TABLE 6-1 Bottlenecks and Barriers That Currently Constrain the Capabilities Considered and Developments That Could Reduce These Constraints[a]

Capability	Bottleneck or Barrier	Relevant Developments to Monitor
Modifying the human microbiome	Limited understanding of microbiome	Improvements in knowledge related to microbiome colonization of host, in situ horizontal transfer of genetic elements, and other relationships between microbiome organisms and host processes
Modifying the human immune system	Engineering of delivery system	Increased knowledge related to the potential for viruses or microbes to deliver immunomodulatory factors
	Limited understanding of complex immune processes	Knowledge related to how to manipulate the immune system, including how to cause autoimmunity and predictability across a population
Modifying the human genome	Means to engineer horizontal transfer	Increased knowledge of techniques to effectively alter the human genome through horizontal transfer of genetic information
	Lack of knowledge about regulation of human gene expression	Increased knowledge related to regulation of human gene expression

[a]Shading indicates developments that are likely to be propelled by commercial drivers. Some approaches, such as combinatorial approaches and directed evolution, may allow bottlenecks and barriers to be widened or overcome with less explicit knowledge or tools.

7

Related Developments That May Impact the Ability to Effect an Attack Using a Synthetic Biology–Enabled Weapon

Synthetic biology is a sophisticated, programmable platform that could in theory enable the development of a wide range of biological and chemical weapons. However, for a capability to warrant concern in the context of this study, it must not only be possible to create an agent in the laboratory but also to use the agent to effect an attack. For many of the potential malicious applications of synthetic biology that were considered, the level of concern raised by technological capabilities is tempered by constraints related to the need to produce the agent in volumes needed to achieve the desired scope of casualty, keep it stable until use, and deliver it to the population in a manner that yields the desired harm. Despite the impressive capabilities afforded by synthetic biology and other modern biotechnologies, these requirements, many of which are the same barriers to weaponization that have constrained the development of bioweapons in the past, are in many cases an important limiting factor in the context of synthetic biology–enabled weapons.

However, these challenges may well be overcome in the future, either by advances in synthetic biology or by developments in other fields. This chapter explores some developments that may become more important in this respect in the coming years. While a comprehensive analysis of technologies being pursued outside of synthetic biology was not conducted as part of this study, these examples are offered to highlight a few areas that will be important to monitor, because they could converge with synthetic biology advancements and ultimately reduce or eliminate barriers to the use of synthetic biology–enabled weapons.

BARRIERS TO THE USE OF BIOWEAPONS

Within the factor usability as a weapon, the report's framework for assessing the potential for the weaponization of agents produced using synthetic biology identifies questions around production, fidelity, stability, delivery, testing, and targeting. Aspects of these attributes as they relate to specific potential applications of synthetic biology are discussed in Chapters 4–6; broader challenges and considerations related to them are described briefly in the following sections. In general, the challenges posed by each attribute largely depend on the potential nature and scope of an intended attack, which could range, for example, from a targeted assassination of one individual to mass casualty across a population. Although a variety of potential circumstances were considered in the assessments presented in this report, it was generally assumed that an actor would seek to develop the bioweapon covertly and minimize the likelihood of attribution once the agent is deployed. However, the possibility of assigning attribu-

tion for a biological attack is not necessarily a deterrent for terror groups, who may choose to affirm their own responsibility or power and who may not fear discovery and subsequent retribution.

Production

Challenges associated with agent production largely depend on the quantity desired. Large-scale production of a bioweapon is extremely challenging because many agents lose infectivity or other features during scale-up. Although synthetic biology technologies may enable improved cell culture methods, innovations in fermentation, and improved ways to mass produce particular chemical and biological components, the large-scale production of bioweapons is still likely to require significant financial and intellectual resources. On the other hand, mass production may not be needed to perpetrate smaller, more narrowly focused attacks or attacks that can be spread by a replicating pathogen.

Fidelity and Testing

Although it is possible to design and build biological constructs or systems without testing, significant synthetic biology achievements are typically rooted in repeated Design-Build-Test cycles, with testing being a crucial step in the process. Testing in computer simulations, cell cultures, or animal models is a labor- and time-intensive process, and learning from the testing process to make design improvements for the next Design-Build-Test iteration can require a great deal of expertise and experience. Success in computer simulations, cell cultures, and animal models does not necessarily guarantee success in humans, because of differences in evolutionary pressures. Fidelity is also not guaranteed, and it can take repeated process improvements to develop a system that will reliably produce the same results every time, especially at scale. Some synthetic biology approaches, such as directed evolution, integrate testing together with other steps in the process, potentially offering a more streamlined option to circumvent resource-intensive testing steps. It is also conceivable that malicious actors would forego some of the rigorous testing that other researchers would perform, since the standard of success—creating an agent capable of doing "enough" harm—is markedly different from the standards involved in publishing results in a scientific journal. Malicious actors may also be able and willing to test in human subjects, unhindered by the moral considerations and ethical frameworks that guide other research efforts. Despite these caveats, however, developing a synthetic biology–enabled bioweapon would likely still require significant testing to achieve a product that is reliable and effective enough for the actor's purposes.

Delivery

A critical consideration in the development of a bioweapon is the capability to deliver it to the intended target population. At smaller scales, delivering a bioweapon can be as simple as contaminating food or water, sticking victims with a needle, or even smearing the agent on victims' skin (CBC, 2017). Larger-scale attacks typically involve some form of aerosol dispersal, such as via a spray or an explosion, which may require that the agent not only be prepared at the optimal particle size for inhalation but also be able to withstand freeze drying, suspension in aerosol preparations, packaging processes, long-term storage, and adverse environmental conditions such as ultraviolet sunlight or extreme temperatures (Frerichs et al., 2004). Such requirements may impose significant barriers to bioweapon development, even with available biotechnologies. While synthetic biology could potentially be used to increase a pathogen's environmental stability, infectivity, transmissibility, or tolerance for weapons delivery systems, maintaining potency or viability throughout the production, storage, and delivery process is still likely to present a significant challenge, particularly for large-scale attacks.

The agent's ability to be transmitted from one individual to another is an important consideration in terms of both production scale and delivery. A communicable agent could theoretically be deployed in small amounts at multiple locations and allowed to spread on its own. Some actors may even find volunteers willing to spread infection by becoming infected themselves, akin to suicide bombers.

Targeting

Attacks may target individual people; groups of people who share a common geography, occupation, ethnicity, or other attribute; or entire populations. Historically, targeting of bioweapons has been based largely on geographic location of the intended victims. Biotechnology advances may offer new opportunities for a malicious actor to influence the overall impact of an attack or the specific individuals affected, such that an agent could be deployed over a broad geographic area but only sicken targeted individuals. For example, actors may consider designing a bioweapon to target particular subpopulations based on their genes or prior exposure to vaccines, or even seek to suppress the immune system of victims to "prime" a population for a subsequent attack. These capabilities, which were feared decades ago but never reached any plausible capability, may be made increasingly feasible by the widespread availability of health and genomic data. While some fundamental barriers still likely limit the success and reliability of such an effort—for example, the United States' genetic diversity may make the U.S. population resistant to targeting based on ethnicity—it is nonetheless crucial to continue to monitor developments that could facilitate targeting of particular populations.

RELEVANT CONVERGENT TECHNOLOGIES

The challenges associated with effecting an attack using a synthetic biology–enabled weapon may be overcome by emergent (new) or convergent capabilities. In the context of technology, convergence occurs when different technologies, often from different fields, create synergies that significantly advance capabilities when they are combined (Roco, 2008). In other contexts, convergence has been described as the formation of a framework to solve scientific and societal challenges that exist at the interfaces of multiple fields (NRC, 2014). In either conceptualization, the merging of diverse areas of expertise can stimulate innovation, from basic science discovery to translational application, which can advance beneficial and malicious goals alike. Convergence can happen through gradual advances over time or occur quite suddenly, taking everyone by surprise. This study considered how developments in multiple fields may converge with biotechnological developments to enable new breakthroughs in the Design-Build-Test cycle or act as "force multipliers" in advancing synthetic biology capabilities. Convergence, of course, can go both ways; as synthetic biology incorporates technologies from other fields, so too will other fields incorporate approaches from synthetic biology, potentially leading to more interdisciplinary collaboration and further breakthroughs. While synergies among technologies are included in the framework within usability of the technology, it is useful to consider how emergent and convergent technologies may allow breakthroughs specifically in aspects relevant to weaponization, since these factors are thought to be in many cases a significant limitation.

To that end, several examples were identified to explore technologies being pursued in fields and toward ends that are not directly related to synthetic biology, yet may converge with biotechnology in ways that help overcome some of the challenges related to creating weapons with synthetic biology. These include gene therapy, nanotechnology, automation, additive manufacturing, genomic data, and health informatics. The potential impacts of these technologies are discussed below and summarized in Table 7-1.

Gene Therapy

Gene therapy has been in development for use in therapeutics for several decades (Moss, 2014), and it can take a number of forms. In an approach known as ex vivo gene therapy, tissues are genetically altered in the cell culture and then transplanted into the body (Hacein-Bey-Abina et al., 2002). Although ex vivo gene therapy is not likely a viable approach for delivering bioweapons, the ability to transduce cells and tissues ex vivo could inform vector improvement and design and provide proof of principle for novel means of delivering substances, thereby providing an in vitro test capability for small-scale bioweapon design and development.

Another approach, known as in vivo gene therapy, might have other implications for bioweapons development. Using this approach, a component (usually a viral vector) is introduced into the body, potentially to a specific target tissue, where it delivers genetic material that creates the desired therapeutic function (Naldini et al., 1996; Kay et al., 2001). Viral vectors are typically chosen as the delivery vehicles because of their naturally evolved ability to

TABLE 7-1 Summary of How Selected Examples of Convergent Technologies May Affect Challenges of Effecting an Attack Using a Synthetic Biology–Enabled Weapon[a]

	Production	Stability	Fidelity	Testing	Targeting	Delivery
Gene therapy			■	■	■	■
Nanotechnology		■			■	■
Automation			■	■	■	
Additive manufacturing	■		■	■		
Health informatics					■	

[a]Shading indicates which attribute each example aligns with most closely.

target specific cells of the human body; their disease-causing genes are removed and replaced with the engineered genetic components. As gene therapy viral vectors continue to be optimized for therapeutic use, their capability to act as delivery vehicles for bioweapons, such as toxin-producing pathways (as discussed in Chapter 5, Making Biochemicals Via In Situ Synthesis) will advance apace.

Gene therapy vectors being researched include adenovirus, adeno-associated viruses, alphaviruses, herpesviruses, retrovirus/lentiviruses, and vaccinia virus (see Table 7-2); gene therapies using retroviruses, adeno-associated virus, and adenoviruses have already advanced to human clinical trials (Edelstein et al., 2007) and in some cases to clinical approval (FDA, 2017a,b; Spark Therapeutics, 2017). The ability of these vectors to transfer genes into cells and the permanence of the edits they make differ from vector to vector. The size of the viral genome is also important, because the size of the engineered gene that can be transferred is limited to what the virus can successfully carry. While problems such as host immune responses, off-target effects, and decay of continued expression have been barriers to successful gene therapy (Verma and Somia, 1997; Mingozzi and High, 2013), work to address these barriers is being conducted and these challenges might not be of concern to an actor seeking to use the approach to deliver a bioweapon as long as the intended victims experience the intended illness or lethality. As gene therapy vectors continue to be made more efficient and coaxed to carry larger transgenes, gene therapy research could pave the way toward circumventing some of the barriers related to delivery of bioweapons.

Most gene therapies today are delivered via injections to target tissues, a route ill-suited to stealthy or widespread delivery of a weaponized gene therapy vector (though perhaps a viable strategy for targeted assassination). The development of inhalable gene therapy is advancing rapidly, however, particularly for treatments of respiratory diseases such as chronic obstructive pulmonary disease and cystic fibrosis (Zarogoulidis et al., 2013). Advances such as these may provide more expanded capability in the future as the aerosol therapy market continues to drive

TABLE 7-2 Characteristics of Viral Vectors Used in Gene Therapies

Characteristic	Adenovirus	Adeno-Associated Virus	Alphavirus	Herpesvirus	Retrovirus/ Lentivirus	Vaccinia (Poxvirus)
Genome	dsDNA	ssDNA	ssRNA (+)	dsDNA	ssRNA (+)	dsDNA
Genome size	39kb	5kb	12kb	120–200kb	3–9kb	130–280kb
Host genome integration	No	No	No	No	Yes	No
Transgene expression	Transient	Potential for long lasting	Transient	Potential for long lasting	Long lasting	Transient
Maximum size of transgene(s)	7.5kb	4.5kb	7.5kb	30kb	8kb	25kb

innovation for therapeutics. Efforts toward aerosolized delivery of vaccines are also advancing rapidly; this research may contribute to innovations in routes of delivery for gene therapies (Low et al., 2015). As these technologies progress and new therapeutics come to market, facilities manufacturing aerosolized therapeutics are likely to proliferate, raising the possibility not only that such approaches may be misused for the creation of bioweapons but also that apparently aboveboard manufacturing facilities could mask subversive programs to develop bioweapons delivery systems.

Although the viral vectors used in gene therapies are heavily engineered to remove the genes that cause disease and these viruses are used under exacting conditions that guard against spread, viruses have a history of evolving around constraints, and it remains possible that a single-use gene therapy vector could become "lytic," leading to the spread of a disease. This is of limited concern for work involving many of the viruses in Table 7-2, which have often been heavily engineered to not propagate in the host. However, there has been a rise in the use of viruses, especially measles and vaccinia, for so-called oncolytic therapies in which the virus replicates in a cancer cell and spreads to surrounding cells (Haddad, 2017). Future studies that chart the evolution of oncolytic viruses in human hosts could potentially become roadmaps for the design and construction of effective bioweapons, if only because they bring into high relief the characteristics of the virus that have the greatest impact on tropism, spread, and pathology.

Nanotechnology

Nanotechnology is driving innovations in the delivery of gene therapies and other therapeutics. Actors with access to nanotechnology tools could adapt these platforms for malicious use, with implications for delivery of pathogens or toxins as well as targeting attacks. Smaller vehicles in general have much better pharmacokinetic and pharmacodynamic properties, making them more effective in penetrating tissues and cells. Nanoparticles used in drug formulations include imprinted polymers, dendrimers, vesicles, nanospheres, nanocapsules, micelles, carbon nanotubes, liposomes, and nanoemulsions (IAP, 2015), and additional nanocarriers are also being researched, including DNA- and viral-based systems.

Engineered nanotechnology could be used to assist in the weaponization of an agent in numerous ways (Kosal, 2009). For example, nanotechnology could be used to create microcapsules or nanocapsules that encase the agent and improve stability or delivery (Koroleva et al., 2016); to make delivery particles more environmentally stable; to create storage devices for biological products; to create specialized nanoparticles that respond to ultraviolet light (Jalani et al., 2016), are activated remotely, or are engineered to evade the immune system (Zolnik et al., 2010; Rodriguez et al., 2013); to confer the ability to penetrate skin or invade into tiny bronchioles in the lung, cross the blood-brain barrier (Saraiva et al., 2016), or target other specific tissues; or to provide advanced aerosolization capability. An example of one nanoparticle formulation and its use as a delivery platform is discussed in Box 7-1.

Automation

Automation is growing rapidly in nearly every field. In biology, the growth of automation is evident in the integration of technologies such as microfluidics, mass spectrometry, bioinformatics, and machine learning into laboratory processes. Automation tools allow researchers to screen ever-larger collections of genetic sequences or physical samples for a wide variety of properties; it is now possible to produce and screen hundreds of thousands of clones and variants in a matter of weeks. Malicious actors could take advantage of these capabilities to, for example, streamline testing of agents, increase fidelity, and fine-tune targeting, potentially while evading mechanisms to detect or screen for malicious activity. Although sequence annotation is becoming more precise, many algorithms must still use unvalidated and unverified data (Poptsova and Gogarten, 2010). This creates "noise" in the system that could inform the design of bioagents or allow malicious actors to undermine legitimate research by, for example, deliberately submitting incorrect genomic data to public databases to mask one's own work or to sabotage the detection efforts of others.

Standard laboratory robotics is now within the reach of virtually any laboratory. By enabling massively scaled-up experimentation and testing, these tools can significantly shorten the time frame of the Design-Build-Test cycle

BOX 7-1
Nanolipoprotein Particles as an In Vivo Delivery Platform

As part of its information-gathering process, the committee received a presentation by Amy Rasley, Ph.D., Lawrence Livermore National Laboratory, on nanolipoprotein particles (NLPs). NLPs are a biomimetic platform enabling in vivo delivery of various nucleic acids, proteins, carbohydrates, and small organic compounds. They are created as a circular lipid bilayer "raft" composed of amphipathic (both hydrophobic and hydrophilic) phospholipids held together by a scaffold composed of amphipathic lipoproteins.

NLPs are created with biocompatible components to avoid the target organism's immune system (i.e., the scaffold proteins are chosen to match the proteins of the target organism). NLP assembly is facile and can be easily scaled up. NLPs can also be lyophilized, thus avoiding the need for cold-chain storage. The size of NLPs can range from 8 to 25 nanometers, permitting them to be tuned for delivery by a variety of routes (e.g., inhalation, injection). They are also versatile, capable of being conjugated with proteins, peptides, oligonucleotides, carbohydrates, or small organic compounds.

All components of NLPs can be produced synthetically without the use of any living systems, and NLPs can be customized for specific applications whose payloads vary drastically in terms of size, charge, hydrophobicity, and functionality. There is thus a wide range of flexibility and possible uses of NLP technology for medical therapeutic purposes and also the potential for misuse of NLP technology as a delivery platform for harmful agents. Detection of bioweapons using NLPs would be difficult, since the scaffold protein would be a native human protein, the NLP half-life in vivo is short, and NLPs are not self-replicating.

SOURCE: Fischer et al., 2013, 2014.

overall and potentially improve the likelihood of producing the desired biological functionality. Microfluidic tools, which provide the capability to handle small volumes, control laminar fluid flows, and measure perturbations and timescales within biological systems, are becoming particularly common and are used in a wide variety of research arenas, including drug development and the development of sensors for detecting biomarkers, biohazards, or pollutants (Dittrich and Manz, 2006; Berkeley Lights, 2017). In synthetic biology, microfluidics tools are being adopted to make the testing of biological products or systems fast, inexpensive, and robust. By facilitating testing of many agents at small scale and potentially low cost, these tools could provide malicious actors the capability to develop bioweapons by systematically incorporating multiple genetic variations to synthesize and screen multiple variants (a combinatorial approach) rather than a precise, knowledge-based approach. In addition, the automation of protein design, enabled by mass spectrometry, potentially allows hundreds of thousands of variants to be tested, assessed, and used for refining the design of protein properties via machine learning algorithms (Huang et al., 2016). The combined use of automated design with microfluidics can potentially enable an actor to rapidly develop and test multiple versions of a potential agent at small scale, at low cost, and with relatively limited prior knowledge of how to engineer the desired phenotypic result. For desired results such as lethality, combinatorial design and screening could also provide enough confidence in the behavior of an agent that the actor may not need to pursue larger-scale testing, as well as provide a way to achieve proof of principle for facilitating fidelity during production scale-up. Finally, microfluidics in particular can also create synergies with other areas such as nanotechnology by facilitating the creation of homogeneous nanoparticles for agent delivery.

Additive Manufacturing

Additive manufacturing technologies, also known as 3D printing, have emerged to create advanced materials with superior performance, lower environmental impacts, or new functionalities. A variety of materials with

complex biological architectures have been successfully emulated in synthetic systems, such as spider silk and leather (Qin et al., 2015). Although the vast majority of commonly available 3D printing technologies have been unable to sustain living cells, this capability is rapidly advancing (Richards et al., 2013). Examples include the development of 3D printers to generate replacement organs or pharmaceutical testing tissues such as livers and hearts (Robbins et al., 2013); the use of a modified inkjet printer to print layers of *Escherichia coli* (Lehner et al., 2017); the printing of viable natto bacteria into clothing (Yao et al., 2015); and the proposed use of 3D printing to generate oncolytic viruses (Swenson, 2015).

It is conceivable that one could produce, with biological 3D printing, engineered microbes, viruses, toxins, or other biological products. This capability could also be used to create biological material that could be used as a platform to test bioagents at relatively low cost, or to explore techniques for ensuring bioagent fidelity. Such activities could likely be pursued surreptitiously, because the creation of a small amount of a highly infectious bioagent using a 3D printer would be hard to detect.

Currently, 3D printers tailored specifically for biologicals are still rather expensive and require high expertise; they are not available to the public in libraries and other common spaces as plastics-based 3D printers are. However, as the technology continues to advance, costs may decrease and these devices may become more widely available.

Health-Associated Data and Bioinformatics

In the era of genomics, it has become increasingly feasible to design medical therapeutics tailored to the genetic makeup of an individual or a population. This approach, known as "precision medicine," relies on the ability to amass large amounts of human genomic data. Sequence data alone are not sufficient, however; it is also necessary to understand genotype–phenotype functional relationships, which often entails tracing epigenetic modifications, metabolism, and changes in protein expression in response to environmental or other factors. The data necessary for such insights can be extracted from blood tests, urinalysis, and a range of other data points stored in individual health records.

Approaches that attempt to link human genomic data with other health metadata are becoming the preferred models for the pharmaceutical industry, making this an extremely active area of research. Not only does this facilitate the pursuit of many more "precise" drug targets, but genomic data, in the context of health metadata, can also allow for reverse engineering approaches for the synthesis of novel small molecules with therapeutic potential (Kim et al., 2016).

None of these approaches is possible without sophisticated bioinformatics and machine learning capabilities that link, correlate, and analyze the data. Such sophisticated techniques also are highly dependent upon having enough correctly annotated data to be able to determine the biomarkers needed to identify specific human conditions of interest. This is likely to present a barrier, particularly for rare or complex multivariant conditions; the existence of more than 5 million known human genetic polymorphisms (Hall, 2011; but GHR [2018] estimates as high as 10 million) hints at the difficulties of trying to determine causative disease factors even with thousands of well-curated patient samples.

While the tailoring of diseases (or spread of diseases) to subpopulations or individuals would not be an exact science, a relatively sophisticated adversary could seek to exploit genomic and health data. The use of genomic data, health metadata, and tailored bioinformatics will continue to advance in the realm of pharmaceutical research, and these advances could enable enhanced targeting capabilities for the development of bioweapons. The vast amount of healthcare data that are now available electronically and the multiple documented incursions into those data, including by foreign powers (Krebs on Security, 2013; Ponemon Institute, 2013; Filkins, 2014; Perakslis, 2014), raises the possibility that an adversary could bypass cybersecurity barriers, identify unique vulnerabilities for specific subpopulations, and then develop bioweapons tailored to target those vulnerabilities. For example, this approach could be used to develop ethnospecific bioweapons. Retroviruses integrate into the genome upon infection, and the integration mechanisms of these viruses could theoretically be altered to greatly favor one genotype over another. Similarly, the existence of population-specific differences in the sequences and structures of receptor proteins suggests that computational modeling, high-throughput screening, or directed evolution could be used to more finely direct an agent to target a specific subpopulation. While such targeting might be more

readily accomplished with known genetic subtypes (such as ethnic subgroups), it may also be possible to target geographic regions or nation-states semiselectively based on allelic distributions in human populations. It may even be possible to drive targeting to an even finer level, raising the specter of "personalized terrorism."

An increasing knowledge of the human immune system and the ranges of individual responses to diseases also may open opportunities for probabilistic targeting of subpopulations. The ethnic prevalence of preexisting pathogens or the national prevalence of immunotypes (due to vaccination strategies in different countries) could, for example, be exploited in the design of bioweapons targeted to individuals with certain disease or vaccine exposures. General engineering of lowered immunity (discussed in Chapter 6, Modifying the Human Immune System) could lead to additional local endogenous viral reactivation. Similarly, given the somewhat regional nature of even highly cross-reactive allergens, knowledge of a subpopulation available from (stolen) health records might provide clues for probabilistic targeting of anaphylactic shock.

