HML-2) was obtained [10] (Figure 3).

This cDNA was used to produce viral RNA, which was then used to program an in vitro extract to produce infectious poliovirus virions. Since then, larger and larger viral genomes have been generated, taking advantage of advances in the ability to synthesize longer and longer segments of DNA. Modern assembly methods have greatly expanded the scale at which DNA can be constructed, to the point that building the genome of virtually any virus—either in the form of the genome itself for a DNA virus or as a cDNA of an RNA virus that can be transcribed into the viral genome is now possible [11]. A notable example is the recent report of the construction of the horsepox genome (consisting of more than 200,000 base pairs) as part of an effort to develop a new smallpox vaccine [12] (It should be noted that while the booting of some viruses, e.g., polio, has been performed using cell-free extracts, most viruses must be booted inside cells, and some viruses, including horsepox, require the use of a helper virus in cells)⁸.

ii) *Re-creating known pathogenic bacteria:*

The genomes of many existing bacteria have been characterized, and the same types of DNA synthesis and booting approaches used for large viral genomes can, in theory, be applied to re-create known pathogenic bacteria. Indeed, JCVI reported the synthesis and booting of *Mycoplasma mycoides* in 2010 [13]. Other microbial genome synthesis projects are well under way, such as for *Escherichia coli* (4 million base pairs) and yeast (11 million base pairs).

B (iii+iv): MAKING EXISTING PATHOGENS MORE DANGEROUS

iii) *Making existing viruses more dangerous:*

The following are selected examples of viral traits, presented to give a sense of the range and type of traits that could theoretically be targeted for modification using biotechnology.

Altered Tropism

Tropism is the capacity of a virus to infect or damage specific cells, tissues, or species. While tropism is primarily influenced by the interaction of the viral cell attachment protein(s) with the receptor(s) present on the cell (thus determining viral entry), the larger property of tropism is determined by multiple viral and host cell factors. Altering tropism could be used to expand the host range of an existing virus or otherwise increase a virus's ability to take hold in a targeted population. Several studies have demonstrated the ability to alter the tropism of viruses. The avian influenza H7N9

strain has been causing isolated human infections since the initial outbreak in China in 2013, but sustained human-to-human transition has not been documented. It has been demonstrated that only three mutational changes in the sequence of the hemagglutinin gene are sufficient to switch the virus's tropism from avian to human and support binding to human tracheal epithelial cells [14]. In earlier studies with avian influenza, researchers used sitedirected mutagenesis to introduce mutations into the hemagglutinin gene to allow wild-type H5N1 virus to bind to human receptors. Researchers have also used synthetic biology to alter tropism in investigations of the respiratory syndromes SARS (severe acute respiratory syndrome) and MERS (Middle East respiratory syndrome). There is considerable evidence indicating that a SARS-like virus in bats was the origin of the 2003 outbreak of SARS in humans. The bat virus, however, does not grow in cell culture. To help elucidate the steps that may have occurred to convert bat SARS-CoV into a virus infecting humans, scientists substituted the human SARS coronavirus receptor binding domain for the equivalent domain in the bat SARS-CoV virus, making the batSARS virus replication competent in cell culture and mice [15]. Similarly, to develop a small-animal model of MERS-CoV, researchers modified both the mouse, to express a chimeric receptor, and the virus [16].

Enhanced Viral Replication

Enhancing viral replication could help increase the impact and spread of a virus-based bioweapon. In experiments with echovirus 7 researchers demonstrated that decreasing the CpG and UpA frequencies in two 1.1- to 1.3-kilobase regions of the viral genome enhanced viral replication in susceptible cells. Conversely, increasing the CpG and UpA frequencies resulted in decreased viral replication. While it is unknown whether these results would be the same in animals—enhanced replication in cell culture does not necessarily correlate with enhanced replication in vivo, and in fact, the reverse is sometimes the case—an actor with sufficient time and resources may be able to generate variants empirically and passage them in a susceptible host to select a variant with enhanced replication ability.

Enhanced Virulence

Virulence measures the relative capacity of a virus to cause actual disease in a host, rather than just infection. Virulence represents the combined effect of multiple genes and determinants that play specific roles in specific settings in vivo. In the best-known example of an engineered virus resulting in enhanced virulence researchers engineered

⁸ Kupferschmidt K. How Canadian researchers reconstituted an extinct poxvirus for \$100,000 using mail-order DNA. Science, News. 2017. July 6. URL: <http://www.sciencemag.org/news/2017/07/how-canadian-researchers-reconstituted-extinct-poxvirus-100000-using-mail-order-dna> (date: 20.01.2021).

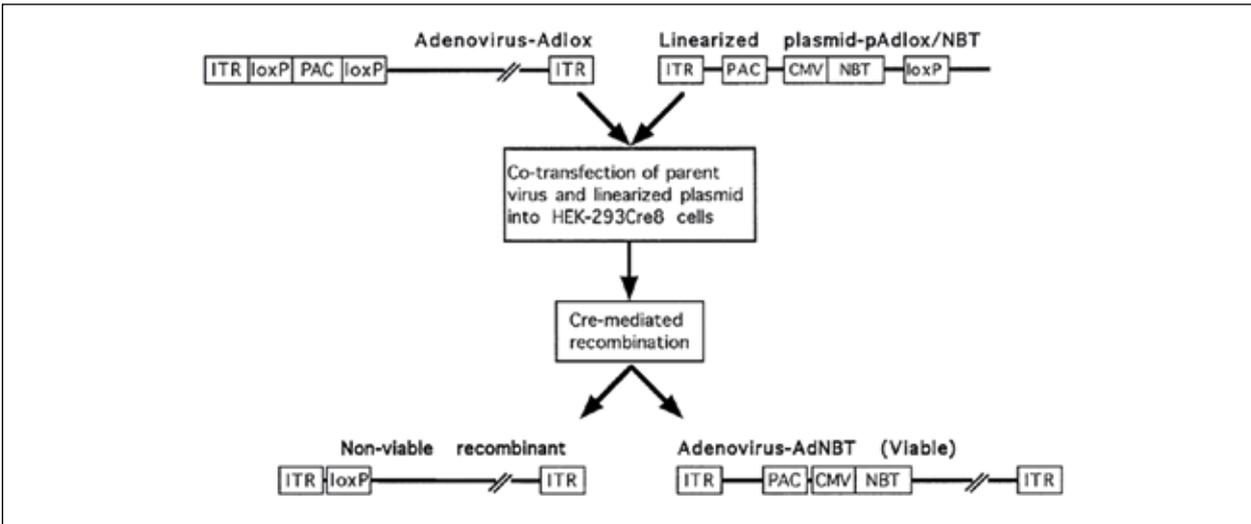


Figure 4 – Construction of recombinant adenovirus encoding neuronal bungarotoxin (Bgt). The schematic shows the *Sfi*I fragment of pAdlox x-Bgt that contains, from left to right, the inverted terminal repeat (ITR), packaging signal (Pac), CMV promoter (CMV), K-Bgt cDNA and loxP site. The fragment was cotransfected into a specially created cell line. HEK-293/CRE8 along with a modified adenovirus that contains two loxP sites on either side of the packaging signal. CRE-mediated intramolecular recombination results in un-packagable viral DNA. Only intermolecular recombination with the plasmid fragment yields functional virus. Hence, proliferation of the correct recombinant is highly favored [19]

ectromelia virus (mousepox), a member of the Orthopoxvirus genus and a natural pathogen of mice, to express mouse interleukin-4 (IL-4), with the goal of producing a contraceptive vaccine to control the mouse overpopulation. In the mouse model, the recombinant virus was shown to suppress primary antiviral cell-mediated immune responses and overcome preexisting immunity [17]. It is also conceivable that actors would seek to manipulate a virus so that it causes disease by different mechanisms than a natural virus might, such as by manipulating neurobiology or altering the host microbiome.

Ability to Evade Immunity

At the root of the increased virulence demonstrated in the mousepox experiments (described under Enhanced Virulence, above) was the recombinant virus's capability to evade immunity. This points to another potential route for actors seeking to produce bioweapons: the development of viruses designed to anticipate and evade the immune response or even to overcome vaccine-based immunity. Detection of viral pathogens by the innate immune system leads to the induction of antiviral mechanisms that are mostly mediated by type-1 interferons. This primary response then leads to the activation of the adaptive immune response that is more directed, antigen-specific, and longer lasting [18].

Many viruses have countermeasures to subvert the innate immune response including interferon-induced antiviral activity. It may be possible to express one or more antagonists of these antiviral

activities in a pathogen that does not already have that particular antagonist. In this way, the arsenal of activities that a virus uses to evade the innate immune response would be expanded and virulence may be enhanced. The creation of chimeric viruses developed by genetically substituting capsid genes has been well documented. These viruses have mainly been developed in the context of, for example, improving adenovirus vectors to target specific tissues and as an approach to circumventing preexisting viral immunity that may limit the use of viral gene therapy vectors. It is conceivable that the latter approach could be used to develop a chimeric viral vector expressing a toxin gene targeted to a particular tissue and used in a population with preexisting immunity to the vector virus [19] (Figure 4).

The molecular determinants of targeting are poorly understood, however, and these approaches generally require significant trial and error to be successful.

Ability to Evade Detection

Some modifications could result in a virus that would be difficult to detect using current outbreak response approaches. The most commonly used methods of laboratory identification of viruses are based on real-time polymerase chain reaction assays in which specific primers and fluorescently labeled probes are designed to bind to conserved and unique regions of the viral DNA or cDNA. Nontargeted methods of detection include array-based assays and next-generation sequencing, but these are not yet in wide use in clinical and commercial laboratories. Cell culture methods

are rapidly disappearing from use. Mutations that target the primer binding sites could therefore result in a virus that is not recognizable.

Ability to Resist Therapeutics

Actors could seek to develop viruses capable of resisting available therapeutics, though the necessity of this approach would depend on whether effective therapeutics exists [20]. Despite the availability of successful antiviral agents such as those used to counter HIV (human immunodeficiency virus), herpes viruses,

iv) *Making existing bacteria more dangerous:*

The following are selected examples of bacterial traits, presented to give a sense of the range and type of traits that could theoretically be targeted for modification using biotechnology.

Altered Tropism

Unlike viruses, which are exclusively intracellular pathogens, bacterial pathogens can be either intracellular or extracellular. Generally, extracellular pathogens are relatively environmentally stable and good at adapting to their environment. Even those that are not spore-forming often have the capacity to replicate and cause damage in multiple tissues and cell types and in different locations in the body. Given their environmental stability, they are difficult to eradicate and may not require host-to-host contact for transmission. Intracellular bacteria, like viruses, rely on host cell nutrients and are often able to evade the host immune system. Intracellular pathogens are usually transmitted via direct contact or aerosol transmission. Both intracellular and extracellular pathogens rely on adherins and colonizing factors, which facilitate contact with host target cells, confer resistance to leukocyte attack, and are significant virulence factors [21].