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 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. As noted in Chapter 4, however, designing such a "new" pathogen is currently far from feasible.

The probabilistic targeting of a disease to unique subpopulations could be used to drive particular military outcomes. Although chicken pox vaccination reduces the importance of this particular example, if a large fraction of a given military cadre is known to have been exposed to a virus such as varicella zoster virus (which causes chicken pox) and is thus at risk to develop a subsequent disease such as shingles, attempting to reactivate and augment this disease might be a viable attack vector. Indeed, the use of probabilistic targeting might prove to be especially important for driving military outcomes in an age where public health measures in the military are virtually universal and can be readily distributed. Probabilistic targeting, combined with targeting via geographic distribution and timed introduction, might be amenable to a larger-scale attack on a region by a more ubiquitous pathogen that could be readily detected and shut down through conventional public health countermeasures.

SUMMARY

- Continued convergence may help overcome some barriers to usability as a weapon for synthetic biology–enabled bioweapons.
- Commercial and other drivers will push developments in these convergent fields, and these advances will also expand opportunities for misuse.
- Medical applications are a key driver for a number of important converging technologies.

While factors such as scale-up, stability, fidelity, and delivery are likely to continue to pose barriers to the weaponization of biological agents, a number of technological developments could create synergies with synthetic biology capabilities that allow malicious actors to overcome these barriers. In this chapter, five examples of convergent technologies at various stages of development (see Table 7-3) are presented that may help reduce barriers in various aspects of weaponization (see Table 7-1). It will be important to monitor future developments in these and other areas to identify and assess vulnerabilities that could facilitate bioweapons development. Such developments might result in significant raising of the level of concern related to the synthetic biology–enabled capabilities examined in this study (see Figure 9-1 and Table 9-1).

TABLE 7-3 Summary of Relative Maturity of Selected Convergent Technologies[a]

Technology	In Development	In Use by Developers of the Technology	In Use by Synthetic Biology Community	In Use by Molecular Biology Community	In Use by Amateur Biologists
Gene therapy					
Nanotechnology					
Automation					
Additive manufacturing					
Health informatics					

[a]For each column, darker shading indicates the technology is in routine use for that community, lighter shading indicates emerging use, and white background indicates little or no use. Adoption flows from left to right in most cases.

8

Options for Mitigating Concerns

The study included consideration of opportunities to mitigate concerns related to the malicious use of biotechnology. The potential for mitigation was an integral part of the framework for assessing concern, as detailed in Chapter 3. As described in Chapters 4–6, considerations relevant to mitigation were included in the assessment of concern for specific potential capabilities, although these assessments did not include an in-depth analysis of current preparedness and response capabilities or speculate about the efficacy of various potential approaches. This chapter explores, from a broader perspective, some current mitigation approaches, how synthetic biology may challenge those approaches, and conversely, how synthetic biology may help address challenges or bolster mitigation approaches. A comprehensive, in-depth review of strengths and weaknesses in current U.S. or international programs was outside the scope of this study; as such, this report does not offer a full analysis of mitigation capabilities and makes no recommendations pertaining to mitigation priorities. Rather, this chapter is intended to provide useful context about fundamental mitigation concepts and approaches that arose during the course of the study, along with a brief exploration of some potential emerging challenges and opportunities.

CURRENT MITIGATION APPROACHES AND INFRASTRUCTURE

The mitigation of synthetic biology–enabled attacks essentially has two broad components: minimizing the chances of an attack and minimizing the negative outcomes once an attack has occurred. As discussed in Chapter 3, Potential for Mitigation, key elements that contribute to the potential for mitigation include deterrence and prevention capabilities, ability to recognize an attack, attribution capabilities, and consequence management capabilities. Broadly speaking, many of the same tools that are used to mitigate natural infectious disease outbreaks or exposure to chemicals (e.g., from environmental spills) are also relevant to mitigation of an intentional biological or chemical attack. In addition, the practices and rules in place to mitigate dual-use research may be relevant to some synthetic biology capabilities. The following sections provide a brief overview of selected existing mitigation approaches and infrastructures related to life sciences research, public health, emergency response, and healthcare capabilities that may be relevant to mitigating synthetic biology–enabled attacks.

Deterrence and Prevention Capabilities

Deterring or preventing the development and use of biological weapons, including those enabled by advances in synthetic biology, is of high priority for the U.S. Department of Defense (DoD) and for the nation. However, there are fundamental challenges to deterring or preventing misuse of biological advances. It has been noted that "the knowledge, materials, and technologies needed to make and use a biological weapon are readily accessible, everywhere in the world" (Gronvall, 2017). While fundamental research and clinical studies are the engines that drive public health and medical treatments, they simultaneously provide dual-use opportunities. Pathogens are ubiquitous, found in hospital and research laboratories, scientific culture collections, infected people and animals, and the environment. The skills and equipment applied to solving challenges in medicine, agriculture, and other disciplines for beneficial purposes are largely the same as those that would be used in making a biological weapon. Advances made in the age of synthetic biology add to the already-broad spectrum of biotechnologies that could be misused.

To support deterrence and prevention of misuse of biotechnology without unnecessarily hindering beneficial research, the prevailing approach has been to implement multiple overlapping tools that, when taken together, can provide greater value. These tools fall into two general categories: norms, and policies and regulations.

Norms and Self-Governance

Norms against the misuse of biology exist and are supported on many levels, from the global to the individual. The Convention on the Prohibition of the Development, Production and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on Their Destruction, commonly known as the Biological Weapons Convention (BWC), is the cornerstone of international-level deterrence for biological weapons, including those created by synthetic biology (UNOG, 2017). The BWC bans such weapons, sets the standard for global norms, binds the nation-states that are party to the treaty, and defines acceptable behavior. There have been violations; for example, the Soviet Union maintained a secret bioweapons program after the treaty was ratified (Alibek, 1998; Cox and Woolf, 2002). However, no country goes against the international norm to flaunt an offensive biological weapons program; even North Korea, which openly flouts international prohibitions against nuclear testing, has denied accusations that the country is developing biological weapons (Sampathkumar, 2017). United Nations Security Council Resolution 1540, which prohibits states from assisting non–state actors in developing biological and other types of weapons, is another relevant international agreement (UN Security Council, 2004).

At the level of institutions and individuals, the scientific community has a tradition of self-governance and established norms entailing what constitutes responsible conduct in science. A landmark example is the 1975 Asilomar conference. With the advent of recombinant DNA technology, leading scientists recommended a moratorium on recombinant DNA experiments involving toxins, oncogenic viruses, and antibiotic resistance until their safety could be assessed (Berg et al., 1974). To facilitate that assessment, scientists and government officials gathered at a conference in Asilomar, California; after further research and national discussion, the moratorium was lifted in 1976, and a new guidance system was created for all recombinant DNA work funded by the U.S. government. What happened at Asilomar has become the template for scientists' responses to scientific discoveries with social and ethical implications and a symbol of the scientific community's capacity to self-govern.

In the decades since, this tradition of self-governance has been applied toward dual-use biotechnologies. In 2004, a National Academies report, known as the "Fink report" after the study's chairman, geneticist Gerald R. Fink (NRC, 2004), made the case that scientists have a moral duty to avoid contributing to the advancement of biowarfare or bioterrorism and outlined types of experiments that would require consideration and review before being undertaken. These experiments—including those relevant to rendering a vaccine ineffective or conferring resistance to available therapeutics, evading detection or diagnosis methods, enhancing or creating virulence, increasing a pathogen's transmissibility or altering its host range, or enabling weaponization—parallel the concerns considered in this report regarding uses of synthetic biology. The Fink report formed the starting point for a federal advisory committee of the U.S. Department of Health and Human Services (HHS) called the National Science Advisory Board for Biosecurity, which defined Dual Use Research of Concern (DURC) (U.S. Government, 2012)

and established the basis for a requirement that U.S. federally funded research involving certain regulated Select Agent pathogens (taken from the Federal Select Agent Program Select Agents and Toxins list; see CDC/APHIS, 2017) undergo DURC research review.

Another important area of self-governance relevant to synthetic biology is the voluntary screening of orders by vendors providing DNA synthesis services. Guided by a framework created by HHS in 2010, DNA providers are encouraged to screen orders for sequences of concern (e.g., DNA encoding Select Agents) and to screen customers to ensure that they are legitimate users of biology (HHS, 2010). Screening is intended to ensure that genetic material of regulated pathogens—including the causative agents of anthrax, smallpox, and rinderpest, for example—cannot be purchased without review and potentially consultation with government agencies. Screening is supported and facilitated by the International Gene Synthesis Consortium (IGSC), an international voluntary coalition of gene synthesis companies, which has adopted the 2010 HHS-recommended screening practices as well as even more stringent measures (IGSC, 2017; Cision PR Newswire, 2018).

Other examples of self-governance include work related to the responsible conduct of scientists (e.g., National Academies of Sciences, Engineering, and Medicine, 2017b,c), bioethics training for students, a life sciences professional code of conduct, and biosafety training for laboratory scientists. While the norms of self-governance are not going to deter or prevent a determined malicious actor from seeking to develop, obtain, or use a biological weapon (whether it is enabled by synthetic biology or not), these norms provide groundwork that could be built upon. At minimum, they offer a basis for social surveillance of unethical or malicious behavior within the scientific community.

U.S. Policies and Regulations

After the 2001 attack involving letters containing anthrax spores, the U.S. Congress strengthened several laws relevant to biosecurity and dual-use research, which resulted in the formal implementation of the Federal Select Agent Program (CDC/APHIS, 2017). In contrast to previous biosafety and containment guidance, which was geared toward equipping laboratory workers to perform experiments on dangerous pathogens without harming themselves or the public, the Select Agent program was designed to protect against unauthorized agent acquisition that might potentially result in the purposeful misuse of those specified agents and toxins deemed most harmful. The regulations require facilities handling listed pathogens to have physical security protections in place and to require individuals to undergo a security assessment before accessing agents on the list. For the most part, Select Agent regulations provide security through denial of access to pathogens, under the assumption that most bad actors would prefer the simplest method of gaining access to pathogens—stealing them from a laboratory.

Additional policies and requirements apply to researchers who receive U.S. federal funding for DURC, and these were recently reviewed by the National Academies (see National Academies of Sciences, Engineering, and Medicine, 2017b). These requirements (U.S. Government, 2012, 2014) stipulate that research using one of 15 pathogens or toxins or that falls within seven identified experimental categories is subject to additional oversight. Research proposals involving highly pathogenic avian influenza H5N1 also are subject to special evaluation by HHS. Although the government recently lifted a moratorium on gain-of-function experiments involving "pathogens of pandemic potential," it specified additional review procedures that must be carried out before such experiments can be conducted (HHS, 2017a).

Some aspects of deterrence and prevention are based in the public health arena. For example, the availability and use of a vaccine or other countermeasure for a particular biological threat, in itself, can be a powerful deterrent—a bad actor is much less likely to use an agent for which the target population is impervious. Even in the absence of a specific medical countermeasure, a robust and healthy population, supported by strong public health infrastructure, can provide resilience against an attack. Conversely, the Ebola outbreak in Guinea, Sierra Leone, and Liberia that killed 11,310 people in 2014–2015 and impacted other countries including the United States is an example of what can happen during a natural outbreak of a serious infectious disease in the absence of a robust public health infrastructure. Kosal (2014) and others have reinforced the importance of strengthening public health infrastructure in all areas of the world as a strong deterrent to misuse of biotechnologies and as a way of enhancing international biosecurity.

Capability to Recognize and Attribute an Attack

Other factors that contribute to mitigation relate to the capability to detect an emerging health threat, recognize it as a purposeful attack, and trace the attack to the actor responsible. Epidemiology, laboratory diagnostics, and environmental monitoring are essential components of systems to detect emerging health threats. Some of the procedures involved in disease surveillance and agent identification can also inform a determination of whether a health threat is the result of an intentional attack or a natural outbreak and potentially provide clues about the actor responsible. Figure 8-1 provides an overview of selected existing procedures and systems in place to identify emerging health threats affecting the U.S. public and military personnel.

In the United States, surveillance and reporting of infectious diseases occur at multiple levels and have both mandatory and voluntary components. Depending on local, state, or territorial jurisdictional requirements, health-care providers, laboratories, hospitals, and other healthcare partners in the civilian arena must report the detection or suspicion of certain agents to their regional public health department and sometimes must submit samples for confirmatory testing at a public health laboratory. Once such a laboratory is involved, an alert is issued to support the identification of other cases of similar disease, and epidemiology becomes an essential factor in disease surveillance. In addition, the identification of certain pathogens (e.g., Select Agents) at these regional public health nodes requires notification of the U.S. Centers for Disease Control and Prevention (CDC) through the Laboratory Response Networks for Chemical and Biological Terrorism (CDC, 2014b,c). The DoD has a similar nodal system of large military reference laboratories, smaller regional laboratories, and local and point-of-contact care centers, referred to as a "soldier-provider-biosurveillance sentinel" approach. The DoD also operates a Global Emerging Infections Surveillance and Response system to monitor emerging infectious diseases (AFHSB, 2017), and DoD laboratories also participate in CDC's Laboratory Response Networks.

To identify a pathogen, a specimen is typically compared against data available from organism banks or sequence databases, such as the Multidrug-Resistant Organism Repository and Surveillance Network (WRAIR, 2017), CDC's MicrobeNet (CDC, 2017b), or GenBank® (NCBI, 2017). Direct antigen tests, supported by both military and civilian healthcare systems, use immunochromatographic methods to identify pathogens and can be conducted in the field or in any physician's office. Increasingly, these tests are being replaced by newer platforms for point-of-care molecular tests, most based on polymerase chain reaction (PCR) technologies, which can rapidly detect bacteria, viruses, and parasites, and require little technical knowledge or sample handling (de Paz et al., 2014; Vidic et al., 2017). While they only target specific known and relatively common pathogens, molecular technologies can quickly rule in or rule out a known pathogen and provide more accurate and sensitive results than direct antigen tests. When tests available at the point of care are inconclusive or confirmatory testing is desired, specimens can be sent to public health, military, or commercial reference laboratories, which have a much more extensive capability based on in-house laboratory-developed tests. These tests, most based on real-time PCR or matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) mass spectroscopy, require laboratory infrastructure and are more complex to perform and analyze, but they are capable of detecting a wider range of pathogens. The molecular identification methods used in the Laboratory Response Network laboratories and thus in the disease surveillance and reporting systems with which they interface are developed nationally and deployed via standardized methods to provide uniformity and comparability of results across each network. These efforts are also supported by extensive National Institute of Allergy and Infectious Diseases research and development efforts to advance methods for tracking, sequencing, and analyzing pathogens.

These surveillance systems support early detection and response when an emerging disease threat presents symptoms that are clearly apparent and can be linked to an identifiable pathogen or toxin. Surveillance networks in countries that have a robust public health system are also a valuable asset toward recognition of an attack, should one occur (see Kosal, 2014). However, such an attack would likely take longer to detect in less-developed countries or in war zones, which generally lack a strong public health infrastructure or for an agent that produces atypical symptoms. Another limitation is the temporal reporting delay between local and national recognition that an outbreak or attack has occurred.

To augment established surveillance and notification systems, public health authorities are exploring the use of a variety of newer networks and potential data sources. For example, the e-mail listserv ProMED-mail acted

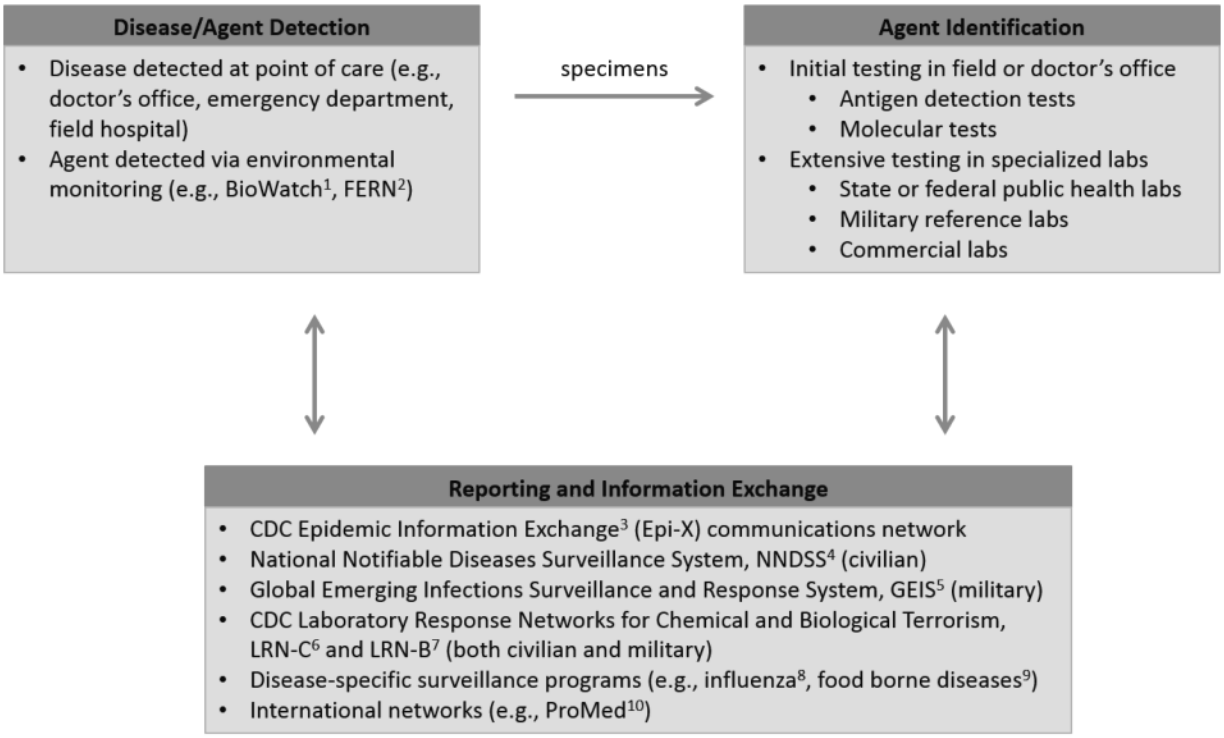


FIGURE 8-1 Examples of elements that contribute to the identification of emerging health threats. When a disease is detected via the healthcare system, initial tests in the field or doctor's office are performed to identify the causative agent. If initial tests are inconclusive, more extensive testing may be carried out in specialized laboratories. If the results meet certain criteria, reporting to one or more surveillance and response networks may be required. These networks in turn adjust testing protocols, reporting requirements, and response guidelines according to current understanding of threats. In general, these steps are carried out under the purview of separate systems in the civilian versus military realm, though there are cross-linkages. There are also systems designed to detect agents directly in the environment in order to provide early warning before affected patients enter the healthcare system.

NOTES: [1] BioWatch is a program of the U.S. Department of Homeland Security that monitors the air in public places for the presence of Select Agents (Firoved, 2016).

[2] The U.S. Department of Agriculture's Food Emergency Response Network (FERN) is responsible for detecting biological, chemical, and radiological contamination of food (FERN, 2017).

[3] CDC, 2017a.

[4] National Notifiable Diseases Surveillance System, https://wwwn.cdc.gov/nndss. Accessed May 11, 2017.

[5] AFHSB, 2017.

[6] CDC, 2014c.

[7] CDC, 2014b.

[8] CDC, 2017c.

[9] CDC, 2017e.

[10] The Program for Monitoring Emerging Diseases is a reporting system maintained by the International Society for Infectious Diseases, http://www.isid.org/promedmail/promedmail.shtml. Accessed January 25, 2018.

as an early warning system during the SARS (severe acute respiratory syndrome) outbreak in China in 2003 (Madoff, 2004); social media has been used to supplement traditional infectious disease surveillance tools (e.g., see Milinovich et al., 2014; Velasco et al., 2014; Charles-Smith et al., 2015; Young, 2015; Fung et al., 2016); and new data sources such as electronic medical records, search engine queries, data on pharmaceutical purchases, or longitudinal seroprevalence or biomonitoring studies (Klompas et al., 2012; Butler, 2013; Fung et al., 2015) could

potentially be mined for real-time disease surveillance purposes. Although these newer platforms are not validated data sources in surveillance and epidemiology—still requiring standards, advanced analytical capabilities, and resolution of privacy concerns (Chiolero et al., 2013; Friedman et al., 2013)—they could be valuable tools for earlier detection of natural or intentional disease events in the future.

Consequence Management Capabilities

Two key capabilities for containing and responding to a chemical or biological attack (consequence management) are the ability to limit the spread of transmissible agents and the ability to counter an agent with vaccines, therapeutics, or other tools.

Methods to Limit the Spread of Transmissible Agents

CDC provides clear definitions of classic infectious disease mitigation measures such as the isolation of infected individuals (CDC, 2014a). Isolation and quarantine, along with contact tracing and travel restrictions, were used to great effect to limit the spread of SARS during the 2003 outbreak (Anderson et al., 2004). The effectiveness of such public health measures is highly dependent on the basic reproduction number, known as R_0, and the serial interval of the pathogen in question. In addition, while such measures tend to work well in a military setting, they can be more difficult to implement in a civilian setting due to poor acceptability and other social factors, as was the case in the United States during the 2015 Ebola outbreak. Other relevant measures to limit the spread of agents include personal protective equipment such as impermeable body suits, gloves, and respirators used to protect emergency workers from contamination when working in the field (FDA, 2017c).

Medical Countermeasures

Medical countermeasures include biological products, drugs, and devices approved by the U.S. Food and Drug Administration (FDA) to prevent, treat, or ameliorate illness in the event of a public health emergency caused by an infectious agent, toxin, or chemical, whether natural or manmade. These include devices such as personal protective equipment, along with vaccines, antibiotics, antivirals, antitoxins, and other drugs and therapeutics.[1]

HHS and the DoD share responsibilities for the development of medical countermeasures, targeted at agents on the Select Agent list, in conjunction with Material Threat Assessments provided by the U.S. Department of Homeland Security (see the Public Health Emergency Medical Countermeasures Enterprise Strategy and Implementation Plans [HHS, 2017b]). Limitations in research capacity, funding, and clinical capabilities necessitate careful decisions about which medical countermeasures can be feasibly developed, from their inception to animal testing, scale-up, clinical testing, and manufacturing. It is also difficult to engage pharmaceutical manufacturers to invest time and platforms into medicines that may not show significant return on investment. Considerations related to how these measures are manufactured (typically on an on-demand basis) and dispensed to populations are also important. Although some countermeasures are placed in the Strategic National Stockpile (maintained by CDC), which supplies state and local public health agencies with medical countermeasures in the event of a national emergency (CDC, 2017f), inventories of many countermeasures are extremely limited and are likely to be sufficient for only the first days of an outbreak situation.

MITIGATION CHALLENGES POSED BY SYNTHETIC BIOLOGY

The mitigation measures described above have strengths and weaknesses despite the advent of synthetic biology. Synthetic biology brings some of those weaknesses into sharper relief, creates new challenges, and creates opportunities for improving mitigation capabilities.

[1] For further information on public health medical countermeasures, see FDA, 2017c.

Challenges to Deterrence and Prevention

Taken together, strategies such as norms and self-governance, voluntary guidance, regulations, and international bans provide numerous barriers to the misuse of biological research that are potentially larger than the sum of their individual parts. However, these strategies, many of which lack formal enforcement mechanisms, have been criticized over the years as insufficient to guard against the purposeful misuse of biology (Palmer et al., 2015). At the international level, for example, the BWC has influenced norms but has few effective enforcement mechanisms. Concerns about the weaknesses of these strategies have gained greater traction with the emergence of synthetic biology. The following sections discuss two areas in which synthetic biology has raised particular concern: the accessibility of modern biotechnology to a wider range of actors and the pitfalls of list-based screening to detect malicious activity.