Enhanced Virulence

Many factors influence bacterial virulence and could potentially be targeted for modification. The primary mechanisms of bacterial pathogenesis include host target cell death, whether by cell lysis (resulting either from the multiplication of intracellular pathogens or as a result of the action of bacterial toxins) or by induction of apoptosis (programmed cell death); mechanical perturbations of host physiology (e.g., blockage of circulatory or respiratory passages due to the size or number of invading bacterium or as a result of mucous production); host cell damage resulting from the host immune response to the bacterial infection; and the action of bacterial toxins. The effects of cell death depend upon the host cells involved and are influenced by the bacterial burden introduced, the route of infection, complicating symptoms induced by host immune response, and the rapidity of the infection process. Colonization potential is influenced by the ability of some pathogenic bacteria (e.g., *Shigella*) to trigger premature or

unscheduled apoptosis in the host cells they infect; the initial phase of this process involves the introduction of enzymatically driven damage to host cell DNA followed by massive disturbances in cell integrity and cell death. Another significant virulence factor is the ability of some bacteria (e.g., *Bacillus anthracis*) to form capsules consisting of polysaccharides and amino acids [22]. Capsules prevent bacteria from being phagocytized by neutrophils and macrophages. Other virulence factors include invasion factors, which are usually encoded chromosomally but may also be plasmid-borne, and siderophores, iron-binding factors that allow bacteria to compete with host cells for iron acquisition [23].

Enhanced Toxin Production

Many bacterial pathogens cause damage to host cells and tissues through the production of toxins. These toxins take two forms: exotoxins and endotoxins. Exotoxins are relatively unstable, highly antigenic proteins that are secreted into host body fluids. Some exotoxins are bound to the bacterial cell wall following their synthesis and are released upon lysis of the invading bacterium. Often highly toxic, exotoxins are produced by both Gram-positive and Gram-negative bacteria. Some exotoxins can act only on certain cell types whereas others affect a broad spectrum of cells and tissues. Some bacterial pathogens make only a single toxin (e.g., cholera, diphtheria, tetanus, botulism) whereas others can synthesize two or more distinct toxins (e.g., *Staphylococcus*, *Streptococcus*). Antitoxin antibodies to exotoxins are usually made rapidly by the host. The genetic determinants of exotoxins are often found on extrachromosomal elements, usually plasmids or bacteriophages. Endotoxins, on the other hand, are relatively stable, lipopolysaccharide components of the outer membrane of some Gram-negative bacteria that can act as toxins under certain circumstances. Lipid A appears to be the toxic component, which can act while in the intact bacteria expressing it. Endotoxins are generally weakly immunogenic, eliciting fever in the host. They can cause hypotension due to increased vascular permeability accompanied by vasodilation, which can in turn result in shock. The genetic determinants for endotoxins are chromosomal. Actors could potentially seek to modify bacteria to enhance their natural toxin production or introduce toxin production into a bacterium that does not naturally produce toxins.

Ability to Evade Immunity

As with viruses, it is possible to engineer bacteria to anticipate or evade the immune response.

Ability to Evade Detection

As with viruses, the most commonly used methods of laboratory identification of bacteria are based on real-time polymerase chain reaction (PCR) assays in which specific primers and fluorescently

labeled probes are designed to bind to conserved and unique regions of the bacterial chromosomal or extrachromosomal DNA. Another widely used method in clinical microbiology laboratories is MALDI-ToF (matrix-assisted laser desorption/ionization time-of-flight), a method of ionizing large molecules and identifying them by mass spectrometry in comparison to reference standards. Nontargeted methods of detection such as array-based assays and next-generation sequencing are available but are not yet in wide use in clinical and commercial laboratories. Culture methods are rapidly disappearing from use [24].

Ability to Resist Therapeutics

In contrast to the relatively small number of antivirals, there are many antibacterial agents available that are capable of acting against a wide variety of bacterial pathogens. However, bacteria can be intrinsically resistant to antibiotics, or can acquire resistance via chromosomal mutation and horizontal gene transfer. There are three main mechanisms of antibiotic resistance [25]. First, the bacterium can prevent the antibiotic from accessing its target, either through reduced permeability of the antibiotic through the cell wall or membrane complex or through increased efflux of the antibiotic back out of the organism and away from its target. Second, the antibiotic target can be altered through genetic mutation, causing the target to become modified or protected. Finally, antibiotic resistance can be acquired by direct modification of the antibiotic itself, either by inactivation by antibiotic hydrolysis or by way of inactivation due to a chemical modification. These mechanisms are well studied and could potentially be adapted for the purposeful creation of antibiotic-resistant pathogenic bacteria.

Enhanced Transmissibility

As with viruses, the property of airborne transmission in bacteria is complex and dependent on multiple host and pathogen factors, in particular environmental stability and tissue tropism. Extracellular bacterial pathogens are extremely adaptable to environmental challenges and may not require host-to-host contact for transmission, making these pathogens difficult to eradicate. In addition, many bacterial pathogens that replicate extracellularly are capable of causing damage to different cells and tissue types. On the other hand, many intracellular bacterial pathogens are communicable (i.e., capable of host-to-host transmission), facilitating rapid spread within a community and thus presenting a greater capacity to threaten public health.

Enhanced Stability

The environmental stability of a bacterium depends on its physiology and life cycle. Gram-positive bacteria are more environmentally stable than Gram-negative bacteria. In addition, when

subjected to harsh environmental conditions such as desiccation, some Gram-positive bacteria form spores capable of remaining viable in the environment for decades, albeit in a metabolically dormant state. For example, spores of *Bacillus anthracis* can remain viable in the environment for up to a century and constitute the infectious form of this pathogen (with vegetative forms not being infectious) [26]. Actors may find it advantageous to engineer bacterial cell walls to more closely resemble Gram-positive organisms to enhance survival during aerosol dissemination and allow the agent to remain viable and available to infect the target host for extended periods of time.