Accessibility of Biotechnology

Biology today is conducted in a markedly different environment than that of the 1975 Asilomar conference, the seminal event that set the model for scientific self-governance. There is now not only an expanded array of tools available, but a far more diverse scientific community. Synthetic biology techniques are accessible to a wide variety of people, including traditional academic and commercial researchers but also amateur biologists, nonbiologist engineers, and manufacturers, not all of whom are steeped in the norms of traditional academic settings. Some have also argued that tacit knowledge is becoming less central to successful biological manipulation thanks to the increasing sophistication of information technologies (Revill and Jefferson, 2014). As noted in Chapter 2, the movement toward making biology "programmable" broadens the array of actors who may be capable of engineering biological components, although the pace and ultimate degree to which biology is and will become "programmable" is a matter of some debate.

In addition to traditional pathways for entering biotechnology—working in academic laboratories, obtaining a graduate degree, and pursuing a traditional postdoctoral fellowship—people can now enter the field through nontraditional ways. For example, do-it-yourself (DIY) models of biological experimentation have gained popularity in recent years, offering nonscientists the tools and guidance for performing biological research. As biotechnology industry analyst Rob Carlson wrote in *Wired* in 2005, "the era of garage biology is upon us," noting that a person could, with a few thousand dollars of investment, get to work "hacking biology" (Carlson, 2005). The community has grown since then; in 2017, a "Global Community BioSummit" was organized at the Massachusetts Institute of Technology (MIT) Media Lab, which brought together "biohackers" and members of independent and community laboratories from dozens of countries (MIT Media Lab, 2017). Many DIY biology activities are expressly educational, fun, or tied to local community needs (e.g., testing food samples). Yet while most of these DIY projects are not sophisticated, the model does make accessible to the general public tools that can be used to do advanced work. For example, for less than $200, reagents and kits can be acquired that enable amateurs to employ gene-editing technologies such as CRISPR/Cas9, although advanced skills and additional laboratory resources would likely be required to use such kits to create a harmful agent. It is also possible that community laboratories could provide a venue for malicious actors or be implicated as misdirection in a perpetrated event.

Another example of a nontraditional group of biotechnologists is the International Genetically Engineered Machine competition (iGEM, 2017b). iGEM began in 2003 as an in-class competition at MIT in which teams of students were challenged to build synthetic biological systems from standard, interchangeable parts, called BioBricks™,and operate them in living cells. Though iGEM projects are carried out by students, many of them entirely new to bioscience, some projects have been quite sophisticated. Now an annual event open to participants outside of MIT, iGEM involves students at the high school, undergraduate, and graduate levels from countries around the globe. Projects routinely involve the engineering of microbial, mammalian, and plant cells; the 2014 grand prize winner, for example, circularized proteins to make them more physically stable.

The fact that a relatively untrained individual could perform complex bioengineering has triggered concerns and mechanisms to improve the safety and knowledge of the amateur community's activities (Kellogg, 2012; Holloway, 2013; Kolodziejczyk, 2017). A "see something, say something" campaign of the Federal Bureau of

Investigation (FBI) performs outreach to both the DIY biology community and to iGEM (Wolinsky, 2016). The FBI and the American Association for the Advancement of Science have also teamed up to increase understanding of the risks and benefits of the field (Lempinen, 2011) and explore ways to "safeguard science."

Pitfalls of List-Based Screening

Advances in synthetic biology capabilities pose a number of challenges to list-based screening as a key tool for deterrence and prevention. In particular, the voluntary screening of orders by DNA providers, a system intended to prevent production of Select Agents, is becoming less useful (Casadevall and Relman, 2010; Carter and Friedman, 2015; DiEuliis et al., 2017b). While screening of customers is and will likely remain an important tool, recent research examples indicate that screening of the sequences ordered by those customers may become less relevant. Using lists may make it easier to implement policy, but a static list-based approach is concerning not only because many pathogens exist in nature, but because synthetic biology now allows for the creation of new pathogens and other potentially harmful biological components that are not found on such lists.

Sequence screening is based on homology to "data from all organisms on the Select Agent list, the Australia Group List, and other national lists of regulated pathogens" (IGSC, 2017), so if an agent is not on the list, it is not flagged. For example, current guidance did not prohibit a DNA provider from fulfilling an order for the genome of the extinct virus horsepox; the recent publication of the synthesis and booting of the horsepox genome (Noyce et al., 2018) raised concerns that some techniques employed to create this pox virus could be applicable to creating smallpox (DiEuliis et al., 2017a; Koblentz, 2017) because horsepox has high sequence similarity to variola virus, the causative agent for smallpox (Tulman et al., 2006). In addition, while there are processes to connect synthesis companies with U.S. law enforcement agencies in the event of a problem, DNA synthesis is performed worldwide, and it is less clear that such processes are in place in all other nations. Importantly, in addition to DNA synthesis screening, lists such as the Select Agent list also form the basis of many of the downstream mitigation tools discussed in this chapter, including detection, diagnostics, and the development and prioritization of medical countermeasures. An overreliance on the Select Agent list is a systemic weakness affecting many aspects of the United States' current biodefense mitigation capability.

Another weakness is that DNA sequences of less than 200 base pairs (known as oligonucleotides) are not screened. This has raised concerns that a determined malicious actor could potentially obtain multiple short sequences from commercial vendors and assemble them to create full-length pathogen DNA, although such a strategy would require significant effort and skill, particularly for pathogens with large genomes. It has been argued that screening oligonucleotide orders is unworkable due to a higher expected false positive rate for any given short sequence, which would be exacerbated by the much higher volume of oligonucleotide orders (Garfinkel et al., 2007; Carter and Friedman, 2015). A counterargument has been put forth that oligonucleotide screening could be performed differently than for longer genes, such as by analyzing groups of oligonucleotides in an order (or across multiple orders) and setting sequence similarity thresholds to higher values. Another concern is that evolving trends in the life sciences enterprise may erode vendors' incentives for screening. As DNA synthesis becomes cheaper, the somewhat fixed cost associated with screening represents an increasingly larger percentage of total costs, creating a disincentive against screening on the part of those companies (DiEuliis et al., 2017b). These costs could be especially acute if oligonucleotide screening were implemented.

Current screening approaches are primarily based on the homology of a sequence order to the sequence of a specified pathogen, as opposed to screening for sequences that confer specific pathogen characteristics. As further understanding is gained connecting sequence to function, there is an opportunity for the types of lists used to evolve. Thus, some form of list-based mitigation could continue to play a role in the deterrence and prevention toolkit, even if this strategy has limitations and will need to be part of a layered approach that includes other strategies (see Opportunities for Improving Deterrence and Prevention Capabilities, below).

Challenges to Recognizing and Attributing an Attack

In a textbook world, approaches to surveillance for disease outbreaks are based on the appearance of clear disease symptoms in a group of individuals connected in place and time and which can be attributed to a causative agent. The recent Zika outbreak in the Americas is a good example of how these "perfect conditions" are not always met. Eighty percent of Zika-infected individuals showed no signs of disease, symptoms were mild even in those who were symptomatic, and the link to microcephaly in infants born to infected women could not have been predicted. Such examples underscore remaining weaknesses in disease surveillance tools for recognizing even natural disease outbreaks; these weaknesses may create particular challenges with regard to some types of synthetic biology–enabled attacks. For example, as discussed in Chapter 6, it may be possible to develop bioweapons that alter the human host and produce health effects that are not immediately obvious as a disease outbreak or attack, such as by reducing immunity or modifying the microbiome.

Synthetic biology could also confound the ability to identify the causative agent in a biological attack. Despite the breadth and depth of available repository resources, there would not always be a reference specimen to use as comparator, particularly if the agent is markedly different from natural pathogens or toxins. Many current mitigation efforts are inherently list based (aimed at detecting Select Agents) and are heavily dependent upon the secrecy of the exact genomic regions used for the PCR primers and probes; should an adversary determine what these regions are, it could be possible to create a functional yet undetectable pathogen by altering those regions using codon-switching techniques.

In addition to challenges related to clinical surveillance, synthetic biology could also further compound weaknesses in environmental surveillance capabilities, which seek to detect agents in the environment to provide early warning before patients present in the healthcare system. For chemical threats, the Laboratory Response Network for Chemical Terrorism utilizes several forms of mass spectroscopy, which makes unbiased detection much more feasible (assuming reference standards are available) than in the biological field, where unbiased detection remains extremely challenging. Although it is feasible to utilize PCR to identify a Select Agent pathogen "needle" from the enormous environmental background "haystack," there is no technology available today that can reliably alert us when a *novel* pathogen, whether natural or engineered, is present in the environmental background. These tools will not be useful in detecting unknowns, genetically engineered chimeras, or agents for which the PCR primer or probe binding site has been altered. Ultra-deep metagenomic sequencing will find vast amounts of uncharacterized sequence in any environmental sample, and sorting it all out to the point where a novel pathogen can be definitively identified is currently too costly and too lengthy a process to be useful. Bioinformatics tools provide powerful means of sifting through seas of sequences, but they rely on assumptions, for example, about what constitutes a taxonomic unit, and the incompleteness of available reference databases affects the accuracy of the results. An additional complication is that the "normal" background microbial composition is poorly characterized for many outdoor and indoor environments and can be affected by many factors (National Academies of Sciences, Engineering, and Medicine, 2017d). Given these challenges, approaches such as metagenomics and environmental surveillance are not likely to completely fulfill the need to provide early identification of agents used in synthetic biology–enabled attacks.

If current environmental surveillance methods are not capable of recognizing a novel agent, the implication is that we are dependent upon the public health system to recognize outbreaks of novel pathogens, whether natural or engineered. Relying on this reactive approach suggests that it would not be possible to act to mitigate or contain an outbreak until patients have developed symptoms that trigger a health community response; as a result of this delay, people would become ill before it is possible to know that an attack has occurred. Isolation of the novel causative agent by culturing (if possible) followed by sequencing or ultra-deep sequencing and painstaking assembly would be needed to characterize the agent and lay the groundwork for analyzing its mechanisms and origin. This initial characterization process might take a few days at best, or considerably longer if the novel agent is a highly engineered version of a normally benign microbe or is no longer present in the patient by the time symptoms are apparent. In cases in which the agent is a pathogen, PCR reagents can be developed quickly once the genome has been obtained, at which point the agent can be added to the list of agents detectable through environmental and clinical surveillance systems.

There is no magic bullet for dealing with all new routes to harm that are made possible by modern biotechnology, including synthetic biology, nor are there magic bullets for handling every natural agent that emerges, as exemplified by experiences with SARS, MERS (Middle East respiratory syndrome), West African Ebola, and other outbreaks. The 2003 SARS outbreak in particular underscored to the international public health and biosurveillance communities the need to have mechanisms in place for rapid characterization and international information sharing to respond adequately to novel and emerging threats. The types of biosecurity concerns related to synthetic biology assessed in this report provide added urgency to that message.

Consequence Management Challenges

If disease surveillance and laboratory infrastructure cannot detect, identify, and characterize the causative agent, it is also possible that current available medical countermeasures—such as vaccines and therapeutics—may be less effective or, in certain cases, ineffective. While existing medical countermeasures may be quite useful for containing or counteracting agents created with synthetic biology that are highly similar to existing pathogens of concern, not all agents may fit this model. For example, if multiple drug resistance mutations are introduced into a bacterium to produce a bioweapon, even a broad-spectrum antibiotic administered before the agent is fully characterized may be ineffective. Similarly, if a viral chimera is engineered bearing novel surface antigens, it is unlikely to be neutralized by immunoglobulin given post-exposure. In short, if the agent is not susceptible to available vaccines, drugs, or antibody-based therapeutics, existing systems are less likely to limit its spread, potentially increasing the scope of casualties. In such scenarios, developing, testing, and approving drugs and vaccines to counter the agent using traditional approaches would entail long delays and an associated likelihood of many people being affected, suggesting a need for novel approaches to rapidly manufacture and test new therapeutics. Effectively implementing such approaches would require not only technological advancement but also rapid regulatory approval processes, such as the Emergency Use Agreement mechanism used by the FDA.

POTENTIAL OPPORTUNITIES TO ADVANCE MITIGATION CAPABILITIES

Despite the challenges posed by the current and anticipated biological threat landscape, there are multiple opportunities to build upon current capabilities and fill some of the gaps. In fact, synthetic biology capabilities may themselves help advance some mitigation efforts. Providing a comprehensive list of technologies with sufficient information to judge their efficacy in dealing with novel outbreaks is outside the scope of this report. This section is intended to highlight some of the ways in which technologies currently in development could improve the ability to handle future outbreaks or attacks, including selected examples of potential opportunities for improving the capacity for deterrence, prevention, attack recognition, attribution, and consequence management.

Opportunities for Improving Deterrence and Prevention Capabilities

Engineering techniques such as abstraction, standardization, modularity, automation, and rational design are likely to enable significant advances in synthetic biology. While the degree of incorporation of computation into the synthetic biology workflow will vary, one opportunity to explore mitigating biodefense concerns, for those approaches that depend on computational engineering, is to explicitly integrate mechanisms to prevent, detect, identify, and store information about malicious activities in the computational infrastructure. This approach could be relevant to all aspects of mitigation but is perhaps most salient for prevention and attribution. Examples of types of approaches that could be further explored are discussed below. Box 8-1 outlines how such approaches might be applied to identify or prevent malicious activity at various stages of two example scenarios.

- *Screening of activities with machine learning:* It may be possible to develop algorithms that learn and recognize patterns, such as DNA segments or sequence transformations, material transfers, or equipment usage, that relate to the creation of a biological threat. This approach could potentially help flag suspicious activity early in the design cycle. However, developing such algorithms requires a large amount of training

data, and data reflecting malicious activity would be hard to come by; as a result, developing a sufficiently accurate algorithm may be infeasible.

- *Systems to constrain design capabilities:* Rules could potentially be encoded directly in software for engineering DNA constructs to make it difficult or impossible to create specific genetic designs, for example, by prohibiting or requiring the addition or removal of specific DNA segments, requiring specific assays, preventing the transfer of materials to specific individuals or entities, or excluding or requiring the use of specific host organisms. Although this approach could help deter or prevent some malicious activities, it would not be sufficient to prevent designs based on specific knowledge or on brute-force combinatorial testing that bypasses biological design tools and could be difficult to implement in a way that prevents user tampering.
- *Maintaining registries of known expertise and materials:* Database infrastructure and supporting tools could be created to track known sources of expertise and materials relevant to the capacity to produce a biological threat, such as information about laboratories, personnel, and sources of material. In addition to identifying relevant players, it could be possible to profile designs coming from them to create known "digital signatures" of the engineering designs of individuals or groups. However, obtaining access to sufficient designs to be able to profile malicious users would be difficult, as distinguishing legitimate activities would be.
- *Maintaining registries of known biological threats:* Despite the inherent limitations of list-based systems in light of synthetic biology capabilities, there may nonetheless be opportunities to enhance the utility of these systems by systematically connecting them to design software and to automated foundries. Furthermore, there is an opportunity for screening procedures to move from a focus on organisms to a focus on DNA functions. It has been argued that emphasizing known pathogenic functions (as opposed to whole genomes of Select Agents; see IARPA, 2017b) would allow the curation of a more meaningful registry, one drawn directly from the DNA components responsible for causing harm. For example, software used for synthetic biology could be required to periodically run "checks" against bioagent registries or to automatically add new biological threats to these registries when they are identified. For such an effort to succeed, it would need to be scalable, searchable, and resistant to hacking. Malicious users would presumably be constrained to other approaches that do not rely on design software, such as experimental approaches like DNA shuffling or mutagenesis.
- *Tracking digital "signatures" in genetic designs:* It may be possible to deploy information technology at key stages in the automation pipeline to identify the source and the creator of synthetic genetic material to ensure that it comes from trusted sources. Were an attack to occur, this information could also help to identify the actor responsible. However, this approach would largely be applicable to strategies employing genetic circuit design tools; attribution of synthetic materials created by other means, such as through directed evolution, would be much more difficult. Watermarks for this purpose could be "biological," for example, if the genetic material (e.g., the DNA sequence) has additional information inserted that uniquely identifies the sample (Heider and Barnekow, 2008), or the watermarks could be "electronic," for example, if the information is added digitally to the electronic file used to communicate the biological information (e.g., in the binary information that encodes a GenBank® file) (Cox et al., 2008). Electronic watermarks are more mature and more likely to be more useful in practice where the biological material is manipulated.

Opportunities for Improving Agent Identification and Attribution Capabilities

Because so much of the natural nucleic acid space has yet to be sequenced and characterized, it remains extremely difficult to determine if a given genetic sequence is of natural or nonnatural origin. However, current analysis methods can help identify situations in which gene sequences appear in unexpected places (e.g., identifying that the toxin gene from *Clostridium botulinum* has been inserted into the genome of *Escherichia coli*). In addition, the products of genetic circuit engineering (see Chapter 4, Figure 4-3) can clearly be recognized as nonnatural and even contain design patterns that may provide attribution clues. Additional tools that enable one to detect that a

BOX 8-1
Workflow Examples to Illustrate Mitigation Opportunities

The following tables highlight examples of how computational approaches to support mitigation might apply to various activities that an actor would perform in pursuing two types of biological threats. These breakdowns are not meant to be exhaustive but rather are presented to illustrate challenges and opportunities. Not all options would apply to all situations, and implementing these options also would likely engender debates over trade-offs regarding issues such as who would get access to tools, materials, and information; how to balance security with a desire to avoid curtailing legitimate research; or societal concerns about privacy and surveillance. Although a full assessment of the opportunity provided by computational biology was outside the committee's scope, shading provides a sense of which activities are considered to present a low (light blue) or medium (darker blue) level of opportunity.

Re-creating a Known Pathogenic Virus

Activity	Potential Computational Approaches to Support Mitigation
Early planning	Accessing literature and protocols relevant to DNA construction, working with a given virus This type of activity is likely to be difficult to distinguish from nonmalicious activity, and attempts to do so would yield many false positives. Implementing mitigation efforts targeted at this step will likely be difficult and likely increase barriers to legitimate activities.
Sequence selection Accessing databases of viral genome sequences	Although database access can be monitored, regulating this process would likely be difficult and could hinder legitimate research. Additionally, any genome sequences removed from databases would likely be available from other sources.
Sourcing materials Ordering reagents and equipment, such as genetic material, DNA synthesis equipment, and cell lines or animals	Material transfer agreements already provide security mechanisms for the legitimate transfer of materials. Illicit transfers would be difficult to prevent and ordering of basic molecular biology reagents and equipment is likely to be too prevalent to monitor.
Design software Software for DNA sequence management, biological manipulation or design, or visualization	Because computation is explicitly involved in this step, the addition of electronic tracking and annotation of the design files can help indicate design origin, destination, and the history of modifications. Electronic watermarking is likely to be more acceptable than biological watermarking.

sequence had been genetically manipulated, or tools to analyze features of a sequence or a resulting organism that contribute to actor attribution, would be valuable additions to mitigation strategies.

Although many U.S. government agencies have expertise and responsibilities relevant to preparing for, preventing, and responding to an attack involving engineered biological components, no single agency has lead

Data management Software used to keep track of the project and the personnel involved	Records such as electronic laboratory notebooks can provide information about the history of a design and those involved in its development; however, malicious users could modify their identity and activities to make this data source less reliable.
General computing Computing that is part of common equipment used for the project, including gel docs, thermocyclers, and incubators	These computing platforms are likely too general purpose to be of much targeted use.

Design of a Metabolic Pathway for In Situ Synthesis of a Toxin via the Gut Microbiome

Activity	Potential Computational Approaches to Support Mitigation
Host selection Choose the chassis/host organism.	The selection of an organism is likely to be too early in the process to determine if malicious activity is intended. Biosafety-level restricted organisms would raise a flag, but the process of obtaining these organisms is already regulated.
Gene selection Identify the genes required to create the needed enzymes.	It would be possible to flag the selection of certain genes, such as those associated with a prohibited toxin. In general, however, gene selection is likely too common a process to reliably detect or prevent malicious activity without unduly curtailing legitimate research.
Design software Construct genetic designs with genes	Electronic watermarking can be used during the design process of interest.
Screening Screen for enzyme activity.	The identification of broad enzyme categories is not likely to detect threats reliably.
Tuning Engineer proteins to modify enzyme activity if needed.	Specifically targeting enzymes for modification may create patterns that can be detected and learned from.
Tuning Swap in regulatory biological components to fine-tune enzyme activity.	The changing of parts is a directed process whereby the resultant activity changes produce a record that can potentially infer desired results.

responsibility in this area. The 2001 Amerithrax letter attacks first brought focus on bioterrorism and the need for the federal government to build standardized software tools and laboratory methods to analyze engineered organisms. Several recent examples are summarized briefly below.

- Safe Genes (DARPA, 2017), a program of the Defense Advanced Research Projects Agency, focuses on developing strategies to better control genome editing activity, such as by inhibiting genome editing in cells or preventing off-target editing activity.
- Functional Genomic and Computational Analysis of Threats (Fun GCAT; IARPA, 2017b), a program of the Intelligence Advanced Research Projects Agency (IARPA), aims to facilitate the design of better tools for screening DNA synthesis orders.
- Finding Engineering-Linked Indicators (FELIX; IARPA, 2017a), another IARPA program, seeks to develop a suite of tools designed to distinguish natural organisms from animals, bacteria, insects, plants, and viruses that have been engineered to potentially cause harm.
- To help reduce risk, the U.S. Department of Homeland Security sponsors the Sequences of Interest database to bring together in a single source nucleic acid and protein data about genetic mechanisms of virulence and resistance, along with protein toxin data and nucleotide data about plasmids and artificial vectors that may signify natural or artificial bacterial genetic change (D. Shepherd, Chemical-Biological Defense Division, Department of Homeland Security, personal communication, 2018).

While these or other programs were not evaluated as part of this study, they represent examples of the kinds of investments that would increase preparedness for the types of synthetic biology–enabled capabilities discussed in this report.

As discussed in Chapters 4–6, synthetic biology techniques can be used to modify pathogens, hosts, and vector species; these agents could possibly be used in complex attacks involving multiple pathogens, hosts, or vectors. Under the public health paradigm, identifying an agent's species and any antimicrobial resistance factors is generally sufficient to guide treatment, for example, with a particular antibiotic. However, that level of information may not be sufficient for forensics and attribution, particularly if a deliberate attack or engineering is suspected. In these cases, responsible federal agencies will want to know how similar the new sample is to strains in the sequence databases, whether it is a common laboratory strain or a strain from a different part of the world, how the new sample compares to strains found at suspected facilities, and the degree of certainty with which we can determine whether the agent is a natural strain or might have been raised in a particular type of culture media, for example. Except in cases in which leftover samples are found in the laboratory where the material was created, proving attribution in the era of synthetic biology appears to be growing increasingly difficult, particularly for complex attacks that could potentially take considerable time to achieve their intended effects. As a result, attribution in the age of synthetic biology is likely to be heavily dependent on computer-based approaches that look for molecular signatures, as well as on intelligence. It is not within the scope of this report to discuss intelligence activities, and it is recognized that highly sophisticated adversaries may be able to evade even the most elaborate attribution approaches.

One of the most significant developments for identifying agents (in the context of treatment as well as detection and attribution) is next-generation sequencing and the drastic reductions in cost and time it enables. The FBI-led analysis of the 2001 Amerithrax attack samples (which took place before the advent of next-generation sequencing) involved the sequencing of a small number of morphologically different isolates at a cost of around $100,000 each in a process taking several years. Were such samples to be analyzed using today's tools, ultra-deep characterization of the sample (about 10 billion sequence reads from a full run on a HiSeq™ sequencing system) could be performed within 1 week with reagent costs of around $10,000. Looking to the future, it is clear that next-generation sequencing will become central to identifying synthetic biology–derived infectious agents. Box 8-2 describes some of the ways in which next-generation sequencing approaches might be used in this context.

Synthetic biology is also likely to lead to the development of new detection technologies. As an example, Pardee et al. (2014) developed a programmable diagnostic assay that is embedded in paper as a low-cost, sensitive diagnostic assay for the presence of Zika virus RNA (Hall and Macdonald, 2016). In another novel approach to diagnostics, Lu et al. (2013) describe the engineering of bacteriophages for diagnostic strategies in which phage-specific antibodies, quantitative PCR, or a reporter molecule are used to detect amplification of engineered phages when the phages encounter target bacteria. Slomovic et al. (2015) describe applications of synthetic biology in the development of both in vitro and in vivo diagnostics, including the development of sensing bacteria in which

BOX 8-2
Opportunities Enabled by Next-Generation Sequencing

The advent of next-generation sequencing opens opportunities for three main approaches that could have implications for identifying synthetic biology–derived agents: next-generation sequencing of cultured isolates, targeted next-generation sequencing, and unbiased metagenomic (or untargeted) next-generation sequencing.

- Next-generation sequencing of cultured isolates generates high-quality complete pathogen genomes (for pathogens where culturing is possible and a complete genome is desired). However, culturing can require days or weeks, depending on the growth rate in culture of the pathogen(s) involved.
- Targeted next-generation sequencing is a scalable hybrid approach where large numbers of informative regions of known pathogens are enriched via amplifications or capture techniques prior to sequencing. Similar to polymerase chain reaction (PCR), however, targeted next-generation sequencing can only find the genomic regions it is designed to look for because the results are queried against existing databases.
- Unbiased metagenomic next-generation sequencing is used to examine complex environmental or clinical samples when targeting of a list of key organisms is not sufficient. Detection of a novel or highly engineered pathogen from a patient is an example of when deep and expensive metagenomics sequencing would be indicated. Although they are still nascent, technologies are being developed[a] to move such approaches closer to the field (e.g., at the point of contact with a patient). Once a new threat is discovered, PCR and targeted next-generation sequencing reagents can be rapidly prepared to permit lower-cost and more rapid detection from other samples or victims.

[a]Examples include nanoscale technologies that support long-read real-time sequencing with analysis done on a laptop computer (Quick et al., 2016) and the broad-spectrum Microbial Detection Array (Jaing et al., 2011; Thissen et al., 2014), which contains 388,000 DNA probes.

"sentinel bacteria could reside in the guts of soldiers or aid workers and serve as short term 'medical records' alerting on the time and scale of contamination or pathogen infection." These studies, while still in a research mode, suggest that synthetic biology tools can help address some of the need for alternative diagnostics that are not based on detecting a specific region of a pathogen by real-time PCR.

Opportunities for Improving Consequence Management Capabilities

Just as synthetic biology expands the types of malicious activities that may be undertaken, it also expands what is possible for beneficial applications. Synthetic biology and related advances (such as the convergent technologies discussed in Chapter 7) open the possibility of new and more systematic approaches to the development of medical countermeasures and other mitigation tools and strategies. Synthetic biology approaches such as rapid DNA synthesis, protein design tools, cell-free expression systems, and automation may significantly advance consequence management capabilities, especially with regard to the development and testing of medical countermeasures. Such approaches could, for example, provide flexibility in the control of protein expression levels, shorten the time to successful countermeasure production, and lower costs. They could potentially even enable the development of countermeasures to newly identified agents without ever culturing the agent itself; through the use of in silico characterization of an agent's key components, antigen components for antibody development could be synthesized, potentially within hours of detection. Such approaches could represent a promising alternative to stockpiling countermeasures when the emergence of novel threats (both natural and engineered) is likely.

In addition, once bioagent and viable culturing conditions have been identified, the large-scale testing capabilities used in synthetic biology could be used to screen candidate countermeasures, for example, by surveying chemical small-molecule libraries to identify drug leads or by testing many organism-relevant phages to identify those that are potentially lethal to the bacterial strain used in an attack.

The following sections discuss ways in which synthetic biology could potentially contribute to the development of diagnostics, vaccines, and other medical countermeasures. However, the technical barriers to the development of synthetic biology–enabled vaccines or therapeutics remain steep, and it is also important to note that there must be a compelling business case for their development and a regulatory process for approval of these countermeasures before they become reality. Almost 4 years after the emergence of the Ebola virus infection in West Africa, we still lack licensed Ebola vaccines, and despite knowing the serious risk of a MERS outbreak outside of the Arabian Peninsula, we are still many years away from a licensed effective MERS vaccine. While outside the scope of this report, a comprehensive understanding of the feasibility of using synthetic biology to develop medical countermeasures would benefit from critical review of both commercial and regulatory considerations.

New "Vaccine Strains" Through Controlled Attenuation of Viruses

The replication cycle of viruses is complex, and the fitness of a given virus depends on many factors. One important factor is the particular codons incorporated into the DNA or RNA; the preferential use of particular codons (or codon pairs), termed codon bias (or codon pair bias), is thought to influence the efficiency of translation (Buchan et al., 2006). Efforts to optimize codon usage almost invariably result in attenuation of the virus, and the more the codon usage bias is disrupted, the more attenuated the resulting virus (Wimmer and Paul, 2011; Martinez et al., 2016).

Burns et al. (2006) and Coleman et al. (2008) proposed to take advantage of this attenuating phenomenon to perform genome-scale manipulation of codon pair bias in poliovirus to develop vaccines in which the degree of attenuation could be controlled by the degree of codon substitution performed. The resulting "vaccine strains" provided protective immunity in mice and, because of the hundreds of substitutions made, did not revert to virulence. Using synthetic biology tools including large-scale, low-cost construction of desired genomic sequences has been proposed as a means of making attenuated vaccines for many other RNA viruses, including influenza virus (Mueller et al., 2010; Yang et al., 2013; Fan et al., 2015), chikungunya virus (Nougairede et al., 2013), respiratory syncytial virus (Meng et al., 2014), simian immunodeficiency virus (as a model for HIV; Vabret et al., 2014), tickborne encephalitis virus (de Fabritus et al., 2015), vesicular stomatitis virus (Wang et al., 2015), and dengue virus (Shen et al., 2015).

Use of DNA Construction to Rapidly Derive Vaccine Stocks

The 2009 H1N1 pandemic made it clear that new methods of developing influenza vaccines were required to speed the response from emergence of a new virus to the development of a vaccine seed stock and production and distribution of the vaccine strain. Toward this goal, Dormitzer et al. (2013) developed a synthetic approach, constructing the hemagglutinin and neuraminidase genes with minimal errors by annealing many staggered oligonucleotides that overlapped by 30 bases with their neighbors and together covered the full length of each gene. Infectious virus was rescued from susceptible cells transfected with the synthetic hemagglutinin and neuraminidase genes and plasmid DNAs encoding viral backbone genes. In a proof-of-concept study performed in collaboration with the Biomedical Advanced Research and Development Authority, an H7N9 vaccine strain was constructed in this manner in 5.5 days; tests demonstrated the antigens expressed by the synthetic genes were immunogenic based on their reaction with ferret sera (Dormitzer et al., 2013). This example demonstrates that synthetic biology tools can facilitate the rapid derivation of vaccine strains to respond to emerging viral threats. However, the commercialization and licensure of vaccines derived in this manner is many years off; having a synthetic biology tool that can facilitate the development of a new countermeasure is a major advance, but it is far short of what is necessary to make that countermeasure safe, effective, and available.

Rapid Development mRNA Vaccines

Another approach to the development of synthetic vaccines is the use of messenger RNA (mRNA). Petsch et al. (2012) demonstrated that mRNAs of influenza hemagglutinin, neuraminidase, and nucleoprotein could be transcribed into proteins in vitro to provide protective immunity against homologous influenza virus. Hekele et al. (2013) used a synthetic self-amplifying mRNA (SAM) to create a vaccine derived from the hemagglutinin gene of the H7N9 influenza virus delivered by a nanoparticle. The vaccine, produced just 8 days after the sequence became available, was immunogenic at low doses. SAM vaccines delivered by nanoparticles have also been developed against HIV-1 (Bogers et al., 2015) and Zika virus (Pardi et al., 2017). In a further development, Richner et al. (2017) also developed a SAM vaccine against Zika virus delivered by nanoparticles but, in that case, a structural gene from the Zika virus was engineered to destroy a conserved epitope to eliminate the production of cross-reactive antibodies against dengue virus, which would exacerbate dengue disease. These examples raise the speculative possibility that self-amplifying mRNAs directly encoding antibody molecules and delivered by nanoparticles could be used as a potential therapeutic approach. However, as with the example in the prior section, because of regulatory and business factors, it would take years before this approach produces therapeutic applications for use.

Use of Synthetic Biology Tools to Develop New Therapeutics

Synthetic biology is also contributing to the development of small-molecule medical countermeasures. The development of a yeast strain capable of producing artemisinic acid, the key precursor to the antimalarial drug artemisinin, demonstrated that complex plant-based natural products can be produced via synthetic biology (Westfall et al., 2012). More recently, compounds such as opioids (Galanie et al., 2015) and penicillin (Awan et al., 2017) have similarly been produced in yeast. Development of existing and novel chemicals and materials remains a primary interest of both the academic and industrial community, making it likely that the cost and time to develop chemical production strains will improve in the future.

Krishnamurthy et al. (2016) summarized the use of synthetic biology tools in the development of new therapeutics, including approaches for the production of new antibiotics and the application of the CRISPR system in developing bacteriophages as targeted therapeutics. The enabling impact of synthetic biology in exploring the great diversity of natural products that can be used as therapeutics is reviewed by Smanski et al. (2016). Platforms for drug discovery can be envisaged using synthetic mammalian genetic circuits, and bacteria, yeasts, and plants engineered with synthetic pathways can be utilized for the large-scale production of drug and drug precursor compounds (Weber and Fussenegger, 2012).

In addition to rapid response with conventional countermeasures, such as antibodies and small-molecule drugs, synthetic biology may also enable the deployment of new types of countermeasures. For example, gene drives and other gene editing methods are being explored for the control of vector populations for illnesses such as malaria and Lyme disease (Harris et al., 2012; Esvelt et al., 2014; Hammond et al., 2016). Microbiome-based interventions for the control of gastrointestinal infections could also provide a programmable platform for combating bacterial threats. For example, Citorik et al. (2014) have described the use of CRISPR/Cas technology to create RNA-guided nucleases that act as antimicrobials by targeting specific DNA sequences. These RNA-guided nucleases enable modulation of complex bacterial populations by selective knockdown of targeted strains.

SUMMARY

A comprehensive, in-depth review of strengths and weaknesses in current U.S. or international programs was outside the scope of this study; as such, this report does not offer a full analysis of mitigation capabilities and makes no recommendations pertaining to mitigation priorities. The following observations indicate areas in which additional attention could help address some of the challenges posed by synthetic biology.

General Observations

- Classical public health measures such as the disease surveillance system are critical to effective mitigation of attacks caused by agents created with synthetic biology. However, synthetic biology provides opportunities to engineer around the current system, and cases are likely to arise in which the current infrastructure will be insufficient and thus in need of enhancement.
- Biological and chemical defense strategies that are nimble, as well as adaptable to a wide range of threats, are needed because of rapid rates of technological change and uncertainty about which approaches an adversary might pursue.

Prevention and Deterrence

- Risk management strategies based on defined lists of biological agents, such as the Federal Select Agent Program Select Agents and Toxins list, will be insufficient for managing risks arising from the application of synthetic biology. Similarly, while measures to control access to physical materials such as synthetic nucleic acids and microbial strains have merits, such approaches will not be effective in mitigating all types of synthetic biology–enabled attacks. Appropriate preparation for these challenges is needed.

Recognition and Attribution

- The development of more flexible, untargeted, and multimodal detection technologies such as next-generation sequencing and mass spectrometry analysis will facilitate improved identification capabilities for synthetic biology–derived agents.
- The development of epidemiological methods (e.g., surveillance and data collection) that would strengthen the ability to detect unusual symptoms or aberrant patterns of disease will be useful.

Consequence Management

- Computer-based approaches may provide a number of tools to support the prevention, detection, attribution, and consequence mitigation of threats posed by synthetic biology. Such approaches represent an area for further exploration.
- Beneficial applications of synthetic biology for countermeasure research and development are expected to provide an opportunity to address concerns raised by synthetic biology, when accompanied by corresponding efforts to facilitate the entire development process, including regulatory considerations.

The ability to respond to a disease outbreak, whether it emerges naturally or from a purposeful attack, is complex and dependent on many social, governmental, and biological factors. Recognizing that an outbreak has occurred is a vital step in this process. Then, the agent must be identified and medical countermeasures made available. The prospect that a causative agent may have been created with synthetic biology and is therefore unknown

and uncharacterized dramatically increases the complexity of these mitigation activities and underscores the need to improve the public health response system.

In light of this context, it will be vital to maintain the current systems used in the military and civilian public health infrastructure. Strengthening this infrastructure in specific areas, including broadening the current approaches to surveillance, is important to better enable the detection of an attack that does not elicit "normal" symptomology.

Although an in-depth analysis of preparedness and response capabilities was outside the scope of this report, identification and characterization of an agent derived by synthetic biology may be a significant gap in the nation's preparedness because many current diagnostic capabilities are based on commonly seen human pathogens and on lists of pathogens designated as high risk. Untargeted approaches to detection that use multiple platforms and integrate the data obtained would be expected to be more effective at identifying and characterizing unknowns. It is also clear that while advances will need to be made in wet-bench detection technologies, computer-based interrogative and forensic methods will become more and more valuable to support prevention, agent identification, and attribution. Large-scale success of computational mitigation requires that the attack strain has been developed by rational engineering design approaches that are not yet ubiquitous; the development of agents with other approaches such as directed evolution will likely remain difficult to prevent or attribute. The difficulty of affirming attribution to the level of certainty required for counteractions or incarceration is considerable, even for "traditional," non-engineered bioweapons.

Finally, synthetic biology is enabling advances in the rapid development and production of medical countermeasures that may be effective against synthetic biology–derived agents. However, many such efforts, which are being pursued in both industry and academia, are still in the research phase, and there remain complex barriers to widespread use of these novel approaches, including regulatory hurdles and hurdles to industry involvement. This field needs to be monitored carefully over time.

9

Moving Forward: Conclusions and Recommendations

The age of synthetic biology has brought with it opportunities to transform approaches to treating disease, manufacturing chemicals, producing fuels, remediating contaminants, and numerous other applications with benefits to humankind. Some synthetic biology capabilities, however, have dual-use potential—that is, they can be misdirected to cause harm to humans, animals, plants, and the environment. This study focuses on the potential for such biotechnologies to be used to attack the U.S. military or the American people and assesses the level of concern warranted on the part of the U.S. Department of Defense and others responsible for protecting public health and national security. The study's deliberative process included the identification of concepts, approaches, and tools that biotechnology comprises in the age of synthetic biology, the identification of specific capabilities that an adversary might potentially gain from the misapplication of synthetic biology, and the development of a framework to guide an assessment of concerns related to these capabilities. This approach was used to provide structure and transparency without being overly prescriptive. The framework was then applied to analyze the state of the art of the technology involved in each capability, the feasibility of using the capability to produce an effective weapon, and the characteristics and resources an actor would require to carry out an attack. After accounting for, in a less in-depth way, proactive and reactive measures that could be taken to mitigate attacks, an overall level of concern was determined for each capability relative to the other capabilities considered. Recognizing that future advances in knowledge or technology may increase the feasibility or impacts of some capabilities and thus raise the level of concern warranted, potential developments were identified that should be monitored and otherwise considered going forward.

Although its primary focus was on the specific capabilities analyzed, the study was carried out with an eye toward the broader backdrop of the history and structures of biological sciences and technology, national defense, and public health in the United States. The misuse of biological sciences to develop biological weapons predates the advent of synthetic biology. A wide range of malicious actors have used or sought to use bioweapons and chemical weapons, including national governments, small groups or cults, and even individuals. Fortunately, actual use of biological weapons has been rare. While there is considerable disagreement among experts about *why* misuse of biology has been rare, or if it is likely to always remain rare, synthetic biology has the potential to change the likelihood and consequences of misuse. Though important for myriad beneficial applications, synthetic biology and related biotechnologies change the defense landscape by making possible new modes of attack and by lowering the barriers to developing and using biological weapons (and to some extent chemical weapons), potentially putting bioweapons within the reach of less-resourced actors. The United States' approach to biodefense was not

designed to counter all the types of weapons (or types of adversaries) that are now possible in the age of synthetic biology. One motivation for this report is to help inform the U.S. defense agencies' efforts to update their approach to biodefense in order to detect and respond to these new threats.

On the positive side, it is expected that synthetic biology and other technologies will enable the development of new methods for detecting biological anomalies, new diagnostic tools, and new therapeutics—developments that could complement and bolster existing biodefense tools. Since 2001, the United States has significantly expanded efforts to counter biological threats, in particular those related to the use of known pathogens to create bioweapons. Among other accomplishments, a multipronged approach has been developed to acquire medical countermeasures, develop a stockpile system for those countermeasures, boost security and safety in the handling of pathogens, and coordinate a response to a biological weapons attack. Given the complicated nature of the biological weapons threat, however, it is not possible to be fully prepared for every contingency. Many pathogens that could be used to create weapons are widely accessible in laboratories around the world and in natural reservoirs such as infected people or animals. The amount of infectious material needed as a seed stock for a weapon is minute, because it is possible to grow a few bacterial cells into quantities capable of effecting a large-scale attack. Furthermore, the infrastructure and laboratory training needed to develop a biological weapon using a known pathogen are dual use and relatively accessible.

The age of synthetic biology adds to these significant challenges. While the existing U.S. biodefense system is designed to defend against specific, naturally occurring pathogens, synthetic biology makes possible the creation of new or altered pathogens, as well as new types of biological weapons, and the relevant technologies are generally accessible all over the world. Synthetic biology also increases the overlap between biological and chemical weapons by enabling the use of biological components to make or deliver chemical agents. In determining how to plan for and respond to these evolving capabilities, defense and public health agencies are challenged to consider these newer threats alongside other risks such as traditional biological weapons threats, threats to national security and stability from naturally occurring biological threats (such as pandemics), and threats related to explosives and nuclear, chemical, and radiological weapons. In resource-constrained environments, users of the framework and assessments presented in this report will need to bear in mind this backdrop of risk in determining how biological threats fit into the broader threat landscape. Comparing the risks related to synthetic biology to those related to these other types of threats was not within the scope of this study.

OVERARCHING RECOMMENDATION

Biotechnology in the age of synthetic biology expands the landscape of potential defense concerns. The U.S. Department of Defense (DoD) and its partnering agencies should continue to pursue ongoing strategies for chemical and biological defense; these strategies remain relevant in the age of synthetic biology. DoD and its partners also need to have approaches to account for the broader capabilities enabled by synthetic biology, now and into the future.

CONCERNS POSED BY SYNTHETIC BIOLOGY–ENABLED CAPABILITIES

The study identified 12 distinct capabilities—ways in which an adversary could potentially pursue an attack using synthetic biology—and grouped these capabilities into three major categories: concerns related to pathogens, concerns related to the production of chemicals or biochemicals, and concerns related to bioweapons that alter the human host. Each capability was analyzed individually, trends and key considerations were identified within each grouping, and each capability was ranked in relation to the other capabilities to determine an overall assessment of concerns. Developments that might affect capabilities and concerns in the future were also considered.

Overall Assessment of Concerns

Figure 9-1 presents a relative ranking of concerns related to the synthetic biology–enabled capabilities that were analyzed. This ranking was generated through an iterative discussion of four factors that increase or decrease

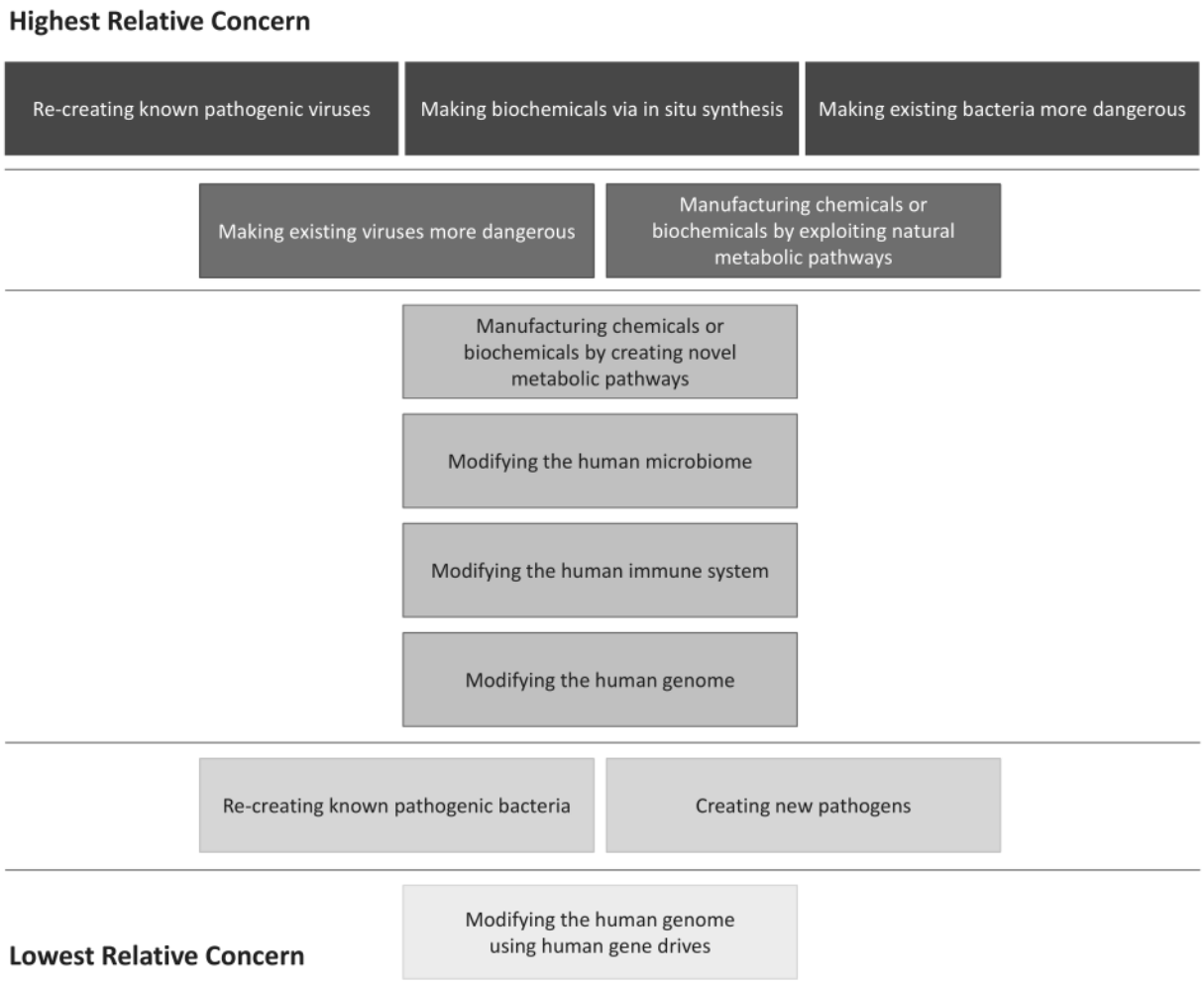


FIGURE 9-1 Relative ranking of concerns related to the synthetic biology–enabled capabilities analyzed. At present, capabilities toward the top warrant a relatively high level of concern while capabilities toward the bottom warrant a relatively low level of concern.

the likelihood or impact of an attack—Usability of the Technology, Usability as a Weapon, Requirements of Actors, and Potential for Mitigation—for each capability as compared to the other capabilities. As discussed in Chapter 3 (Applying the Framework in the Assessment of Concern), this assessment is based on a holistic view of the factors and capabilities assessed and is not a formulaic approach. Table 9-1 summarizes the assessment of the specific factors considered when analyzing the individual capabilities and Figure 9-2 shows the relative concern for each capability, organized by factor.