C (v): CREATING NEW PATHOGENS

v) *Creating new pathogens:*

A major aspiration within the field of synthetic biology is the design and creation of new organisms with beneficial uses. In the context of bioweapons, the possibility that this aspiration may potentially be directed toward producing pathogens that are entirely new was considered. In contrast with the discussion of modifying existing pathogens, the term «new» is used here to describe novel combinations of genetic parts from multiple organisms for which the product is not recognizable as primarily from one source. This can include genetic parts designed computationally with no near relative in the natural world. The resulting range of potential bioweapons in this category is extremely broad but serves to illustrate the more challenging applications that may be possible at some point in the future. One example of a new pathogen would be a virus constructed from parts of many different natural viruses. This mix-and-match approach might be used to combine the replication properties of one virus, the stability of another virus, and the host-tissue tropism of a third, for example. A variety of experimental approaches would be applicable to this goal. Directed-evolution approaches could be used to sample random combinations of viral DNA parts; while each individual combination would have a small chance of success, sampling a very large number of combinations would increase the chances of success. More explicit design approaches might be to develop software to model and predict the properties of specific designs, which would then be built, tested, and improved through multiple iterations of the Design-Build-Test cycle. Even simple changes to existing viruses can produce drastic deficiencies in key viral properties, making any such effort especially difficult. Nonetheless, work involving recomposing the structure of a bacteriophage genome into modular pieces suggests that radical new combinations of viral sequences may be viable, although tools to design viruses with high confidence of success are currently lacking. A different example of a new pathogen would be one based on synthetic «genetic circuits». A major

pursuit within synthetic biology is the capability to arbitrarily program specific functions using genetic material. These efforts are exemplified by the engineering of DNA-encoded programs, relying heavily on concepts derived from information theory and computer science, such as constructing logic gates from individual switching functions. Importantly, the genetic material encoding those functions can in principle come from anywhere – from any branch of the tree of life or from an entirely new DNA sequence that has never been observed in nature. The designs for genetic circuits have greatly increased in complexity over time through increased reliance on component abstractions and standardization. A number of genetic circuits have been designed to function in human cell lines in culture however, applications using genetic circuits in the human body are still in their infancy. The potential for using such technology to cause harm in the human body is thus a subject of broad speculation. Novel circuits could (in theory) be used to convert a healthy cell into a cancerous one or to provoke an autoimmune response. Such circuits might be designed to act on the host DNA using engineered factors that turn host genes on or off, such as at the level of transcription or translation. A variety of mechanisms have been demonstrated for such general-purpose switching. They include the use of natural or artificial microRNA molecules and the use of CRISPR/dCas9-type programmable gene repression or activation [27]. Importantly, these are examples of mechanisms that have displayed a high degree of programmability in terms of which host DNA sequences can be targeted. In a similar vein, the potential programmability of genetic effectors may also lead to genetic circuits that sense and compute based on the state or type of cell or even specific genetic identity. In some cases, genetic circuits could be delivered to a small number of host cells using nonreplicating delivery mechanisms, which could be either virus-derived, such as those used in some gene therapies or based on nonbiological materials. At the extreme end of difficulty (and feasibility) lies the engineering of life forms that are particularly dissimilar from known life on this planet. «Xenobiology» offers some possibilities – for example, a bacterium employing a different combination of deoxyribonucleotides and ribonucleotides to encode its genetic information [28]. There is a wide range of expert opinion as to the long-term plausibility of such efforts.

D (vi+vii+viii): PRODUCTION OF CHEMICALS OR BIOCHEMICALS

vi) *Manufacturing chemicals or biochemicals by exploiting natural metabolic pathways:*

Biochemical compounds naturally produced by plant and microbial cells have been used for

centuries as medicinal compounds. These products have been prepared as both plant extracts, in which the active ingredient is one of numerous chemical structures in the formulation, and as high-purity single compounds, made by cultivating the producing organism in large-scale bioreactors and then purifying the output. Such products have been used to treat diseases ranging from microbial infection to hypertension. The opioids, used as analgesics, are now accessible by microbial fermentation, as well, though optimization of the «home-brewing» process has not been rigorously explored. Each naturally occurring biochemical is the result of a series of chemical reactions that transform simple feedstocks such as glucose into the end products of interest. These transformations are mediated by enzymes encoded by the host organism's DNA. Because biotechnologies allow the DNA encoding the necessary enzymes to be exploited independent of the original host, it is now possible to make such products without relying on the organism that naturally produces them.

vii) *Manufacturing chemicals or biochemicals by creating novel metabolic pathways:*

While nature has provided a wide array of biochemical compounds that could be exploited for targeted synthesis, enzyme-mediated conversions also can be used to produce chemicals that organisms do not naturally create. Biocatalysis has long been used to produce pharmaceutical intermediates and active ingredients not found in nature. It is not always necessary to use living microbial organisms in these processes; instead, purified enzymes can be used in reaction vessels in a manner analogous to traditional organic synthesis. At its core, designing a new biosynthetic pathway involves specifying a series of enzymatic steps that can convert a set starting substrate to the desired end product. In practice, the starting substrate is often a known primary metabolite (e.g., acetyl-CoA), and the proposed reaction steps are based on known enzymatic chemistry. Engineered metabolic pathways that do not follow an existing natural blueprint have been exploited to commercialize biological production of chemical compounds. The true limits of biological synthesis are unknown, and advances in protein design and engineering are rapidly expanding the repertoire of enzyme-catalyzed reactions⁹. Researchers have also shown that materials typically present in very small amounts in biological systems, such as halogens, can be incorporated into natural products by merging plant and microbial biosynthesis machinery. These examples suggest that the range of molecules that may be accessible by biological synthesis is far larger than what has been demonstrated to date.