While the ranking of concerns has a strong foundation based on the expertise of the committee members and the breadth and depth of the committee's discussions, there are a few important limitations to note. One is that the study process did not involve accessing intelligence or other classified information. The study also did not consider information related to the capabilities or intents of specific adversaries. Others may use such information, along with details about government programs aimed at deterring, detecting, attributing, and addressing the consequences of biological attacks, to complement and expand upon this report's analysis. Likewise, additional

TABLE 9-1 Relative Level of Concern Related to Each Factor for Each Capability Considered

	Usability of the Technology	Usability as a Weapon	Requirements of Actors	Potential for Mitigation
Level of concern for re-creating known pathogenic viruses	High	Medium-high	Medium	Medium-low
Level of concern for re-creating known pathogenic bacteria	Low	Medium	Low	Medium-low
Level of concern for making existing viruses more dangerous	Medium-low	Medium-high	Medium	Medium
Level of concern for making existing bacteria more dangerous	High	Medium	Medium	Medium
Level of concern for creating new pathogens	Low	Medium-high	Low	Medium-high
Level of concern for manufacturing chemicals or biochemicals by exploiting natural metabolic pathways	High	High	Medium	Medium-high
Level of concern for manufacturing chemicals or biochemicals by creating novel metabolic pathways	Medium-low	High	Medium-low	Medium-high
Level of concern for making chemicals or biochemicals via in situ synthesis	Medium-high	Medium	Medium	High
Level of concern for modifying the human microbiome	Medium-low	Medium	Medium	Medium-high
Level of concern for modifying the human immune system	Medium	Medium-low	Low	High
Level of concern for modifying the human genome	Medium-low	Low	Medium-low	High
Level of concern for modifying the human genome using human gene drives	Low			

details about potential mitigation options could be used to expand upon the report's analysis. In addition, there was no attempt to weigh the likelihood that an actor would choose to use synthetic biology instead of a more "traditional" approach when pursuing an outcome that could be achieved with or without synthetic biology. For example, an actor seeking to deploy a known pathogen in an attack could acquire the pathogen by re-creating it using synthetic biology or by stealing existing cultures of the pathogen from a legitimate research laboratory. Similarly, an actor seeking to acquire a given chemical or toxin may choose to engineer a microbe to produce it or may produce it through traditional chemical synthesis. In such cases, determining which method is more likely would require information about an actor's intentions, resources, and capabilities, which was beyond the scope of this study. The rankings are therefore agnostic to the availability of these alternative routes and are based solely on the capabilities that synthetic biology provides to an actor. It also follows that as technologies advance, an actor's proclivity to pursue a given route may change.

The capabilities were ranked in relation to each other and grouped into five major levels of concern, relative to each other. There was no attempt to quantify the relative levels of concern; as such, the dividing lines within Figure 9-1 are not intended to indicate that one capability poses twice (or any numerical multiple of) the level of concern compared to the capability below it. In addition, the grouping of two capabilities into the same category

of concern does not indicate that those capabilities are identical in terms of the factors considered or the relative values placed on those factors. For example, re-creating known pathogenic bacteria and creating new pathogens are associated with a similar overall level of concern, but for different reasons. Finally, it is important to note that this assessment represents a snapshot in time and represents the range of concern associated with each capability, with particular exceptions or special cases noted in Chapters 4–6, and will change as knowledge and technologies advance.

Capabilities currently warranting the highest relative level of concern include re-creating known pathogenic viruses, making biochemicals via in situ synthesis, and the use of synthetic biology to make existing bacteria more dangerous. These capabilities are based on technologies and knowledge that are readily available to a wide array of actors. The ability to mitigate attacks related to these capabilities would depend on the effectiveness of existing countermeasures, such as antibiotics or vaccines, toward the agents used.

Capabilities posing a moderate-to-high relative level of concern include manufacturing chemicals or biochemicals by exploiting natural metabolic pathways and making existing viruses more dangerous. These capabilities are also supported by available technologies and knowledge but involve more constraints and would likely be limited by factors related to both biology and skill. For example, while viral genomes are easily manipulated on a molecular basis, constraints on what types of change those genomes can accommodate limit capability in this area. Similarly, at present, it takes a fair amount of skill to engineer a bacterium to express a pathway to efficiently produce a chemical or biochemical. While both capabilities are considered to be in the same grouping, modifying viral characteristics intentionally using rational design remains a substantial challenge, making the modification of an existing virus slightly less concerning at present. Similar to the capabilities in the top category of relative concern, mitigation options for these capabilities depend largely on existing infrastructure.

Capabilities posing a moderate relative level of concern include manufacturing chemicals or biochemicals by creating novel metabolic pathways, efforts to modify the human microbiome to cause harm, efforts to modify the human immune system, and efforts to modify the human genome. Although conceivable, these capabilities are more futuristic—likely limited by available knowledge and technology, as described in Chapters 5 and 6. However, there are significant forces driving rapid advancement in all of these areas. Manufacturing chemicals or biochemicals by creating novel metabolic pathways was placed highest in this grouping because once a synthesis pathway for a chemical or biochemical is known, the tools for engineering a bacterial (or other) cell to produce it are fairly well developed. While the detailed pathways by which certain chemicals may be synthesized in a biological organism are not yet known, commercial applications are driving progress in this area. The modification of the human microbiome is placed next in this grouping. Although current understanding of the complex and dynamic system that is our microbiome is relatively low, there are significant efforts to increase this knowledge because of the desire to modulate the microbiome to improve human health. Modification of the immune system and modification of the human genome are the third and fourth capabilities in this grouping, largely due to the limits of available knowledge related to the mechanisms of action and means of delivery that would be involved in developing and using bioweapons based on these capabilities. However, these areas are also being vigorously pursued because of clear biomedical applications.

Capabilities warranting a lower relative level of concern include re-creating known pathogenic bacteria and creating new pathogens. These capabilities involve major challenges from the standpoint of both design and implementation. In particular, while the technology for synthesizing and assembling larger segments of DNA continues to advance, the synthesis of bacteria is currently limited by constraints on synthesizing, manipulating, and booting an entire bacterial genome. In addition, antibiotics and other therapeutics are available to counter many bacterial pathogens. Constructing a totally novel pathogen has tremendous challenges. If it is difficult to build a known bacterium, it is all the more challenging to design one from scratch. In this regard, an actor may decide to try to design a virus, but in this case one would be working against the large barrier of evolutionary constraints created by hundreds of millions of years of co-evolution between viruses and their hosts. That said, combinatorial approaches could enable the exploration of sequence space that nature has not yet achieved.

The use of human gene drives warrants a minimal level of concern because it would be impractical to rely on sexual reproduction for a gene drive to spread through a human population.

In addition to the relative level of concern posed by individual capabilities, the study included consideration of

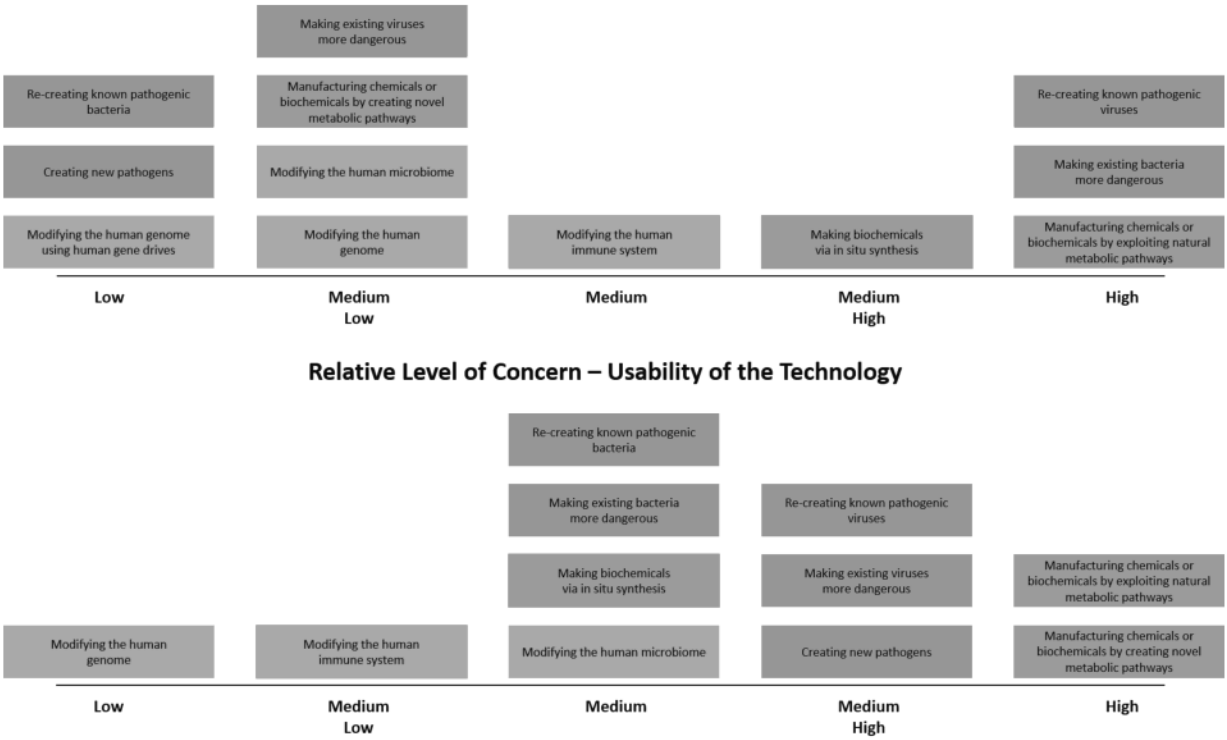


FIGURE 9-2 Relative level of concern related to each factor for each capability considered. NOTE: Coloring indicates the chapter in which the assessment for each capability is presented: Chapter 4 (orange), Chapter 5 (blue), or Chapter 6 (green).

how two or more capabilities may be used in combination. Such an approach could create synergies that result in either a more dangerous weapon or using one capability to overcome barriers that currently hinder another capability. For example, a pathway for the production of a toxin could potentially be implanted in the human microbiome, an "intersectional" approach considered to warrant a high level of concern. Similarly, particular genes or RNA molecules that modulate the immune system could potentially be mounted on a virus to lead to greater harm than either the genes or the virus would on their own. Going forward, it will be important to continue to consider how scientific and technological advances may synergize to improve existing approaches or create novel ones.

Assessment of Specific Types of Capabilities

The assessment of overall concerns draws upon the analysis of each of the 12 specific capabilities considered. In addition to conclusions related to the *relative* assessment of concerns, underlying themes and conclusions emerged when each individual capability was examined in the context of other capabilities in the same category (e.g., when assessing all approaches that involve pathogens). Underlying themes and conclusions related to pathogens, the production of chemicals or biochemicals, and bioweapons that alter the human host are discussed below.

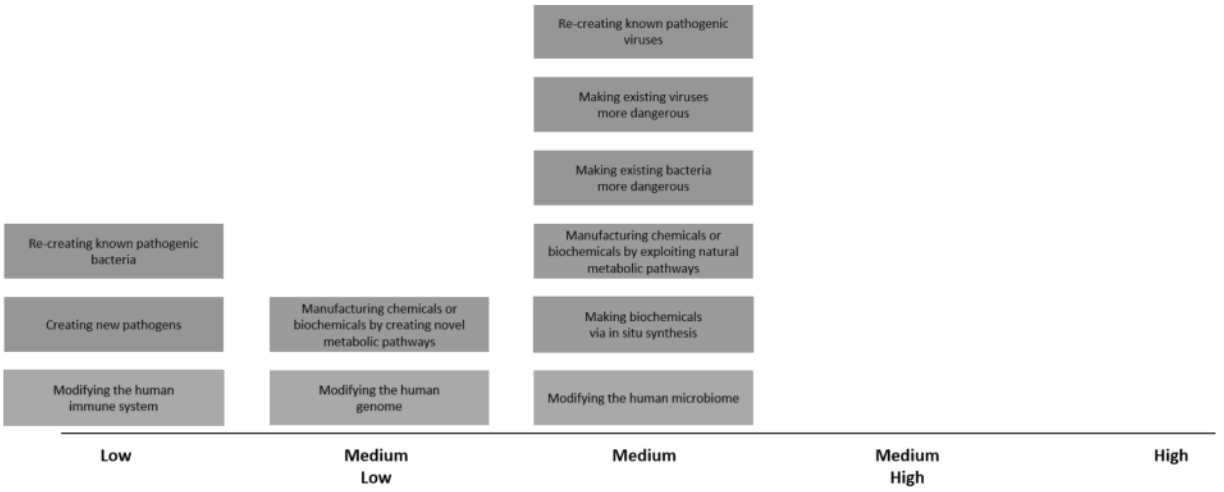


FIGURE 9-2 Continued.

Pathogens

Chapter 4 focuses on the use of biotechnology to create pathogenic agents, including the possibility of re-creating known pathogens, modifying both pathogenic and nonpathogenic microbes to enhance their capability to cause harm, and creating new pathogens. Rapid advances in DNA synthesis technology have made it possible to obtain a pathogen without direct access to the infectious agent itself. Today, any viral genome can be synthesized based on published sequences, and booting that sequence into a replicating form is also feasible for most viruses. Similar approaches to creating bacteria are currently more difficult due to the size of their genomes and the fact that they are living organisms and not obligate intracellular parasites like viruses, though these technical bottlenecks will likely be reduced over time. Because known pathogens have been studied extensively, and because the existence (or lack) of medical countermeasures is also known, there is a relatively high level of confidence in assigning relative levels of concern to the re-creation of known viruses and bacteria. For example, it is currently easier to re-create a virus than a bacterium in the laboratory, though prophylactics and therapeutics against these agents sometimes, but not always, mitigate the level of concern.

The technologies to manipulate microbial genomes to add new phenotypes such as drug resistance have been available for decades and continue to be made simpler. Here again, there are differences in the feasibility of applying these approaches to bacteria and viruses; whereas adding genes to bacteria does not usually significantly

affect the ability of the bacteria to grow and divide, the way viral genomes have evolved makes them more sensitive to changes, such that altering viral genomes often reduces their virulence and replication abilities. Generally speaking, phenotypic modifications to pathogens may lessen the capability for mitigation. One notable example is adding antibiotic resistance to bacteria or adding antiviral resistance to those few viruses for which antivirals exist. Engineering bacteria or viruses to resist existing therapeutics would likely be relatively straightforward to accomplish and could seriously undermine the ability to mitigate an attack by treating infected individuals.

Production of Chemicals or Biochemicals

As discussed in Chapter 5, engineering organisms to produce chemicals or biochemicals is becoming more feasible as researchers learn more about the natural pathways used to produce these substances and as better tools are developed to build predictable synthetic pathways. Just as drug resistance can be engineered into bacteria, so can simple or even complex biosynthetic pathways. This capability is being driven largely by a desire to use biotechnology to produce useful molecules, but can be subverted by those with malicious intent. The commercial drivers behind these approaches will certainly widen the bottlenecks over time. Moreover, combinatorial approaches and the use of computer algorithms to aid in pathway design will bring down barriers to building new synthetic pathways.

Mitigation of attacks based on these modified organisms could be difficult to achieve. Currently, when presented with the signs of a chemical attack, first responders and medical professionals are not trained to suspect that the chemical was produced or delivered biologically. Similarly, having a bacterium that normally does not produce a toxin act as the delivery vehicle for that toxin could thwart existing diagnostic tests.[1] Therefore, while at present there are barriers to effectively developing these capabilities, the potential deficiencies in mitigation raise the level of concern.

Bioweapons That Alter the Human Host

Chapter 6 focuses on the possible vulnerabilities and means of attack that are more closely related to the human body itself. Here, one focus was on engineering the microbiomes of the gut, skin, oral cavity, or nasopharyngeal space. Such manipulations could be used, for example, to directly affect the function of the gastrointestinal tract or the skin, cause dysbiosis, or even potentially affect other aspects of human physiology such as the immune or nervous systems. If such manipulations can be achieved, the level of concern would be high because the opportunities for mitigation could be quite limited. The detailed interactions that occur in the microbiome environment are being studied intensively, and knowledge in this area is constantly increasing.

The study also included consideration of approaches that could potentially be used to modify the human immune system by inducing immune suppression or hyperreactivity or by using immunosuppressive agents in combination with existing pathogens. Potential approaches that use genes or RNAs as weapons, use genome editing, or use human gene drives were also considered. In general, these approaches pose a lower level of concern with respect to the technologies, actors' capabilities, and organizational footprints, because of the uncertainties associated with obtaining a useful weapon given the immature state of these areas of research. However, due to the novelty of these approaches, it is possible that if such approaches were used successfully, options for mitigation could be fairly limited, thus somewhat increasing the level of concern. The notable exception to these concerns is the use of human gene drives to alter the human genome. Because gene drives require sexual reproduction to spread, it would be exceedingly difficult to affect change to large populations of humans without waiting many, many generations. This capability was therefore placed in the lowest level of concern. It is noted, however, that using gene drives to alter other organisms such as mosquito vectors, in an effort to improve their ability to transmit pathogens (or to broaden the list of pathogens they can transmit) may become a concern as more is learned about the interactions between pathogens and insect vectors.

[1] Depending on the site or type of infection, diagnostics are often based on species identification, and therefore the presence of a toxin might be missed if the species is not one that normally produces a toxin.

Potential Developments to Monitor

This report's analysis necessarily reflects a snapshot in time, given understanding of current technologies and capabilities. As knowledge and biotechnology continue to evolve, it can be expected that current bottlenecks will open and current barriers will be broken. To consider how such developments might affect biodefense concerns, key bottlenecks and barriers were identified that, if overcome, could substantially increase the feasibility or impact of a potential attack and thus increase the level of concern warranted. It is impossible to predict precisely when the next fundamental breakthrough in technology with wide-ranging applications (and implications), akin to PCR tools or the gene editing platform CRISPR/Cas9, will arise or even what that technology might be. Such developments are influenced by the drivers of commercial and academic research, as well as by possible converging or synergistic technologies that may come from outside the field of synthetic biology. The use of a framework such as the one presented in this report facilitates the identification of bottlenecks and barriers, as well as the ability to recognize when bottlenecks and barriers have been overcome, by identifying the types of technological capabilities that would facilitate the production and use of synthetic biology–enabled bioweapons. A summary of key bottlenecks and barriers and areas worth monitoring is provided in Table 9-2. Based on knowledge of the synthetic biology field, the table notes areas of commercial activity that could speed the process toward overcoming these bottlenecks and barriers.

Conclusions and recommendations were developed based on the analysis of individual synthetic biology–enabled capabilities, the holistic assessment of relative levels of concern for all capabilities considered, and identification of bottlenecks and barriers that, if overcome, could affect the level of concern in the future.

TABLE 9-2 Bottlenecks and Barriers That Currently Constrain the Capabilities Considered and Developments That Could Reduce These Constraints[a]

Capability	Bottleneck or Barrier	Relevant Developments to Monitor
Re-creating known pathogenic viruses (Chapter 4)	Booting	Demonstrations of booting viruses with synthesized genomes
Re-creating known pathogenic bacteria (Chapter 4)	DNA synthesis and assembly	Improvements in synthesis and assembly technology for handling larger DNA constructs
	Booting	Demonstrations of booting bacteria with synthesized genomes
Making existing viruses more dangerous (Chapter 4)	Constraints on viral genome organization	Increased knowledge of viral genome organization and/or demonstration of combinatorial approaches capable of facilitating larger-scale modifications to viral genome
	Engineering complex viral traits	Increased knowledge of determinants of complex viral traits, as well as how to engineer pathways to produce them
Making existing bacteria more dangerous (Chapter 4)	Engineering complex bacterial traits	Advances in combinatorial approaches and/or increased knowledge of determinants of complex bacterial traits, as well as how to engineer pathways to produce them
Creating new pathogens (Chapter 4)	Limited knowledge regarding minimal requirements for viability (in both viruses and bacteria)	Increased knowledge of requirements for viability in viruses or bacteria
	Constraints on viral genome organization	Increased knowledge of viral genome organization and/or demonstration of combinatorial approaches capable of facilitating larger-scale modifications to viral genome

continued

TABLE 9-2 Continued

Capability	Bottleneck or Barrier	Relevant Developments to Monitor
Manufacturing chemicals or biochemicals by exploiting natural metabolic pathways (Chapter 5)	Tolerability of toxins to the host organism synthesizing the toxin	Pathway elucidation, improvements in circuit design, and improvements in host ("chassis") engineering to make toxins tolerable to the host organism synthesizing the toxin
	Pathway not known	Pathway elucidation and/or demonstrations of combinatorial approaches
	Challenges to large-scale production	Improvements in intracellular and industrial productivity
Manufacturing chemicals or biochemicals by creating novel metabolic pathways (Chapter 5)	Tolerability of toxins to the host organism synthesizing the toxin	Pathway elucidation and/or improvements in circuit design and/or improvements in host ("chassis") engineering to make toxins tolerable to the host organism synthesizing the toxin
	Engineering enzyme activity	Increased knowledge of how to modify enzymatic functions to make specific products
	Limited knowledge of requirements for designing novel pathways	Improvements in directed evolution and/or increased knowledge of how to build pathways from disparate organisms
	Challenges to large-scale production	Improvements in intracellular and industrial productivity
Making biochemicals via in situ synthesis (Chapter 5)	Limited understanding of microbiome	Improvements in knowledge related to microbiome colonization of host, in situ horizontal transfer of genetic elements, and other relationships between microbiome organisms and host processes
Modifying the human microbiome (Chapter 6)	Limited understanding of microbiome	Improvements in knowledge related to microbiome colonization of host, in situ horizontal transfer of genetic elements, and other relationships between microbiome organisms and host processes
Modifying the human immune system (Chapter 6)	Engineering of delivery system	Increased knowledge related to the potential for viruses or microbes to deliver immunomodulatory factors
	Limited understanding of complex immune processes	Knowledge related to how to manipulate the immune system, including how to cause autoimmunity and predictability across a population
Modifying the human genome (Chapter 6)	Means to engineer horizontal transfer	Increased knowledge of techniques to effectively alter the human genome through horizontal transfer of genetic information
	Lack of knowledge about regulation of human gene expression	Increased knowledge related to regulation of human gene expression

[a]Shading indicates developments thought to be propelled by commercial drivers. Some approaches, such as combinatorial approaches and directed evolution, may allow bottlenecks and barriers to be widened or overcome with less explicit knowledge or tools.

Conclusions and Recommendations: Synthetic Biology Expands What Is Possible

Synthetic biology expands what is possible in creating new weapons. It also expands the range of actors who could undertake such efforts and decreases the time required. Based on this study's analysis of the potential ways in which synthetic biology approaches and tools may be misused to cause harm, the following specific observations were made:

(a) **Of the potential capabilities assessed, three currently warrant the most concern: (1) re-creating known pathogenic viruses, (2) making existing bacteria more dangerous, and (3) making harmful biochemicals via in situ synthesis.** The first two capabilities are of high concern due to usability of the technology. The third capability, which involves using microbes to produce harmful biochemicals in humans, is of high concern because its novelty challenges potential mitigation options.

(b) **With regard to *pathogens*, synthetic biology is expected to (1) expand the range of what could be produced, including making bacteria and viruses more harmful; (2) decrease the amount of time required to engineer such organisms; and (3) expand the range of actors who could undertake such efforts.** The creation and manipulation of pathogens is facilitated by increasingly accessible technologies and starting materials, including DNA sequences in public databases. A wide range of pathogen characteristics could be explored as part of such efforts.

(c) **With regard to *chemicals*, *biochemicals*, and *toxins*, synthetic biology blurs the line between chemical and biological weapons.** High-potency molecules that can be produced through simple genetic pathways are of greatest concern, because they could conceivably be developed with modest resources and organizational footprint.

(d) **It may be possible to use synthetic biology to *modulate human physiology* in novel ways.** These ways include physiological changes that differ from the typical effects of known pathogens and chemical agents. Synthetic biology expands the landscape by potentially allowing the delivery of biochemicals by a biological agent and by potentially allowing the engineering of the microbiome or immune system. Although unlikely today, these types of manipulations may become more feasible as knowledge of complex systems, such as the immune system and microbiome, grows.

(e) **Some malicious applications of synthetic biology may not seem plausible now but could become achievable if certain barriers are overcome.** These barriers include knowledge barriers, as is the case for building a novel pathogen, or technological barriers, as in engineering complex biosynthetic pathways into bacteria or re-creating known bacterial pathogens. It is important to continue to monitor advances in biotechnology that may lower these barriers.

FUTURE USE OF THE FRAMEWORK

A framework that can be both relatively straightforward and enduring in its utility is valuable. There are many different types of frameworks that have been applied to issues related to the misuse of biological agents, each of which has its advantages and disadvantages. The framework presented in this report specifies a process to facilitate the consideration of expert opinions regarding the level of concern about specific synthetic biology–enabled capabilities or combinations of capabilities. The subjective nature of the framework requires that its users have familiarity with the field of biotechnology and, as appropriate, that domain experts are enlisted to provide and evaluate pertinent data and fill in any gaps in expertise. The technical depth and breadth of this study committee, along with the processes used to facilitate its discussions, helped to provide a thorough assessment while preventing individual perspectives from dominating the discussions.

Nonetheless, there are limitations to the framework's use in the context of this study. Specifically, the study task did not include consideration of intelligence information about the intents or capabilities of potential actors who may seek to misuse life sciences, nor did it include a comprehensive analysis of the U.S. government's capabilities related to preparedness for and mitigation of attacks. Therefore, this report does not represent a threat assessment. By combining this report's assessment of concern with intelligence and other information, others could, in the future, assess vulnerabilities and risks to inform decision making.

Conclusions and Recommendations: A Framework for Assessing Concern Contributes to Planning

The DoD and its interagency partners should use a framework in assessing synthetic biology capabilities and their implications.

(a) **A framework is a valuable tool for parsing the changing biotechnology landscape.**
(b) **Using a framework facilitates the identification of bottlenecks and barriers, as well as efforts to monitor advances in technology and knowledge that change what is possible.**
(c) **A framework provides a mechanism for incorporating the necessary technical expertise into the assessment.** A framework enables the participation of technical experts in synthetic biology and biotechnology along with experts in complementary areas (e.g., intelligence and public health).

BIODEFENSE IMPLICATIONS OF THE AGE OF SYNTHETIC BIOLOGY

It has been stated on numerous occasions, by both scientific and political leaders, that the 21st century is the century of the life sciences (U.S. Congress, 2000). Much of the excitement and anticipation comes from the promise that advances in biotechnology offer to society. But, as with previous expansions in technological capabilities, the potential for benefit also comes along with potential risks that the technology could be misused to cause harm. It is therefore wise for the U.S. government to pay close attention to rapidly advancing fields such as synthetic biology, just as it did to advances in chemistry and physics during the Cold War era. Approaches modeled after those taken to counter Cold War threats are not sufficient for biological and biologically–enabled chemical weapons in the age of synthetic biology. On the other hand, the nation's experience preparing for naturally occurring diseases provides a strong foundation to build upon in developing strategies to prevent and respond to emerging biological threats and biologically–enabled chemical threats. While this study does not constitute a threat assessment and does not make specific recommendations regarding addressing current vulnerabilities, several areas were identified that warrant attention as the nation seeks to bolster its preparedness and defense capabilities.

Conclusions and Recommendations: A Range of Strategies Is Needed to Prepare and Respond

Many of the traditional approaches to biological and chemical defense preparedness will be relevant to synthetic biology, but synthetic biology will also present new challenges. The DoD and partner agencies will need approaches to biological and chemical weapons defense to meet these new challenges.

(a) **The DoD and its partners in the chemical and biological defense enterprise should continue exploring strategies that are applicable to a wide range of chemical and biodefense threats.** Nimble biological and chemical defense strategies are needed because of rapid rates of technological change, as well as strategies adaptable to a wide range of threats because of uncertainty about which approaches an adversary might pursue.
(b) **The potential unpredictability related to how a synthetic biology–enabled weapon could manifest creates an added challenge to monitoring and detection. The DoD and its partners should evaluate the national military and civilian infrastructure that informs population-based surveillance, identification, and notification of both natural and purposeful health threats.** An evaluation should consider whether and how the public health infrastructure needs to be strengthened to adequately recognize a synthetic biology–enabled attack. Ongoing evaluation will support responsive and adaptive management as technology advances.
(c) **The U.S. government, in conjunction with the scientific community, should consider strategies that manage emerging risk better than current agent-based lists and access control approaches.** Strategies based on lists, such as the Federal Select Agent Program Select Agents and Toxins list, will be insufficient for managing risks arising from the application of synthetic biology. While measures to control access to physical materials such as synthetic nucleic acids and microbial strains have merits, such approaches will not be effective in mitigating all types of synthetic biology–enabled attacks.

Exploration Areas

Although it was outside the scope of this study to comprehensively assess the preparedness and response capabilities of existing military and civilian defense and public health enterprises or determine how to address gaps, **exploration of the following areas is suggested to address some of the challenges posed by synthetic biology:**

(a) **Developing capabilities to detect unusual ways in which a synthetic biology–enabled weapon may manifest.** For consequence management, expanding the development of epidemiological methods (e.g., surveillance and data collection) would strengthen the ability to detect unusual symptoms or aberrant patterns of disease. Enhancing epidemiological methods will have an additional benefit of strengthening the ability to respond to natural disease outbreaks.
(b) **Harnessing computational approaches for mitigation.** The role of computational approaches for prevention, detection, control, and attribution will become more important with the increasing reliance of synthetic biology on computational design and computational infrastructure.
(c) **Leveraging synthetic biology to advance detection, therapeutics, vaccines, and other medical countermeasures.** Taking advantage of beneficial applications of synthetic biology for countermeasure research and development is expected to prove valuable, along with corresponding efforts to facilitate the entire development process, including regulatory considerations.

A great deal of the scientific knowledge, materials, and techniques required for beneficial biological research or development could be misused. It is extremely challenging to prevent this, however, because the scientific community relies upon access to publications, genetic sequences, and biological materials to advance the state of science and, importantly, to reproduce the results of others to verify findings and build upon them. Biotechnology presents a "dual-use dilemma" (NRC, 2004), and synthetic biology is part of this dilemma. Although dual-use research is going to remain a challenge for scientists and for the nation's defense, there is reason for optimism that, with continued monitoring of biotechnology capabilities and strategic biodefense investments, the United States can foster fruitful scientific and technological advances while minimizing the risk that these same advances will be used for harm.

References

ACAAI (American College of Allergy, Asthma, and Immunology). 2017. Allergy Symptoms. Available at: http://acaai.org/allergies/symptoms. Accessed November 3, 2017.

AFHSB (Armed Forces Health Surveillance Branch). 2017. Global Emerging Infections Surveillance and Response System. Available at: https://health.mil/Military-Health-Topics/Health-Readiness/Armed-Forces-Health-Surveillance-Branch/Global-Emerging-Infections-Surveillance-and-Response. Accessed November 14, 2017.

Alibek, K. 1998. Behind the Mask: Biological Warfare. Perspective 9(1). Available at: http://www.bu.edu/iscip/vol9/Alibek.html. Accessed November 14, 2017.

Anderson, R.M., C. Fraser, A.C. Ghani, C.A. Donnelly, S. Riley, N.M. Ferguson, G.M. Leung, T.H. Lam, and A.J. Hedley. 2004. Epidemiology, transmission dynamics and control of SARS: The 2002–2003 epidemic. *Philosophical Transactions of the Royal Society London B* 359(1447):1091–1105.

Atkinson, N.J., J. Willeveldt, D.J. Evans, and P. Simmonds. 2014. The influence of CpG and UpA dinucleotide frequencies on RNA virus replication and characterization of the innate cellular pathways underlying virus attenuation and enhanced replication. *Nucleic Acids Research* 42(7):4527–4545.

Aubry, F., A. Nougairède, L. de Fabritus, G. Querat, E.A. Gould, and X. de Lamballerie. 2014. Single-stranded positive-sense RNA viruses generated in days using infectious subgenomic amplicons. *Journal of General Virology* 95(Pt. 11):2462–2467.

Awan, A.R., B.A. Blount, D.J. Bell, W.M. Shaw, J.C.H. Ho, R.M. McKiernan, and T. Ellis. 2017. Biosynthesis of the antibiotic nonribosomal peptide penicillin in baker's yeast. *Nature Communications* 8:15202.

Baker Lab. 2017. Rosetta. University of Washington. Available at: http://boinc.bakerlab.org/rosetta. Accessed November 8, 2017.

Barquist, L., C.J. Boinett, and A.K. Cain. 2013. Approaches to querying bacterial genomes with transposon-insertion sequencing. *RNA Biology* 10(7):1161–1169.

Barton, G.M., and R. Medzhitov. 2002. Retroviral delivery of small interfering RNA into primary cells. *Proceedings of the National Academy of Sciences of the United States of America* 99(23):14943–14945.

Becker, M.M., R.L. Graham, E.F. Donaldson, B. Rockx, A. Sims, T. Sheahan, R.J. Pickles, D. Corti, R.E. Johnston, R.S. Baric, and M.R. Denison. 2008. Synthetic recombinant bat SARS-like coronavirus is infectious in cultured cells and in mice. *Proceedings of the National Academy of Sciences of the United States of America* 105(50):19944–19949.

Benner, S.A., and A.M. Sismour. 2005. Synthetic biology. *Nature Reviews Genetics* 6(7):533–543.

Bennett, R.L., and J.D. Licht. 2018. Targeting epigenetics in cancer. *Annual Reviews of Pharmacology and Toxicology* 58:187–207.

Benson, R.A., I.B. McInnes, P. Garside, and J.M. Brewer. 2018. Model answers: Rational application of murine models in arthritis research. *European Journal of Immunology* 48(1):32–38.

Berg, P., D. Baltimore, H.W. Boyer, S.N. Cohen, R.W. Davis, D.S. Hogness, D. Nathans, R. Roblin, J.D. Watson, S. Weissman, and N.D. Zinder. 1974. Potential biohazards of recombinant DNA molecules. *Science* 185(4148):303.

Berkeley Lights. 2017. Berkeley Lights automates biological processing to reduce drug development timelines and bring therapies to market faster. News: September 12. Available at: https://www.berkeleylights.com/berkeley-lights-automates-biological-processing-reduce-drug-development-timelines-bring-therapies-market-faster. Accessed January 31, 2018.

Black, J.B., P. Perez-Pinera, and C.A. Gersbach. 2017. Mammalian synthetic biology: Engineering biological systems. *Annual Review of Biomedical Engineering* 19:249–277.

Blair, J.M., M.A. Webber. A.J. Baylay, D.O. Ogbolu, and L.J. Piddock. 2015. Molecular mechanisms of antibiotic resistance. *Nature Reviews Microbiology* 13:41–51.

Blue Ribbon Study Panel on Biodefense. 2015. A National Blueprint for Biodefense: Leadership and Major Reform Needed to Optimize Efforts—Bipartisan Report of the Blue Ribbon Study Panel on Biodefense. Washington, DC: Hudson Institute. October. Available at: http://www.biodefensestudy.org/a-national-blueprint-for-biodefense. Accessed February 20, 2018.

Bogers, W.M., H. Oostermeijer, P. Mooij, G. Koopman, E.J. Verschoor, D. Davis, J.B. Ulmer, L.A. Brito, Y. Cu, K. Banerjee, G.R. Otten, B. Burke, A. Dey, J.L. Heeney, X. Shen, G.D. Tomaras, C. Labranche, D.C. Montefiori, H.X. Liao, B. Haynes, A.J. Geall, and S.W. Barnett. 2015. Potent immune responses in rhesus macaques induced by nonviral delivery of a self-amplifying RNA vaccine expressing HIV Type 1 envelope with a cationic nanoemulsion. *Journal of Infectious Diseases* 211(6):947–955.

Böhme, L., and T. Rudel. 2009. Host cell death machinery as a target for bacterial pathogens. *Microbes and Infection* 11(13):1063–1070.

Bornscheuer, U.T., G.W. Huisman, R.J. Kazlauskas, S. Lutz, J.C. Moore, and K. Robins. 2012. Engineering the third wave of biocatalysis. *Nature* 485(7397):185–194.

Breaker, R. 2017. Presentation at the First Meeting of the Committee on Strategies for Identifying and Addressing Biodefense Vulnerabilities Posed by Synthetic Biology, January 26, Washington, DC.

Brocken, D.J.W., M. Tark-Dame, and R.T. Dame. 2017. dCas9: A versatile tool for epigenome editing. *Current Issues in Molecular Biology* 26:15–32.

Brojatsch, J., A. Casadevall, and D. Goldman. 2014. Molecular determinants for a cardiovascular collapse in anthrax. *Frontiers in Bioscience* 6:139–147.

Brophy, J.A., and C.A. Voigt. 2014. Principles of genetic circuit design. *Nature Methods* 11(5):508–520.

Brubaker, S.W., K.S. Bonham, I. Zanoni, and J.C. Kagan. 2015. Innate immune pattern recognition: A cell biological perspective. *Annual Review of Immunology* 33:257–290.

Buchan, J.R., L.S. Aucott, and I. Stansfield. 2006. tRNA properties help shape codon pair preferences in open reading frames. *Nucleic Acids Research* 34(3):1015–1027.

Burns, C.C., J. Shaw, R. Campagnoli, J. Jorba, A. Vincent, J. Quay, and O.M. Kew. 2006 Modulation of polio virus replicative fitness in HeLa cells by deoptimization of synonymous codon usage in the capsid region. *Journal of Virology* 80(7):3259–3272.

Burstein, D., L.B. Harrington, S.C. Strutt, A.J. Probst, K. Anantharaman, B.C. Thomas, J.A. Doudna, and J.F. Banfield. 2017. New CRISPR-Cas systems from uncultivated microbes. *Nature* 542(7640):237–241.

Butler, D. 2013. When Google got flu wrong: US outbreak foxes a leading web-based method for tracking seasonal flu. *Nature* 494(7436):155–156.

Butterfield, G.L., M.J. Lajoie, H.H. Gustafson, D.L. Sellers, U. Nattermann, D. Ellis, J.B. Bale, S. Ke, G.H. Lenz, A. Yehdego, R. Ravichandran, S.H. Pun, N.P. King, and D. Baker. 2017. Evolution of a designed protein assembly encapsulating its own RNA genome. *Nature* 552(7685):415–420.

BWC (Biological Weapons Convention). 1972. On the Prohibition of the Development, Production and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on Their Destruction. Available at: https://www.unog.ch/80256EDD006B8954/(httpAssets)/C4048678A93B6934C1257188004848D0/$file/BWC-text-English.pdf. Accessed January 26, 2017.

Byerly, C.R. 2010. The U.S. military and the influenza pandemic of 1918–1919. *Public Health Reports* 125(Suppl. 3):82–91.

Candela, M., E. Biagi, S. Maccaferri, S. Turroni, and P. Brigidi. 2012. Intestinal microbiota is a plastic factor responding to environmental changes. *Trends in Microbiology* 20(8):385–391.

Carbonell, P., A. Currin, A.J. Jervis, N.J.W. Rattray, N. Swainston, C. Yan, E. Takano, and R. Breitling. 2016. Bioinformatics for the synthetic biology of natural products: Integrating across the Design–Build–Test cycle. *Natural Product Reports* 33(8):925–932.

Carleton, H.A., and P. Gerner-Smidt. 2016. Whole-genome sequencing is taking over foodborne disease surveillance. *Microbe* 11(7):311–317.

Carlson, R. 2005. Splice it yourself. *Wired*, May 1. Available at: https://www.wired.com/2005/05/splice-it-yourself. Accessed November 14, 2017.

Carter, S.R., and R.M. Friedman. 2015. *DNA Synthesis and Biosecurity: Lessons Learned and Options for the Future*. J. Craig Venter Institute. Available at: http://www.jcvi.org/cms/fileadmin/site/research/projects/dna-synthesis-biosecurity-report/report-complete.pdf. Accessed January 31, 2018.

Carus, W.S. 2017. A Short History of Biological Warfare: From Pre-History to the 21st Century. CSWMD Occasional Paper 12. Center for the Study of Weapons of Mass Destruction, National Defense University. August 7. Available at: http://wmdcenter.ndu.edu/Publications/Publication-View/Article/1270642/a-short-history-of-biological-warfare-from-pre-history-to-the-21st-century. Accessed on November 13, 2017.

Casadevall, A., and D.A. Relman. 2010. Microbial threat lists: Obstacles in the quest for biosecurity? *Nature Reviews Microbiology* 8(2):149–154.

Cavallo, R., G. Antonelli, and H.H. Hirsch. 2013. Viruses and immunity in transplant patients. *Clinical and Developmental Immunology* 2013:492352.

CBC. 2017. Women accused of poisoning North Korea's Kim Jong-nam arrive at Malaysia court. CBC News: July 27. Available at: http://www.cbc.ca/news/world/north-korea-kim-jong-nam-malaysia-1.4225369. Accessed on November 2, 2017.

CDC (Centers for Disease Control and Prevention). 2001. The Public Health Response to Biological and Chemical Terrorism: Interim Planning Guidance for State Public Health Officials. July 2001. Available at: https://emergency.cdc.gov/documents/planning/planningguidance.pdf. Accessed May 12, 2017.

CDC. 2014a. About Quarantine and Isolation. Available at: https://www.cdc.gov/quarantine/quarantineisolation.html. Accessed November 14, 2017.

CDC. 2014b. Laboratory Network for Biological Terrorism. Available at: https://emergency.cdc.gov/lrn/biological.asp. Accessed November 14, 2017.

CDC. 2014c. Laboratory Response Network for Chemical Threats (LRN-C). Available at: https://emergency.cdc.gov/lrn/chemical.asp. Accessed November 14, 2017.

CDC. 2017a. Epi-X. Available at: https://www.cdc.gov/mmwr/epix/epix.html. Accessed November 14, 2017.

CDC. 2017b. MicrobeNet. Available at: https://www.cdc.gov/microbenet/index.html. Accessed November 14, 2017.

CDC. 2017c. Overview of Influenza Surveillance in the United States. Available at: https://www.cdc.gov/flu/weekly/overview.htm. Accessed November 14, 2017.

CDC. 2017d. Preparation and Planning for Bioterrorism Emergencies. Available at: https://emergency.cdc.gov/bioterrorism/prep.asp. Accessed on May 12, 2017.

CDC. 2017e. PulseNet. Available at: https://www.cdc.gov/pulsenet/index.html. Accessed November 14, 2017.

CDC. 2017f. Strategic National Stockpile. Available at: https://www.cdc.gov/phpr/stockpile/index.htm. Accessed November 14, 2017.

CDC/APHIS (Centers for Disease Control and Prevention and U.S. Department of Agriculture Animal and Plant Health Inspection Service). 2017. Select Agents and Toxins List. Available at: https://www.selectagents.gov/selectagentsandtoxinslist.html. Accessed November 6, 2017.

Cello, J., A.V. Paul, and E. Wimmer. 2002. Chemical synthesis of poliovirus cDNA: Generation of infectious virus in the absence of natural template. *Science* 297(5583):1016–1018.

Champer, J., R. Reeves, S.Y. Oh, C. Liu, J. Liu, A.G. Clark, and P.W. Messer. 2017. Novel CRISPR/Cas9 gene drive constructs reveal insights into mechanisms of resistance allele formation and drive efficiency in genetically diverse populations. *PLoS Genetics* 13(7):e1006796.

Chan, L.Y., S. Kosuri, and D. Endy. 2005. Refactoring bacteriophage T7. *Molecular Systems Biology* 1(1):2005.0018.

Chan, Y.K., and M.U. Gack. 2016. Viral evasion of intracellular DNA and RNA sensing. *Nature Reviews in Microbiology* 14(6):360–373.

Chao, R., Y. Yuan, and H. Zhao. 2015. Building biological foundries for next-generation synthetic biology. *Science China Life Science* 58(7):658–665.

Charles-Smith, L.E., T.L. Reynolds, M.A. Cameron, M. Conway, E.H.Y. Lau, J.M. Olsen, J.A. Pavlin, M. Shigematsu, L.C. Streichert, K.J. Suda, and C.D. Corle. 2015. Using social media for actionable disease surveillance and outbreak management: A systematic literature review. *PLoS ONE* 10(10):e0139701.

Chinnadurai, G., S. Chinnadurai, and J. Brusca. 1979. Physical mapping of a large-plaque mutation of adenovirus type 2. *Journal of Virology* 32(2):623–628.

Chiolero, A., V. Santschi, and F. Paccaud. 2013. Public health surveillance with electronic medical records: At risk of surveillance bias and overdiagnosis. *European Journal of Public Health* 23(3):350–351.

CIDAR Lab. 2016. Eugene. Boston University. Available at: http://cidarlab.org/eugene. Accessed November 8, 2017.

Cision PR Newswire. 2018. International Gene Synthesis Consortium updates screening protocols for synthetic DNA products and services. News: January 3. Available at: https://www.prnewswire.com/news-releases/international-gene-synthesis-consortium-updates-screening-protocols-for-synthetic-dna-products-and-services-300576867.html. Accessed January 31, 2018.

Citorik, R.J., M. Mimee, and T.K. Lu. 2014. Sequence-specific antimicrobials using efficiently delivered RNA-guided nucleases. *Nature Biotechnology* 32(11):1141–1145.

Clapper, J.E. 2016. Worldwide Threat Assessment of the U.S. Intelligence Community. Statement for the Record by James R. Clapper, Director of National Intelligence. Senate Armed Service Committee, February 16, 2016. Available at: https://www.dni.gov/files/documents/SASC_Unclassified_2016_ATA_SFR_FINAL.pdf. Accessed May 19, 2017.

Clemen, R. 1991. *Making Hard Decisions: An Introduction to Decision Analysis*. Boston: PWS-Kent.

Cobb, R.E., N. Sun, and H. Zhao. 2013. Directed evolution as a powerful synthetic biology tool. *Methods* 60(1):81–90.

Cockrell, A.S., B.L Yount, T. Scobey, K. Jensen, M. Douglas, A. Beall, X.C. Tang, W.A. Marasco, M.T. Heise, and R.S. Baric. 2016. A mouse model for MERS coronavirus induced acute respiratory distress syndrome. *Nature Microbiology* 2:16226.

Coen, D.M., and D.D. Richman. 2013. Antiviral agents. Pp. 338–373 in *Field's Virology,* 6th Ed., D.M. Knipe and P.M. Howley, eds. Philadelphia, PA: Lippincott Williams and Wilkins.

Coleman, J.R., D. Papamichail, S. Skiena, B. Futcher, E. Wimmer, and S. Mueller. 2008. Virus attenuation by genome-scale changes in codon pair bias. *Science* 320(5884):1784–1787.

Cong, L., F.A. Ran, D. Cox, S. Lin, R. Barretto, N. Habib, P.D. Hsu, X. Wu, W. Jiang, L.A. Marraffini, and F. Zhang. 2013. Multiplex genome engineering using CRISPR/Cas systems. *Science* 339(6121):819–823.

Cox, I.J., M.L. Miller, J.A. Bloom, J. Fridrich, and T. Kalker 2008. *Digital Watermarking and Steganography,* 2nd Ed. Burlington, MA: Morgan Kaufmann.