⁹ NRC. 2015. Industrialization of Biology: A Roadmap to Accelerate the Advanced Manufacturing of Chemicals. Washington, DC: The National Academies Press.

viii) *Making biochemicals via in situ synthesis:*

The human microbiome, particularly the gut microbiome, has been a target for metabolic engineering. Gut microbes influence the metabolism of their host and are capable of producing a wide variety of biochemicals. While the extent of the influence of the microbiome on host metabolism remains an active research area, there has already been significant progress toward engineering gut microbes for therapeutic purposes. Engineered microbes are currently being prepared for clinical trials for the treatment of metabolic disorders¹⁰, although engineering high flux through a metabolic pathway remains undemonstrated. As this research gains steam, it is worth considering whether the human microbiota could be exploited to make biochemicals (within the cells of commensal organisms) and deliver them to human hosts to cause harm. In addition to the gut microbiome, the skin microbiome could be another potential avenue for in situ synthesis of such compounds. Related concepts include the manipulation of the human microbiome to cause dysbioses or as an avenue for horizontal gene transfer. Environmental dispersion of a microorganism capable of producing toxins, antimetabolites, or controlled chemicals may also be considered a potential in situ delivery mechanism, one whose outcome would be difficult to predict. The basic principles of pathway engineering in a microbe are the same whether the intention is to culture the organisms in large vessels followed by purification of the molecules of interest or to introduce the organisms into the environment or a human host for in situ production and release of a biochemical.

E (ix+x+xi): BIOWEAPONS THAT ALTER THE HUMAN HOST

ix) *Modifying the human microbiome:*

Human health is highly dependent upon the human microbiome—the microorganisms that live on and within us, especially those associated with the gut, oral cavity, nasopharyngeal space, and skin. These populations of microbes are likely far easier to manipulate than the human host itself, making the microbiome a potentially accessible vector for attack. The human microbiome is the focus of a great deal of academic and commercial research, and microbiome manipulation is an area that is rapidly developing (for more information see also¹¹ i.e. Human Microbiome Project).

Delivery of harmful cargo via the microbiome.

The microbiome could be used as a vector for other types of harmful cargoes, as well. For example, microbes could be modified to produce functional small RNAs (e.g., microRNAs [miRNAs]) that could be transferred to the host via the gut or skin

microbiome to cause a variety of health impacts. Microbes also could potentially be engineered to horizontally transfer a genetic cargo to the native microbiome to, for example, cause a host's own well-established microbes to produce a harmful biochemical. In such a scenario the harmful agent would be manufactured by organisms in the established microbiome, so the engineered microbe would need to infiltrate and persist within the microbiome only long enough to transfer its cargo to a sufficient number of native microbes. Thus, this approach would circumvent the challenges associated with establishing engineered microbes in otherwise occupied niches. It may be possible to harm a population by enhancing the spread of vectors or phage carrying such genetic cargoes [29]. Synthetic biology methods could advance such a capability, for example, through the engineering of toxin:antitoxin couples that would help ensure retention of plasmids. It is also conceivable that microbes could one day be engineered to horizontally transfer genes directly to human cells.

Use of the microbiome to increase the impact of an attack. The microbiome can also potentially be exploited to design a more effective bioweapon or increase the impact of an attack. Knowledge of the human microbiome could be used to modify pathogens or their delivery mechanisms to allow more efficient propagation within or between populations, for example, by taking advantage of the frequent exchange of bacteria between humans and animals. In particular, domestic animals could be used as carriers for engineered agents transmitted via the microbiome. For example, engineered dog or cat microbiomes could be established via adulterated feedstocks or via purposeful contamination of populations in animal shelters or pet stores and then subsequently transmitted to humans. Natural transfers resulting from animal-human contact, such as the transfer of the parasite *Toxoplasma gondii* from cats to humans and the transfer of *Campylobacter* from dogs to humans, illustrate the feasibility of this approach. Similarly, research into the role of the microbiome in pathogenesis could provide a roadmap as to how to generate improved pathogens that are better supported by their microbial peers. Studies involving wide-ranging transposon- or CRISPR-based deletion libraries of pathogens have provided many insights into pathogenesis that might have dual-use implications, and such libraries could prove useful in identifying which genes productively or specifically interact with endogenous flora to better establish a pathogen [30]. In addition to using the microbiome to spread toxins and pathogens, manipulating the microbiome might also prove

¹⁰ Synlogic. 2017. IND-Enabling Studies. URL: <https://www.synlogictx.com/pipeline/pipeline/> (date: 20.01.2021).

¹¹ Human Microbiome Project. NIN. 2020. August 20. URL: <https://commonfund.nih.gov/hmp> (date: 12.01.2021).

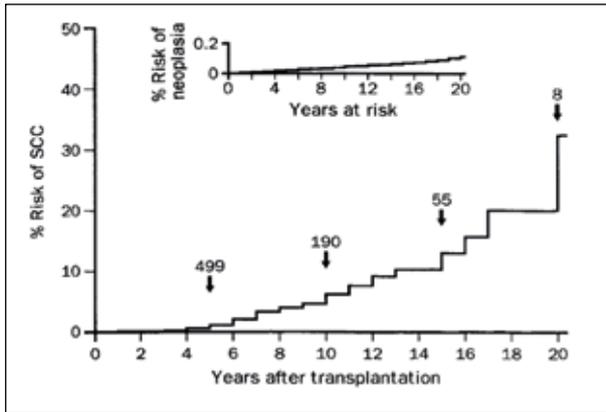


Figure 5 – Cumulative risk of neoplasia after organ transplantation. The risk was 13.6% after ten years and 40% after 20 years. Figures above are numbers of recipients at risk for 5-years period [36]

to be a useful adjunct for other biological threats. Recent research shows, for example, that eukaryotic viruses utilize bacteria to improve their chances of infection. It is also conceivable that an actor could introduce an initial agent into a population in order to trigger widespread treatment with broad-spectrum antibiotics and then take advantage of the treated population’s «clean slate» to introduce or expand an engineered organism via the (now disrupted) microbiome. An actor taking this two-step approach could even incorporate antibiotic or antiviral resistance elements into the initial attack.

Engineered dysbiosis. Our ever-increasing understanding of the human microbiome may lead to opportunities for engineered dysbiosis—that is, the purposeful perturbation of the normally healthy microbiome. This could be accomplished either by causing a known dysbiosis or engineering a new one, and in either case would likely involve introducing otherwise nonpathogenic microorganisms that then lead to diminutions in human health and performance. Since the microbiome likely plays a key role in human dysbioses could also potentially be used to cause longer-term debilitation of a population’s ability to defend against disease. Gut, oral, nasal, and skin microbiomes could be targets for such an approach. The degradation of military readiness due to continued operations in harsh climes is an ongoing issue. This situation could be made much worse by targeted additions to or alterations of the skin microbiome that lead to heightened chafing, rashes, windburn, and itchiness. While these are seemingly minor concerns, over time they could degrade military capabilities to the point of impacting readiness.

x) *Modifying the human immune system*

Human immunity is the bulwark for protection against infectious disease (For quick review see ref. [31–33]). Two basic systems respond to the vast array

of threats in the natural environment. The first is the innate immune system, a collection of nonspecific protective mechanisms triggered by pathogen-associated molecular patterns, such as lipoteichoic acid from Gram-positive bacteria or unmethylated CpG sequences in viral DNA. The second is the adaptive immune system, which generates highly specific antibody and T-cell responses tailored to individual diseases and disease variants. Many natural pathogens manipulate the human immune system, both by suppressing the immune response (e.g., immunodeficiency viruses) and by upregulating certain responses (e.g., respiratory syncytial virus, which induces the immune system to favor a response involving Type 2 T helper cells [Th2] and subsequently increases the proclivity toward asthma. These examples suggest that it may be feasible to develop a bioweapon capable of manipulating or «engineering» the immune response. Several potential forms for such a bioweapon were considered:

Engineering immunodeficiency. Manipulating a target population to have decreased immunity could increase the impact of a biological attack [17, 34]. This goal could be pursued either by manipulating a pathogen to simultaneously reduce immunity and cause disease or by separately introducing an immune-suppressing agent and a bioweapon into a target population. Agents used to cause immunodeficiency could be pathogens (e.g., the insidious spread of HIV (human immunodeficiency virus) or chemicals). It is also possible that a disease agent could be tailored to the immune state of a population, either by engineering the agent to avoid extant adaptive or innate immune barriers or by actually taking advantage of those barriers. Chronic (artificial) immunodeficiency as in patients after solid organ transplants can lead to «spontaneous» tumors (Figure 5) which occurs in 40% recipients in 20 years after surgery [35, 36].

Engineering hyperreactivity. The flip side of engineering immune deficiencies would be to attempt to cause immune hyperreactivity. Both pathogens and chemicals have been demonstrated to create a cytokine storm, a dangerous state that results from a positive feedback loop in the immune response. It may be possible to engineer an agent to purposefully trigger such a cascade. For example, some researchers have suggested that the introduction of anthrax lethal toxin into a more benign disease vector could trigger a cytokine storm. There are already widespread responses in the human population to a limited number of well-known allergens and that may provide a means of engineering biological threats that would trigger life-threatening IgE-mediated immune responses. The development and testing of new immunotherapies could also provide a roadmap for potentially engineering threats; for example, actors

could learn from clinical studies in which anti-CD28 antibodies caused life-threatening cytokine storms.

Engineering autoimmunity. Natural autoimmune diseases cause significant disability and death. It may be possible to engineer a disease that causes the body to turn on itself. Mouse models for the stimulation of autoimmunity now exist. For example, Experimental Autoimmune Encephalomyelitis, which mimics the symptoms of the human malady multiple sclerosis, has been induced in mice by immunization with antigens that cause an immune response [37]. Normally, such self-immunization is prevented by the mechanisms that ensure exclusion of antibodies and T-cells that are self-reactive, but some pathogens may present antigens that are similar enough to the body's own proteins that the original immune response spreads from the pathogen to the new human target. Research into checkpoint inhibitors, compounds designed to unleash the human immune system to eradicate tumors, could also potentially inform efforts to purposely engineer autoimmunity. By overstimulating the immune system, checkpoint inhibitors have been shown to lead to autoimmunity, often in the form of colitis. In addition, particular compounds have been shown to lead to an autoimmune disease of the liver. One potential route of attack could be to introduce such compounds via the microbiome.

xi) *Modifying the human genome:*

In addition to using synthetic genes to impact human physiology through pathogens or modifications to the microbiome, it may also be possible to insert engineered genes directly into the human genome via horizontal transfer, in other words, to use «genes as weapons». Recent improvements in the ability to deliver genetic information via horizontal transfer, for example, through tools such as CRISPR/Cas9, potentially opened the way for synthetic or cross-species transfer of genetic information into human hosts. In addition to protein-encoding genes, genes that encode RNA products such as short hairpin RNAs (shRNAs) or miRNAs could potentially be exploited as weapons in their own right. In combination with technologies for the modification of genes or their expression, deepening insights into systems biology could open new opportunities for causing diseases that are outside the rubric of the types of threats typically focused on in biodefense. Several ways in which synthetic biology approaches could be used to horizontally transfer genetic information to a human target to cause harm were considered:

Deletions or additions of genes. If researchers can create mouse models of particular disease states based on the deletion or addition of particular genes, it follows that if the genomes of human beings could

be similarly modified, such modifications could potentially cause a wide variety of noninfectious diseases. In particular, decades of research on genes associated with oncogenesis – oncogenes – have yielded many examples of gene changes that lead to cancer, including via infection by viruses and bacteria. Oncogenes could potentially be horizontally transferred to human cells via unnatural means. In this vein, CRISPR/Cas9 has been used to create point mutations, deletions, and complex chromosomal rearrangements in germline and somatic cells to develop mouse models for cancer [38].