Cox, M.S., and A.F. Woolf. 2002. Preventing Proliferation of Biological Weapons: U.S. Assistance to the Former Soviet States. CRC Report for Congress RL31368. Available at: https://fas.org/sgp/crs/nuke/RL31368.pdf. Accessed November 14, 2017.

Cress, B.F., J.A. Englaender, W. He, D. Kasper, R.J. Linhardt, and M.A. Koffas. 2014. Masquerading microbial pathogens: Capsular polysaccharides mimic host-tissue molecules. *FEMS Microbiology Reviews* 38(4):660–697.

Cui, J., Y. Yin, Q. Ma, G. Wang, C. Olman, Y. Zhang, W. Chou, C. S. Hong, C. Zhang, S. Cao, X. Mao, Y. Li, S. Qin, S. Zhao, J. Jiang, P. Hastings, F. Li, and Y. Xu. 2015. Comprehensive characterization of the genomic alterations in human gastric cancer. *International Journal of Cancer* 137(1):86–95.

Cullen, B.R. 2013. MicroRNAs as mediators of viral immune evasion. *Nature Immunology* 14(3):205–210.

Cummings, C.L., and J. Kuzma. 2017. Societal Risk Evaluation Scheme (SRES): Scenario-based multi-criteria evaluation of synthetic biology applications. *PLoS ONE* 12(1):e0168564.

Daigle, K.A., C.M. Logan, and R.S. Kotwal. 2015. Comprehensive performance nutrition for Special Operations Forces. *Journal of Special Operations Medicine* 15(4):40–53.

DARPA (Defense Advanced Research Projects Agency). 2017. Safe Genes. Available at: https://www.darpa.mil/program/safe-genes. Accessed November 14, 2017.

Datlinger, P., A.F. Rendeiro, C. Schmidl, T. Krausgruber, P. Traxler, J. Klughammer, L. C. Schuster, A. Kuchler, D. Alpar, and C. Bock. 2017. Pooled CRISPR screening with single-cell transcriptome readout. *Nature Methods* 14:297–301.

Davey, R.T., L. Dodd, M.A. Proschan, J. Neaton, J.N. Nordwall, J.S. Koopmeiners, J. Beigel, J. Tierney, H.C. Lane, A.S. Fauci, M.B.F. Massaquoi, F. Sahr, and D. Malvy. 2016. A randomized, controlled trial of ZMapp for Ebola virus infection. *New England Journal of Medicine* 375(15):1448–1456.

de Fabritus, L., A. Nougairède, F. Aubry, E.A. Gould, and X. de Lamballerie. 2015. Attenuation of tick-borne encephalitis virus using large-scale random codon re-encoding. *PLoS Pathogens* 11(3):e1004738.

de Paz, H.D., P. Brotons, and C. Muñoz-Almagro. 2014. Molecular isothermal techniques for combating infectious diseases: Towards low-cost point-of-care diagnostics. *Expert Review of Molecular Diagnostics* 14(7):827–843.

de Vries, R.P., W. Peng, O.C. Grant, A.J. Thompson, X. Zhu, K.M. Bouwman, A.T.T. de la Pena, M.J. van Breemen, I.N.A. Wickramasinghe, C.A.M. de Haan, W. Yu, R. McBride, R.W. Sanders, R.J. Woods, M.H. Verheije, I.A. Wilson, and J.C. Paulson. 2017. Three mutations switch H7N9 influenza to human-type receptor specificity. *PLoS Pathogens* 13(6):e1006390.

Dhar, P.K., and R. Weiss. 2007. Enabling the new biology of the 21st century. *Systems and Synthetic Biology* 1(1):1–2.

DiEuliis, D., and J. Giordano. 2017. Why gene editors like CRISPR/Cas may be a game-changer for neuroweapons. *Health Security* 15(3):296–302.

DiEuliis, D., K. Berger, and G. Gronvall. 2017a. Biosecurity implications for the synthesis of horsepox, an orthopoxvirus. *Health Security* 15(6):629–637.

DiEuliis, D., S.R. Carter, and G.K. Gronvall. 2017b. Options for synthetic DNA order screening, revisited. *mSphere* 2(4):e00319–17.

Dittrich, P.S., and A. Manz. 2006. Lab-on-a-chip: Microfluidics in drug discovery. *Nature Reviews Drug Discovery* 5(3):210–218.

DOJ (U.S. Department of Justice). 2010. Amerithrax Investigative Summary, February 19. Available at: https://www.justice.gov/archive/amerithrax/docs/amx-investigative-summary.pdf. Accessed November 6, 2017.

Donia, M.S., and M.A. Fischbach. 2015. Small molecules from the human microbiota. *Science* 349(6246):1254766.

Dormitzer, P.R., P. Suphaphiphat, D.G. Gibson, D.E. Wentworth, T.B. Stockwell, M.A. Algire, N. Alperovich, M. Barro, D.M. Brown, S. Craig, B.M. Dattilo, E.A. Denisova, I. De Souza, M. Eickmann, V.G. Dugan, A. Ferrari, R.C. Gomilla, L. Han, C. Judge, S. Mane, M. Matrosovich, C. Merryman, G. Palladino, G.A. Palmer, T. Spencer, T. Strecker, H. Trusheim, J. Uhlendorff, Y. Wen, A.C. Yee, J. Zaveri, B. Zhou, S. Becker, A. Donabedian, P.W. Mason, J.I. Glass, R. Rappouli, and J.C. Venter. 2013. Synthetic generation of influenza vaccine viruses for rapid response to pandemics. *Science and Translational Medicine* 5(185):185ra68.

Doudna, J.A., and E. Charpentier. 2014. The new frontier of genome engineering with CRISPR-Cas9. *Science* 346(6213):1258096.

Duffy, J. 1951. Smallpox and the Indians in the American colonies. *Bulletin of the History of Medicine* 25(4):324–341.

Edelstein, M.L., M.R. Abedi, and J. Wixon. 2007. Gene therapy clinical trials worldwide to 2007—an update. *Journal of Gene Medicine* 9(10):833–842.

Elowitz, M.B., and S. Leibler. 2000. A synthetic oscillatory network of transcriptional regulators. *Nature* 403(6767):335–338.

Endy, D. 2005. Foundations for engineering biology. *Nature* 438(7067):449–453.

Endy, D., S. Galanie, and C.D. Smolke. 2015. Complete absence of thebaine biosynthesis under home-brew fermentation conditions. bioRχiv, doi:10.1101/024299.

Eppinger, M., M.K. Mammel, J.E. Leclerc, J. Ravel, and T.A. Cebula. 2011. Genomic anatomy of *Escherichia coli* O157:H7 outbreaks. *Proceedings of the National Academy of Sciences of the United States of America* 108(50):20142–20147.

Esvelt, K.M., A.L. Smidler, F. Catteruccia, and G.M. Church. 2014. Concerning RNA-guided gene drives for the alteration of wild populations. *eLife* 3:e03401.

Fan, R.L., S.A. Valkenburg, C.K. Wong, O.T. Li, J.M. Nicholls, R. Rabadan, J.S. Peiris, and L.L. Poon. 2015. Generation of live attenuated influenza virus by using codon usage bias. *Journal of Virology* 89(21):10762–10773.

FDA (U.S. Food and Drug Administration). 2017a. FDA approval brings first gene therapy to the United States. FDA News: August 30. Available at: https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm574058.htm. Accessed January 30, 2017.

FDA. 2017b. FDA approves CAR-T cell therapy to treat adults with certain types of large B-cell lymphoma. FDA News: October 18. Available at: https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm581216.htm. Accessed January 31, 2017.

FDA. 2017c. What Are Medical Countermeasures? Available at: https://www.fda.gov/EmergencyPreparedness/Counterterrorism/MedicalCountermeasures/AboutMCMi/ucm431268.htm. Accessed November 14, 2017.

FERN (Food Emergency Response Network). 2017. Uniting Federal State and Local Laboratories for Food Emergency Response. Available at: https://www.fernlab.org. Accessed November 14, 2017.

Filkins, B. 2014. Health Care Cyberthreat Report: Widespread Compromises Detected, Compliance Nightmare on Horizon. SANS Institute, February. Available at: https://www.sans.org/reading-room/whitepapers/analyst/health-care-cyberthreat-report-widespread-compromises-detected-compliance-nightmare-horizon-34735. Accessed November 2, 2017.

Finlay, B.B., and G. McFadden. 2006. Anti-immunology: Evasion of the host immune system by bacterial and viral pathogens. *Cell* 124(4):767–782.

Firoved, A. 2016. The Federal Perspective on the State of Our Nation's Biodefense. Testimony of Aaron Firoved, Acting Director, OHA National Bio-surveillance Integration Center, Before the Senate Committee on Homeland Security and Governmental Affairs, April 14. Available at: https://www.dhs.gov/news/2016/04/14/written-testimony-oha-senate-committee-homeland-security-and-governmental-affairs. Accessed November 14, 2017.

Fischer, N.O., A. Rasley, M. Corzett, M.H. Hwang, P.D. Hoeprich, and C.D. Blanchette. 2013. Colocalized delivery of adjuvant and antigen using nanolipoprotein particles enhances the immune response to recombinant antigens. *Journal of the American Chemical Society* 135(6):2044–2047.

Fischer, N.O., D.R. Weilhammer, A. Dunkle, C. Thomas, M. Hwang, M. Corzett, C. Lychak, W. Mayer, S. Urbin, N. Collette, J.C. Chang, G.G. Loots, A. Rasley, and C.D. Blanchette. 2014. Evaluation of nanolipoprotein particles (NLPs) as an in vivo delivery platform. *PLoS ONE* 9(3):e93342.

Fouchier, R.A. 2015. Studies on influenza virus transmission between ferrets: The public health risks revisited. *mBio* 6(1): e02560-14.

Frerichs, R.L., R.M. Salerno, K.M. Vogel, N.B. Barnett, J. Gaudioso, L.T. Hickok, D. Estes, and D.F. Jung. 2004. *Historical Precedence and Technical Requirements of Biological Weapons Use: A Threat Assessment*. SAND2004-1854. Albuquerque, NM: Sandia National Laboratories. Available at: http://prod.sandia.gov/techlib/access-control.cgi/2004/041854.pdf. Accessed January 31, 2018.

Friedman, D.J., R.G. Parrish, and D.A. Ross. 2013. Electronic health records and U.S. public health: Current realities and future promise. *American Journal of Public Health* 103(9):1560–1567.

Friedmann, E.I. 1994. Viable microorganisms in permafrost. Pp. 21–26 in *Permafrost as Microbial Habitat,* D.A. Gilichinsky, ed. Pushchino: Russian Academy of Sciences.

Fung, I.C.H., Z.C.H. Tse, and K.W. Fu. 2015. The use of social media in public health surveillance. *Western Pacific Surveillance and Response Journal* 6(2):3–6.

Fung, I.C.H., C.H. Duke, K.C. Finch, K.R. Snook, P.L. Tseng, A.C. Hernandez, M. Gambhir, K.W. Fu, and Z.T.H. Tse. 2016. Ebola virus disease and social media: A systematic review. *American Journal of Infection Control* 44(12):1660–1671.

Galanie, S., K. Thodey, I.J. Trenchard, M.F. Interrante, and C.D. Smolke. 2015. Complete biosynthesis of opioids in yeast. *Science* 349(6252):1095–1100.

Gao, L., and Y.A. Kwaik. 2000. The modulation of host cell apoptosis by intracellular bacterial pathogens. *Trends in Microbiology* 8(7):306–313.

Gardner, T.S., C.R. Cantor, and J.J. Collins. 2000. Construction of a genetic toggle switch in *Escherichia coli*. *Nature* 403(6767):339–342.

Garfinkel, M.S., D. Endy, G.L. Epstein, and R.M. Friedman. 2007. Synthetic genomics: Options for governance. *Biosecurity and Bioterrorism: Biodefense Strategy, Practice, and Science* 5(4):359–362.

Ghaisas, S., J. Maher, and A. Kanthasamy. 2016. Gut microbiome in health and disease: Linking the microbiome-gut-brain axis and environmental factors in the pathogenesis of systemic and neurodegenerative diseases. *Pharmacology and Therapeutics* 158:52–62.

GHR (Genome Home Reference). 2018. What are single nucleotide polymorphisms (SNPs)? Available at: https://ghr.nlm.nih.gov/primer/genomicresearch/snp. Accessed January 29, 2018.

Gibson, D.G., L. Young, R. Chuang, J.C. Venter, C.A. Hutchison III, and H.O. Smith. 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nature Methods* 6(5):343–345.

Gibson, D.G., J.I. Glass, C. Lartigue, V.N. Noskov, R.Y. Chuang, M.A. Algire, G.A. Benders, M.G. Montague, L. Ma, M.M. Moodie, C. Merryman, S. Vashee, R. Krishnakumar, N. Assad-Garcia, C. Andrews-Pfannkoch, E.A. Denisova, L. Young, Z.Q. Qi, T.H. Segall-Shapiro, C.H. Calvey, P.P. Parmar, C.A. Hutchison III, H.O. Smith, and J.C. Venter. 2010. Creation of a bacterial cell controlled by a chemically synthesized genome. *Science* 329(5987):52–56.

Gronvall, G.K. 2017. Prevention of the development or use of biological weapons. *Health Security* 15(1):36–37.

Guenther, C.M., B.E. Kuypers, M.T. Lam, T.M. Robinson, J. Zhao, and J. Suh. 2014. Synthetic virology: Engineering viruses for gene delivery. *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology* 6(6):548–558.

Guichard, A., V. Nizet, and E. Bier. 2012. New insights into the biological effects of anthrax toxins: Linking cellular to organismal responses. *Microbes and Infection* 14(2):97–118.

Guillemin, J. 2006. *Biological Weapons: From the Invention of State-Sponsored Programs to Contemporary Bioterrorism*. New York: Columbia University Press.

Hacein-Bey-Abina, S., F. Le Deist, F. Carlier, C. Bouneaud, C. Hue, J.P. De Villartay, A.J. Thrasher, N. Wulffraat, R. Sorensen, S. Dupuis-Girod, A. Fischer, E.G. Davies, W. Kuis, L. Leiva, and M. Cavazzana-Calvo. 2002. Sustained correction of X-linked severe combined immunodeficiency by ex vivo gene therapy. *New England Journal of Medicine* 346(16):1185–1193.

Hadadi, N., J. Hafner, A. Shajkofci, A. Zisaki, and V. Hatzimanikatis. 2016. ATLAS of biochemistry: A repository of all possible biochemical reactions for synthetic biology and metabolic engineering studies. *ACS Synthetic Biology* 5(10):1155–1166.

Haddad, D. 2017. Genetically engineered vaccinia viruses as agents for cancer treatment, imaging, and transgene delivery. *Frontiers in Oncology* 7:96.

Hall, B. 2011. *Evolution: Principles and Processes*. Sudbury, MA: Jones and Bartlett.

Hall, R.A., and J. Macdonald. 2016. Synthetic biology provides a toehold in the fight against Zika. *Cell Host and Microbe* 19(6):752–754.

Hammond, A., R. Galizi, K. Kyrou, A. Simoni, C. Siniscalchi, D. Katsanos, M. Gribble, D. Baker, E. Marois, S. Russell, A. Burt, N. Windbichler, A. Crisanti, and T. Nolan. 2016. A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector *Anopheles gambiae*. *Nature Biotechnology* 34(1):78–83.

Harris, A., A.R. McKerney, D. Nimmo, Z. Curtis, I. Black, S.A. Morgan, M.N. Oviedo, R. Lacroix, N. Naish, N.I. Morrison, A. Collado, J. Stevenson, S. Scaife, T. Dafa'alla, G. Fu, C. Phillips, A. Miles, N. Raduan, N. Kelly, C. Beech, C.A. Donnelly, W.D. Petrie, and L. Alphey. 2012. Successful suppression of a field mosquito population by sustained release of engineered male mosquitoes. *Nature Biotechnology* 30(9):828–830.

He, X.J., T. Chen, and J.K. Zhu. 2011. Regulation and function of DNA methylation in plants and animals. *Cell Research* 21(3):442–465.

Heider, D., and A. Barnekow. 2008. DNA watermarks: A Proof of concept. *BMC Molecular Biology* 9:40.

Heise, M.T., and H.W. Virgin. 2013. Pathogenesis of viral infection. Pp. 254–285 in *Field's Virology,* 6th Ed., D.M. Knipe and P.M. Howley, eds. Philadelphia, PA: Lippincott Williams and Wilkins.

Hekele, S., S. Bertholet, J. Archer, D.G. Gibson, G. Palladino, L.A. Brito, G.R. Otten, M. Brazzoli, S. Buccato, A. Bonci, D. Casini, D. Maione, Z.Q. Qi, J.E. Gill, N.C. Caiazza, J. Urano, B. Hubby, G.F. Gao, Y. Shu, E. de Gregorio, C.W. Mandl, P.W. Mason, E.C. Settembre, J.B. Ulmer, J.C. Venter, P.R. Dormitzer, R. Rappuoli, and A.J. Geall. 2013. Rapidly produced SAM® vaccine against H7N9 influenza is immunogenic in mice. *Emerging Microbes and Infections* 2(8):e52.

Herfst, S., E.J. Schrauwen, M. Linster, S. Chatinimitkul, E. de Wit, V.J. Munster, E.M. Sorrell, T.M. Bestebroer, D.F. Burke, D.J. Smith, G.F. Rimmelzwaan, A.D. Osterhaus, and R.A. Fouchier. 2012. Airborne transmission of influenza A/H5N1 virus between ferrets. *Science* 336(6088):1534–1541.

Herfst, S., M. Böhringer, B. Karo, P. Lawrence, N.S. Lewis, M.J. Mina, C.J. Russell, J. Steel, R.L. de Swart, and C. Menge. 2017. Drivers of airborne human-to-human pathogen transmission. *Current Opinion in Virology* 22:22–29.

HHS (U.S. Department of Health and Human Services). 2010. Screening Framework Guidance for Providers of Synthetic Double-Stranded DNA. Available at: https://www.phe.gov/Preparedness/legal/guidance/syndna/Documents/syndna-guidance.pdf. Accessed on November 14, 2017.

HHS. 2013. Framework for Guiding Funding Decisions about Research Proposals with the Potential for Generating Highly Pathogenic Avian Influenza H5N1 Viruses That Are Transmissible Among Mammals by Respiratory Droplets. February. Available at: https://www.phe.gov/s3/dualuse/Documents/funding-hpai-h5n1.pdf. Accessed May 11, 2017.

HHS. 2017a. Framework for Guiding Funding Decisions About Proposed Research Involving Enhanced Potential Pandemic Pathogens. Available at: https://www.phe.gov/s3/dualuse/Documents/p3co.pdf. Accessed January 25, 2018.

HHS. 2017b. PHEMCE Strategy and Implementation Plan: 2017–2018. Available at: https://www.phe.gov/Preparedness/mcm/phemce/Pages/strategy.aspx. Accessed January 31, 2018.

Holloway, D.T. 2013. Regulating amateurs. *The Scientist*, March. Available at: http://www.the-scientist.com/?articles.view/articleNo/34444/title/Regulating-Amateurs. Accessed January 25, 2018.

Holmes, E.C. 2003. Error thresholds and the constraints to RNA virus evolution. *Trends in Microbiology* 11(12):543–546.

Huang, P., S.E. Boyken, and D. Baker. 2016. The coming of age of *de novo* protein design. *Nature* 537:320–327.

Huang, Q., K. Gumireddy, M. Schrier, C. le Sage, R. Nagel, S. Nair, D.A. Egan, A. Li, G. Huang, A.J. Klein-Szanto, P.A. Gimotty, D. Katsaros, G. Coukos, L. Zhang, E. Puré, and R. Agami. 2008. The microRNAs miR-373 and miR-520c promote tumour invasion and metastasis. *Nature Cell Biology* 10(2):202–210.

Hughes, J., B. Shelton, and T. Hughes. 2010. Suspected dietary supplement injuries in Special Operations soldiers. *Journal of Special Operations Medicine* 10(3):14–24.

IAP (InterAcademy Partnership). 2015. The Biological and Toxin Weapons Convention: Implications of Advances in Science and Technology. Available at: https://royalsociety.org/~/media/policy/projects/biological-toxin-weapons-convention/biological-weapons-technical-document.pdf. Accessed January 31, 2018.

IARPA (Intelligence Advanced Research Projects Agency). 2017a. Finding Engineering-Linked Indicators (FELIX). Available at: https://www.iarpa.gov/index.php/research-programs/felix. Accessed November 14, 2017.

IARPA. 2017b. Functional Genomic and Computational Assessment of Threats (FunGCAT). Available at: https://www.iarpa.gov/index.php/research-programs/fun-gcat. Accessed November 14, 2017.

iGEM (International Genetically Engineered Machine). 2017a. Registry of Standard Biological Parts. Available at: http://parts.igem.org/Main_Page. Accessed on May 19, 2017.

iGEM. 2017b. Synthetic Biology Based on Standard Parts. Available at: http://igem.org/Main_Page. Accessed on November 14, 2017.

IGSC (International Gene Synthesis Consortium). 2017. Harmonized Screening Protocol© v2.0 Gene Sequence & Customer Screening to Promote Biosecurity, November 19. Available at: https://genesynthesisconsortium.org/wp-content/uploads/IGSCHarmonizedProtocol11-21-17.pdf. Accessed January 25, 2018.

Imai, M., T. Watanabe, M. Hatta, S.C. Das, M. Ozawa, K. Shinya, G. Zhong, A. Hanson, H. Katsura, S. Watanabe, C. Li, E. Kawakami, S. Yamada, M. Kiso, Y. Suzuki, E.A. Maher, G. Neumann, and Y. Kawaoka. 2012. Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets. *Nature* 486(7403):420–428.

IOM/NRC (Institute of Medicine and National Research Council). 2006. *Globalization, Biosecurity, and the Future of the Life Sciences*. Washington, DC: The National Academies Press.

IPCS (International Programme on Chemical Safety). 1996. Principles and Methods for Assessing Direct Immunotoxicity Associated with Exposure to Chemicals. Environmental Health Criteria 180. Geneva: World Health Organization. Available at: http://www.inchem.org/documents/ehc/ehc/ehc180.htm. Accessed November 3, 2017.

Iwasaki, A., and R. Medzhitov. 2013. Innate responses to viral infection. Pp. 189–213 in *Field's Virology,* 6th Ed., D.M. Knipe and P.M. Howley, eds. Philadelphia, PA: Lippincott Williams and Wilkins.

Jackson, R.J., A.J. Ramsay, C.D. Christensen, S. Beaton, D.F. Hall, and I.A. Ramshaw. 2001. Expression of mouse interleukin-4 by a recombinant ectromelia virus suppresses cytotoxic lymphocyte responses and overcomes genetic resistance to mousepox. *Journal of Virology* 75(3):1205–1210.

Jaing, C., S. Gardner, K. McLoughlin, J.B. Thissen, and T. Slezak. 2011. Detection of adventitious viruses from biologicals using a broad-spectrum microbial detection array. *PDA Journal of Pharmaceutical Science and Technology* 65(6):668–674.

Jalani, G., R. Naccache, D.H. Rosenzweig, L. Haglund, F. Vetrone, and M. Cerruti. 2016. Photocleavable hydrogel-coated upconverting nanoparticles: A multifunctional theranostic platform for NIR imaging and on-demand macromolecular delivery. *Journal of the American Chemical Society* 138(3):1078–1083.

JCVI (J. Craig Venter Institute). 2010. First self-replicating synthetic bacterial cell. J. Craig Venter Institute News: May 20. Available at: https://www.jcvi.org/first-self-replicating-synthetic-bacterial-cell-constructed-j%C2%A0craig-venter-institute-researchers. Accessed January 18, 2018.