Epigenetic modifications. Just as programmed genetic modifications are possible, it may also prove possible to use horizontal transfer to alter the epigenetic state of an organism in a way that causes harm. Epigenetic modifications are clearly of immense importance in gene expression and are implicated in disease states and pathogenicity. For example, it is possible to predict the course of oncogenesis based on the epigenetic state of a tumor. Sequence-specific epigenetic modifications can be carried out by small RNAs in other species, such as plants, but are not extensive in humans. However, the sequence-specific binding capabilities of Cas9 and other CRISPR elements may allow fusion proteins to carry out sequence-specific epigenetic modifications [39]. There are also chemicals that yield relatively nonspecific epigenetic changes [40].

Small RNAs. Small RNAs are another example of functional genetic information that could be horizontally transferred. Small RNAs, although not a genome modification per se, are important because they may prove capable of modifying gene expression and bringing about phenotypic change. The large number of small interfering RNA (siRNA), short hairpin RNA (shRNA), micro RNA (miRNA) and other small-RNA library studies in a variety of species and cells from different species, including human, provides a potential roadmap of what sequences may lead to what disease states or to modulation of defenses against disease (for review see [41]). Similarly, there are already numerous viral and other vectors that can encode and express small RNAs. The known fact that many viral pathogens already seem to encode small RNAs that aid in their pathogenicity further underlines this possibility [42]. For example, the oncogenic gamma herpesviruses Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV) encode miRNAs that clearly act as mediators of immune suppression [43]. While most gene delivery mechanisms would likely be facilitated by CRISPR elements, direct delivery of small RNAs via liposomes or other vehicles has proven possible in many cell types. More recently the delivery of entire messenger RNAs (mRNAs) has proven useful for vaccination and cellular reprogramming [44]. Naked RNA is generally considered to be fragile

due its susceptibility to ribonuclease in the cell, and its delivery is largely confined to laboratory settings, but there are approaches for stabilizing RNAs (e.g., using liposomes, nanoparticles, synthetic polymers, cyclodextrins, ribonucleoproteins, and viral capsids). RNA can be expressed from genes delivered as simple expression vectors, as low fitness-burden cargoes on viral pathogens, or via CRISPR element insertion. One reason that RNA delivery is potentially a viable biological threat is that even a small initial skew in gene expression (such as the changes in gene expression normally caused by miRNAs) could greatly alter the probability of an initial cellular alteration. Even small amounts of a targeted RNA would not modify the genome per se but might allow or encourage cells to begin the process of self-transformation to tumors, as evidenced by the fact that a large number of pro-oncogenic miRNAs have already been discovered [45]. In addition to RNAs produced by viruses, bacteria produce numerous small regulatory RNAs; introduction of these into the endogenous microbiome could lead to dysbiosis. Larger mRNAs can also be delivered via liposomes and nanoparticles or by RNA replication strategies being developed for vaccine production; these methods could potentially be used to express deleterious cargo such as toxins or oncogenes, similar to threats related to DNA vectors.

CRISPR/Cas9. CRISPR elements can be harnessed for site-specific cleavage of genes, followed by homologous recombination via double-strand break repair or other mechanisms. This technology has revolutionized genome engineering. The fact that DNA recognition can be programmed by simple modification of an RNA element makes precision targeting of genome change much easier than previous technologies such as zinc finger endonucleases and TAL effector nuclease (TALEN)-mediated sequence specific recognition of DNA. Another advantage of CRISPR technology is its broad host range; CRISPR elements are able to recognize and bind to DNA sequences in species other than those in which they originally evolved. Thus, the fact that gene editing technologies such as CRISPR make possible genomic changes in animal models that directly impact health and pathogenesis further implies that it may be possible to manipulate either germline or somatic cells to make such changes in humans. Significantly, the sequence specificity of CRISPR elements might also make possible ethno specific targeting of gene-based weapons depending on the distributions of

alleles. In terms of delivery, CRISPR elements could potentially be loaded onto a pathogen or delivered via the microbiome to modify human genomes in a way that would pose harm to individuals or populations.

Human gene drives. Because of the ability of CRISPR elements to modify genomes, they can be repurposed as selfish genetic elements in their own right, wherein their introduction into a naïve genome leads to their site-specific establishment. In sexually reproducing organisms, an appropriately modified CRISPR element or other homing endonuclease gene, when used as a gene drive, can spread throughout a population. Gene drives are well known in nature, such as the *Drosophila P* element, which moves nonspecifically through naïve populations based on sexual (vertical) transfer. Gene drives have recently proven to be extremely useful for engineering mosquito populations for infertility [46]. They have been proposed for the attenuation of fitness in other undesirable species, as well¹².

It should be noted that exome sequence data are being generated at an exponential rate, the introduction of CRISPR elements in humans or other higher organisms would likely be identified quickly and immediately recognized as cause for alarm. The presence of previously unknown oncogenes in viruses not normally known to harbor oncogenes would also be an immediate cause for alarm. However, the surreptitious spread of an oncogenic small-RNA sequence, especially if it is embedded within a protein-encoding gene, might be less noticeable and thus evade detection. In addition, threats related to horizontal gene transfer in synergy with the threats posed by pathogens may lead to new modes of attack. Just as clinical trials of immunotherapies are increasingly a roadmap for engineering cytokine storms, the increasing knowledge on gene deletions, gene additions, and small-RNA modifications of human cells may provide a roadmap for the induction of noninfectious disease states that could be abetted by pathogen engineering (and, conversely, that could abet the spread of the pathogens themselves, such as via immunodeficiency viruses).

Finally, let us fully cite from the book *Biodefense in the Age of Synthetic Biology* p. 92, printed in 2018:¹³

«More insidiously, it is possible that some diseases could be engineered not only to target but to actively take advantage of known immune prevalences, in particular

¹² National Academies of Sciences, Engineering, and Medicine. *Gene Drives on the Horizon: Advancing Science, Navigating Uncertainty, and Aligning Research with Public Values*. Washington, DC: The National Academies Press. 2016.

¹³ *Biodefense in the Age of Synthetic Biology* 2018. ISBN 978-0-309-46518-2. <https://doi.org/10.17226/24890> (Committee on Strategies for Identifying and Addressing Potential Biodefense Vulnerabilities Posed by Synthetic Biology, Board on Chemical Sciences and Technology, Board on Life Sciences).

those related to vaccination. An extremely sophisticated adversary, knowing in advance the likely fitness landscape of a given pathogen, could release an engineered pathogen that is «designed to evolve» in particular ways upon encountering the most likely human immune response. For example, if an immunodominant epitope is known, and if previous modeling or experimentation had indicated the range of likely sequence substitutions in response to the antibodies already present due to vaccination,

and if some of these sequence substitutions lead to increased engagement with a cell surface receptor, then the sequence of the pathogen could be poised in advance to evolve greater lethality or transmissibility. The advantage of this approach, from a malicious actor's perspective, is that a milder form of a disease could spread broadly and then «self-activate» as a result of «designed evolution» to become a pandemic».

It is not very much that one can add to this prophetic paragraph.

Author Contribution

Elaboration of the concept of the paper; collection, analysis, and systematization of scientific literature; writing and edition of paper.

Conflict of interest statement

I am declaring that I prepared the article from sources freely available on the Internet and free available publications, figures and other possible legal sources. I, as a sole author declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

Peer review information

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Синтетическая биология – друг или враг? Каких угроз нам следует ожидать?

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Синтетическая биология – недавно появившаяся ветвь технологий двойного назначения, новая область применения инженерных принципов в биологии. *Цель работы* – показать основные технологические приемы этой технологии и привести конкретные примеры ее использования для создания новых видов биологических поражающих агентов и приемов ведения биологической войны, ранее немислимых и представленных только в научно-фантастических книгах. Основные инструменты и методические приемы синтетической биологии: синтез и секвенирование больших фрагментов ДНК; разработка «платформы» («шасси») – т.е. системы-хозяина, несущей генетический набор инструментов для экспрессии желаемых генов сконструированного биологического пути, доставляемых подходящими векторами; разработка систем транскрипции, не истощающих ресурсы клетки (синтетические промоторы и факторы транскрипции); инструменты модификации генома (нуклеаза CRISPR/Cas9, нуклеазы цинковых пальцев, TALE нуклеазы, мегануклеазы); и компьютерные инструменты (участвующие в базовом структурном проектировании и синтезе; в проектировании сети; в прогнозировании поведения/функции/реакции). Синтетическая биология уже показала большие возможности в воссоздании известных патогенных вирусов и патогенных бактерий; в повышении опасности для людей существующих патогенных бактерий и вирусов (например, путем повышения их вирулентности или способности преодолевать иммунитет); создании патогенов, ранее не существовавших в природе; производства токсичных химикатов или биохимических веществ с использованием естественных и искусственных метаболических путей; изготовлении токсических веществ посредством синтеза *in situ*; изменение микробиома человека; изменения иммунной системы человека; модификации генома человека путем добавления, удаления или модификации генов или посредством эпигенетических изменений, которые изменяют экспрессию генов и могут передаваться от родителя к ребенку во время репродукции, распространяя генетические изменения в популяции. В работе подробно рассмотрены возможности синтетической биологии для разработки новых средств и способов ведения биологической войны. Например, введение в геном вируса экстремелии (оспы

мышей) гена интерлейкина-4 значительно повысило его вирулентность для мышей. Он подавлял первичные иммунные ответы и преодолевал ранее существовавший иммунитет к исходному вирусу. Аналогичный результат был получен с рекомбинантным аденовирусом, кодирующим нейрональный токсин индийской змеи бунгаруса – бунгаротоксин. Путем введения в составе инъекционных препаратов антигенов, похожих на собственные белки человека, можно за короткий срок вызвать аутоиммунное состояние у тысяч людей, которое проявится у них через годы рассеянным склерозом и другими энцефалопатиями. Приводится работа, опубликованная в 2018 г., в которой утверждается, что современные технологии синтетической биологии и математического моделирования эпидемий, если уже известен иммунодоминантный эпитоп патогена, позволяют сконструировать такой его вариант, который при «столкновении» с наиболее вероятным иммунным ответом человека будет эволюционировать в сторону большей контагиозности и способности преодолевать иммунитет, сформировавшийся в результате уже перенесенной болезни или вакцинации. Автор считает, что необходимо не только постоянно отслеживать эти новые биотехнологии двойного назначения, но и совершенствовать традиционные и научные методы мониторинга их использования.

Ключевые слова: синтетическая биология; угроза; возбудитель; бактерии; вирус; человек.

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Вклад автора

Разработка концепции статьи; сбор, анализ и систематизация научной литературы; написание и издание статьи.

Заявление о конфликте интересов

Я заявляю, что подготовил статью на основании источников, свободно доступных в Интернете, бесплатных публикаций, рисунков и других легальных источников. Я, как единственный автор, заявляю, что исследование проводилось при отсутствии каких-либо коммерческих или финансовых отношений, которые могут быть истолкованы как потенциальный конфликт интересов.

Сведения о рецензировании

Статья прошла открытое рецензирование двумя рецензентами, специалистами в данной области. Рецензии находятся в редакции журнала и в РИНЦе.

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