Jefferson, C., F. Lentzos, and C. Marris. 2014. Synthetic biology and biosecurity: Challenging the "myths." *Frontiers in Public Health* 2:115.

Jinek, M., K. Chylinski, I. Fonfara, M. Hauer, J.A. Doudna, and E. Charpentier. 2012. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337(6096):816–821.

Jinek, M., A. East, A. Cheng, S. Lin, E. Ma, and J. Doudna. 2013. RNA-programmed genome editing in human cells. *eLife* 2:e00471.

Jochem, G. 2017. Pet store puppies linked to *Campylobacter* outbreak in people. NPR News: September 12. Available at: http://www.npr.org/sections/health-shots/2017/09/12/550423656/pet-store-puppies-linked-to-campylobacter-outbreak-in-people. Accessed November 3, 2017.

Jones, P.A., and S.B. Baylin. 2007. The epigenomics of cancer. *Cell* 128(4):683–692.

June, C.H., J.T. Warshauer, and J.A. Bluestone. 2017. Is autoimmunity the Achilles' heel of cancer immunotherapy? *Nature Medicine* 23:540–547.

Kadlec, R.P., and A.P. Zelicoff. 2000. Implications of the biotechnology revolution for weapons development and arms control. Pp. 11–26 in *Biological Warfare: Modern Offense and Defense,* R. Zilinskas, ed. Boulder, CO: Lynne Rienner.

Kampranis, S.C., and A.M. Makris. 2012. Developing a yeast cell factory for the production of terpenoids. *Computational and Structural Biotechnology Journal* 3:e201210006.

Kan, S.B., X. Huang, Y. Gumulya, K. Chen, and F.H. Arnold. 2017. Genetically programmed chiral organoborane synthesis. *Nature* 552(7683):132–136.

Kaper, G.B., J.P. Nataro, and H.L. Mobley. 2004. Pathogenic *Escherichia coli*. *Nature Reviews Microbiology* 2(2):123–140.

Kaplan, G., J. Lubinski, A. Dasgupta, and V.R. Racaniello. 1985. In vitro synthesis of infectious poliovirus RNA. *Proceedings of the National Academy of Sciences of the United States of America* 82(24):8424–8428.

Kau, A.L., P.P. Ahern, N.W. Griffin, A.L. Goodman, and J.I. Gordon. 2011. Human nutrition, the gut microbiome and the immune system. *Nature* 474(7351):327–336.

Kay, M.A., J.C. Glorioso, and L. Naldini. 2001. Viral vectors for gene therapy: The art of turning infectious agents into vehicles of therapeutics. *Nature Medicine* 7(1):33–40.

Keeney, R. 1992. *Value-Focused Thinking: A Path to Creative Decisionmaking*. Cambridge, MA: Harvard University Press.

Keeney, R., and H. Raiffa. 1993. *Decisions with Multiple Objectives: Preferences and Tradeoffs*. Cambridge, UK: Cambridge University Press.

Kellogg, S. 2012. The rise of DIY scientists: Is it time for regulation? *Washington Lawyer*, May. Available at: https://www.dcbar.org/bar-resources/publications/washington-lawyer/articles/may-2012-diy-scientist.cfm. Accessed January 25, 2018.

Khalil, R.K., C. Skinner, S. Patfield, and X. He. 2016. Phage-mediated Shiga toxin (*Stx*) horizontal gene transfer and expression in non-Shiga toxigenic *Enterobacter* and *Escherichia coli* strains. *Pathogens and Disease* 74(5):ftw037.

Kim, J.Y., J.Y. Shin, M.R. Kim, S.K. Hann, and S.H. Oh. 2012. siRNA-mediated knock-down of COX-2 in melanocytes suppresses melanogenesis. *Experimental Dermatology* 21(6):420–425.

Kim, R.S., N. Goossens, and Y. Hoshida. 2016. Use of big data in drug development for precision medicine. *Expert Review of Precision Medicine and Drug Development* 1(3):245–253.

Klompas, M., J. McVetta, R. Lazarus, E. Eggleston, G. Haney, B.A. Kruskal, W.K. Yih, P. Daly, P. Oppedisano, B. Beagan, M. Lee, C. Kirby, D. Heisey-Grove, A. DeMaria, Jr., and R. Platt. 2012. Integrating clinical practice and public health surveillance using electronic medical record systems. *American Journal of Public Health* 102(Suppl. 3):S325–S332.

Knight, T. 2003. Idempotent Vector Design for Standard Assembly of Biobricks. MIT Synthetic Biology Working Group Technical Reports. Available at: https://dspace.mit.edu/bitstream/handle/1721.1/21168/biobricks.pdf. Accessed July 14, 2017.

Koblentz, G.D. 2017. The *de novo* synthesis of horsepox virus: Implications for biosecurity and recommendations for preventing the reemergence of smallpox. *Health Security* 15(6):620–628.

Kolodziejczyk, B. 2017. Do-it-yourself biology shows safety risks of an open innovation movement. Brookings TechTank, October 9. Available at: https://www.brookings.edu/blog/techtank/2017/10/09/do-it-yourself-biology-shows-safety-risks-of-an-open-innovation-movement. Accessed November 14, 2017.

Koroleva, M.Y., T.Y. Nagovitsina, D.A. Bidanov, O.S. Gorbachevski, and E.V. Yurtov. 2016. Nano- and microcapsules as drug-delivery systems. *Resource-Efficient Technologies* 2(4):233–239.

Kosal, M.E. 2009. *Nanotechnology for Chemical and Biological Defense*. New York: Springer.

Kosal, M.E. 2014. A new role for public health in bioterrorism deterrence. *Frontiers in Public Health* 2:278.

Kotula, J.W., S.J. Kerns, L.A. Shaket, L. Siraj, J.J. Collins, J.C. Way, and P.A. Silver. 2014. Programmable bacteria detect and record an environmental signal in the mammalian gut. *Proceedings of the National Academy of Sciences of the United States of America* 111(13):4838–4843.

Kouranova, E., K. Forbes, G. Zhao, J. Warren, A. Bartels, Y. Wu, and X. Cui. 2016. CRISPRs for optimal targeting: Delivery of CRISPR components as DNA, RNA, and protein into cultured cells and single-cell embryos. *Human Gene Therapy* 27(6):464–475.

Kranz, L.M., M. Diken, H. Haas, S. Kreiter, C. Loquai, K.C. Reuter, M. Meng, D. Fritz, F. Vascotto, H. Hefesha, C. Grunwitz, M. Vormehr, Y. Husemann, A. Selmi, A.N. Kuhn, J. Buck, E. Derhovanessian, R. Rae, S. Attig, J. Diekmann, R.A. Jabulowsky, S. Heesch, J. Hassell, P. Langguth, S. Grabbe, C. Huber, O. Tureci, and U. Sahin. 2016. Systemic RNA delivery to dendritic cells exploits antiviral defence for cancer immunotherapy. *Nature* 534(7607):396–401.

Krebs on Security. 2013. Wash. Hospital Hit by $1.03 Million Cyberheist. April. Available at: https://krebsonsecurity.com/2013/04/wash-hospital-hit-by-1-03-million-cyberheist. Accessed November 2, 2017.

Kreuzer-Martin, H.W., and K.H. Jarman. 2007. Stable isotope ratios and forensic analysis of microorganisms. *Applied and Environmental Microbiology* 73(12):3896–3908.

Krishnamurthy, M., R.T. Moore, S. Rajamani, and R.G. Panchal. 2016. Bacterial genome engineering and synthetic biology: Combating pathogens. *BMC Microbiology* 16:258.

Kupferschmidt, K. 2017. How Canadian researchers reconstituted an extinct poxvirus for $100,000 using mail-order DNA. *Science*, News: July 6. Available at: http://www.sciencemag.org/news/2017/07/how-canadian-researchers-reconstituted-extinct-poxvirus-100000-using-mail-order-dna. Accessed November 6, 2017.

Kuss, S.K., G.T. Best, C.A. Etheredge, A.J. Pruijssers, J.M. Frierson, L.V. Hooper, T.S. Dermody, and J.K. Pfeiffer. 2011. Intestinal microbiota promote enteric virus replication and systemic pathogenesis. *Science* 334(6053):249–252.

Lauring, A.S., A. Acevado, S.B. Cooper, and R. Andino. 2012. Codon usage determines the mutational robustness, evolutionary capacity and virulence of an RNA virus. *Cell Host and Microbe* 12(5):623–632.

Lehner, B.A.E., D.T. Schmieden, and A.S. Meyer. 2017. A straightforward approach for 3D bacterial printing. *ACS Synthetic Biology* 6(7):1124–1130.

Lempinen, E.W. 2011. FBI, AAAS collaborate on ambitious outreach to biotech researchers and DIY biologists. American Association for the Advancement of Science News: April 1. Available at: https://www.aaas.org/news/fbi-aaas-collaborate-ambitious-outreach-biotech-researchers-and-diy-biologists. Accessed November 14, 2017.

Li, W., Z. Shi, M. Yu, W. Ren, C. Smith, J.H. Epstein, H. Wang, C. Crameri, Z. Hu, H. Zhang, J. Zhang, J. McEachern, H. Field, P. Daszak, B.T. Eaton, S. Zhang, and L.F. Wang. 2005. Bats are natural reservoirs of SARS-like coronaviruses. *Science* 310(5748):676–679.

Lim, W.A., and C.H. June. 2017. The principles of engineering immune cells to treat cancer. *Cell* 168:724–740.

Lindler, L.E., E. Choffnes, and G.W. Korch. 2005. Definition and overview of emerging threats. Pp. 351–359 in *Biological Weapons Defense: Infectious Disease and Counterbioterrorism, Part III. Emerging Threats and Future Preparation,* L.E. Lindler, F.J. Lebeda, and G. Korch, eds. Totowa, NJ: Humana Press.

Linster, M., S. van Boheemen, M. de Graaf, E.J.A. Schrauwen, P. Lexmond, B. Mänz, T.M. Bestebroer, J. Baumann, D. van Riel, G.F. Rimmelzwaan, A.D.M.E. Osterhaus, M. Matrosovich, R.A.M. Fouchier, and S. Herfst. 2014. Identification, characterization, and natural selection of mutations driving airborne transmission of A/H5N1 virus. *Cell* 157(2):329–339.

Lipsitch, M. 2014. Can limited scientific value of potential pandemic pathogen experiments justify the risks? *mBio* 5(5):e02008-14.

Liu, J., T. Gaj, Y. Yang, N. Wang, S. Shui, S. Kim, C. N. Kanchiswamy, J. Kim, and C.F. Barbas III. 2015. Efficient delivery of nuclease proteins for genome editing in human stem cells and primary cells. *Nature Protocols* 10:1842–1859.

Lotz, M.T., and R.S. Peebles, Jr. 2012. Mechanisms of respiratory syncytial virus modulation of airway immune responses. *Current Allergy and Asthma Reports* 12(5):380–387.

Low, N., A. Bavdekar, L. Jeyaseelan, S. Hirve, K. Ramanathan, N.J. Andrews, N. Shaikh, R.S. Jadi, A. Rajagopal, K.E. Brown, D. Brown, J.B. Fink, O. John, P. Scott, X. Riveros-Balta, M. Greco, R. Dhere, P.S. Kulkarni, and A.M.H. Restrepo. 2015. A randomized, controlled trial of an aerosolized vaccine against measles. *New England Journal of Medicine* 372(16):1519–1529.

Lu, T.K., A.S. Khalil, and J.J. Collins. 2009. Next-generation synthetic gene networks. *Nature Biotechnology* 27(12):1139–1150.

Lu, T.K., J. Bowers, and M.S. Koeris. 2013. Advancing bacteriophage-based microbial diagnostics with synthetic biology. *Trends in Biotechnology* 31(6):325–327.

Luo, M.L., A.S. Mullis, R.T. Leenay, and C.L. Beisel. 2015. Repurposing endogenous type I CRISPR-Cas systems for programmable gene repression. *Nucleic Acids Research* 43(1):674–681.

Macho, A.P., and C. Zipfel. 2015. Targeting of plant pattern recognition receptor-triggered immunity by bacteria type-III secretion system effectors. *Current Opinions in Microbiology* 23:14–22.

Madoff, L.C. 2004. ProMED-mail: An early warning system for emerging diseases. *Clinical Infectious Diseases* 39(2):227–232.

Mali, P., L.H. Yang, K.M. Esvelt, J. Aach, M. Guell, J.E. DiCarlo, J.E. Norville, and G.M. Church. 2013. RNA-guided human genome engineering via Cas9. *Science* 339(6121):823–826.

Martinez, M.A., A. Jordan-Paiz, S. Franco, and M. Nevot. 2016. Synonymous virus genome recoding as a tool to impact viral fitness. *Trends in Microbiology* 24(2):134–147.

Matheson, R. 2016. Reprogramming gut bacteria as "living therapeutics." MIT News: April 6. Available at: http://news.mit.edu/2016/startup-synlogic-reprogramming-gut-bacteria-living-therapeutics-0405. Accessed November 9, 2017.

Mendoza, B.J., and C.T. Trinh. 2018. Enhanced guide–RNA design and targeting analysis for precise CRISPR genome editing of single and consortia of industrially relevant and non-model organisms. *Bioinformatics* 34(1):16–23.

Meng, J., S. Lee, A.L. Hotard, and M.L. Moore. 2014. Refining the balance of attenuation and immunogenicity of respiratory syncytial virus by targeted codon deoptimization of virulence genes. *mBio* 5(5):e01704–14.

Mercy, G., J. Mozziconacci, V.F. Scolari, K. Yang, G. Zhao, A. Thierry, Y. Luo, L.A. Mitchell, M. Shen, Y. Shen, R. Walker, W. Zhang, Y. Wu, Z.X. Xie, Z. Luo, Y. Cai, J. Dai, H. Yang, Y.J. Yuan, J.D. Boeke, J.S. Bader, H. Muller, and R. Koszul. 2017. 3D organization of synthetic and scrambled chromosomes. *Science* 355(6329):eaaf4597.

Miele, E., G.P. Spinelli, E. Miele, E. Di Fabrizio, E. Ferretti, S. Tomao, and A. Gulino. 2012. Nanoparticle-based delivery of small interfering RNA: Challenges for cancer therapy. *International Journal of Nanomedicine* 7:3637–3657.

Milinovich, G.J., G.M. Williams, A.C. Clements, and W. Hu. 2014. Internet-based surveillance systems for monitoring emerging infectious diseases. *Lancet Infectious Diseases* 14(2):160–168.

Miller, S.D., W.J. Karpus, and T.S. Davidson. 2007. Experimental autoimmune encephalomyelitis in the mouse. *Current Protocols in Immunology* 15–1.

Mimee, M., A.C. Tucker, C.A. Voigt, and T.K. Lu. 2015. Programming a human commensal bacterium, *Bacteroides thetaiotaomicron*, to sense and respond to stimuli in the murine gut microbiota. *Cell Systems* 1(1):62–71.

Mingozzi, F., and K.A. High. 2013. Immune responses to AAV vectors: Overcoming barriers to successful gene therapy. *Blood* 122(1):23–36.

MIT Media Lab. 2017. Global Summit on Community Biotechnology, September 22–24. Community Biotechnology Initiative, Massachusetts Institute of Technology. Available at: https://www.biosummit.org. Accessed November 14, 2017.

Mitchell, L.A., A. Wang, G. Stracquadanio, Z. Kuang, X. Wang, K. Yang, S. Richardson, J.A. Martin, Y. Zhao, R. Walker, Y. Luo, H. Dai, K. Dong, Z. Tang, Y. Yang, Y. Cai, A. Heguy, B. Ueberheide, D. Fenyö, J. Dai, J.S. Bader, and J.D. Boeke. 2017. Synthesis, debugging, and effects of synthetic chromosome consolidation: synVI and beyond. *Science* 355(6329):eaaf4831.

Moon, T.S., C. Lou, A. Tamsir, B.C. Stanton, and C.A. Voigt. 2012. Genetic programs constructed from layered logic gates in single cells. *Nature* 491(7423):249–253.

Morgan, K.M., M.L. DeKay, P.S. Fischbeck, M.G. Morgan, B. Fischhoff, and H.K. Florig. 2001. A deliberative method for ranking risks (II): Evaluation of validity and agreement among risk managers. *Risk Analysis* 21(5):923–937.

Morgan, M.G., and M. Henrion. 1990. *Uncertainty: A Guide to Dealing with Uncertainty in Quantitative Risk and Policy Analysis*. Cambridge, UK: Cambridge University Press.

Moss, J.A. 2014. Gene therapy review. *Radiologic Technology* 86(2):155–180.

Mou, H., Z. Kennedy, D.G. Anderson, H. Yin, and W. Xue. 2015. Precision cancer mouse models through genome editing with CRISPR-Cas9. *Genome Medicine* 7:53.

Müllbacher, A., and M. Lobigs. 2001. Creation of killer poxvirus could have been predicted. *Journal of Virology* 75(18):8353–8355.

Muehlbauer, S.M., T.H. Evering, G. Bonuccelli, R.C. Squires, A.W. Ashton, S.A. Porcelli, M.P. Lisanti, and J. Brojatsch. 2007. Anthrax lethal toxin kills macrophages in a strain-specific manner by apoptosis or caspase-1-mediated necrosis. *Cell Cycle* 6:758–766.

Mueller, S., J.R. Coleman, D. Papamichail, C.B. Ward, A. Nimnual, B. Futcher, S. Skiena, and E. Wimmer. 2010. Live attenuated influenza virus vaccines by computer-aided rational design. *Nature Biotechnology* 28(7):723–726.

Nafissi, N., and R. Slavcev. 2012. Construction and characterization of an *in-vivo* linear covalently closed DNA vector production system. *Microbial Cell Factories* 11:154.

Naldini, L., U. Blömer, P. Gallay, D. Ory, R. Mulligan, F.H. Gage, I.M. Verma, and D. Trono. 1996. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* 272(5259):263–267.

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

National Academies of Sciences, Engineering, and Medicine. 2017a. *A Proposed Framework for Identifying Potential Biodefense Vulnerabilities Posed by Synthetic Biology: Interim Report*. Washington, DC: The National Academies Press.

National Academies of Sciences, Engineering, and Medicine. 2017b. *Dual Use Research of Concern in the Life Sciences: Current Issues and Controversies*. Washington, DC: The National Academies Press.

National Academies of Sciences, Engineering, and Medicine. 2017c. *Fostering Integrity in Research*. Washington, DC: The National Academies Press.

National Academies of Sciences, Engineering, and Medicine. 2017d. *Microbiomes of the Built Environment: From Research to Application*. Washington, DC: The National Academies Press.

Nature Biotechnology. 2009. What's in a name? 27(12):1071–1073.

NCBI (National Center for Biotechnology Information). 2017. GenBank. Available at: https://www.ncbi.nlm.nih.gov/genbank. Accessed November 25, 2017.

Neumann, G., T. Watanabe, H. Ito, S. Watanabe, H. Goto, P. Gao, M. Hughes, D.R. Perez, R. Donis, E. Hoffmann, G. Hobom, and Y. Kawaoka. 1999. Generation of influenza A viruses entirely from cloned cDNAs. *Proceedings of the National Academy of Sciences of the United States of America* 96(16):9345–9350.

Nicklin, S.A., and A.H. Baker. 2002. Tropism-modified adenoviral and adeno-associated viral vectors for gene therapy. *Current Gene Therapy* 2(3):273–293.

Nielsen, A.A., B.S. Der, J. Shin, P. Vaidyanathan, V. Paralanov, E.A. Strychalski, D. Ross, D. Densmore, and C.A. Voigt. 2016. Genetic circuit design automation. *Science* 352(6281):aac7341.

Niu, Y.Y., B. Shen, Y.Q. Cui, Y.C. Chen, J.Y. Wang, L. Wang, Y. Kang, X.Y. Zhao, W. Si, W. Li, A.P. Xiang, J.K. Zhou, X.J. Guo, Y. Bi, C.Y. Si, B. Hu, G.Y. Dong, H. Wang, Z.M. Zhou, T.Q. Li, T. Tan, X.Q. Pu, F. Wang, S.H. Ji, Q. Zhou, X.X. Huang, W.Z. Ji, and J.H. Sha. 2014. Generation of gene-modified cynomolgus monkey via Cas9/RNA-mediated gene targeting in one-cell embryos. *Cell* 156(4):836–843.

Nougairede, A., L. De Fabritus, F. Aubry, E.A. Gould, E.C. Holmes, and X. de Lamballerie. 2013. Random codon re-encoding induces stable reduction of replicative fitness of Chikungunya virus in primate and mosquito cells. *PLoS Pathogens* 9(2):e1003172.

Noyce, R.S., S. Lederman, and D.H. Evans. 2018. Construction of an infectious horsepox virus vaccine from chemically synthesized DNA fragments. *PLoS ONE* 13(1):e0188453.

NRC (National Research Council). 1992. The capacity of toxic agents to compromise the immune system (biologic markers of immunosuppression). Pp. 63–82 in *Biological Markers in Immunotoxicology*. Washington, DC: National Academy Press.

NRC. 2004. *Biotechnology Research in an Age of Terrorism*. Washington, DC: The National Academies Press.

NRC. 2014. *Convergence: Facilitating Transdisciplinary Integration of Life Sciences, Physical Sciences, Engineering, and Beyond*. Washington, DC: The National Academies Press.

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

O'Bryan, S., S. Dong, J.M. Mathis, and S.K. Alahari. 2017. The roles of oncogenic miRNAs and their therapeutic importance in breast cancer. *European Journal of Cancer* 72:1–11.

Ostrov, N., M. Landon, M. Guell, G. Kuznetsov, J. Teramoto, N. Cervantes, M. Zhou, K. Singh, M.G. Napolitano, M. Moosburner, E. Shrock, B.W. Pruitt, N. Conway, D.B. Goodman, C.L. Gardner, G. Tyree, A. Gonzales, B.L. Wanner, J.L. Norville, M.J. Lajoie, and G.M. Church. 2016. Design, synthesis, and testing toward a 57-codon genome. *Science* 353(6301):819–822.

Palmer, M.J., F. Fukuyama, and D.A. Relman. 2015. A more systematic approach to biological risk. *Science* 350(6267):1471–1473.

Palsson Lab. 2017. COBRA Tools. University of California. Available at: http://systemsbiology.ucsd.edu/COBRATools. Accessed November 8, 2017.

Pan, X., Y. Yang, and J. Zhang. 2014. Molecular basis of host specificity in human pathogenic bacteria. *Emerging Microbes & Infections* 3(3):e23.

Pardee, K., A.A. Green, T. Ferrante, D.E. Cameron, A.D. Keyser, P. Yin, and J.J. Collins. 2014. Paper-based synthetic gene networks. *Cell* 159(4):940–954.

Pardi, N., M.J. Hogan, R.S. Pelc, H. Muramatsu, H. Andersen, C.R. DeMaso, K.A. Dowd, L.L. Sutherland, R.M Scearce, R. Parks, W. Wagner, A. Granados, J. Greenhouse, M. Walker, E. Willis, J. Yu, C.E. McGee, G.D. Sempowski, B.L. Mui, Y.K. Tam, Y. Huang, D. Vanlandingham, V.M. Holmes, H. Balachandran, S. Sahu, M. Lifton, S. Higgs, S.E. Hensley, T.D. Madden, M.J. Hope, K. Kariko, S. Santra, B.S. Graham, M.G. Lewis, T.C. Pierson, B.F. Haynes, and D. Weissman. 2017. Zika virus protection by a single low-dose nucleoside-modified mRNA vaccination. *Nature* 543(7644):248–251.

PCAST (President's Council of Advisors on Science and Technology). 2016. Action Needed to Protect Against Biological Attack. November 2016. Available at: https://obamawhitehouse.archives.gov/sites/default/files/microsites/ostp/PCAST/pcast_biodefense_letter_report_final.pdf. Accessed May 11, 2017.

Peng, Y., S. Allen, R.J. Millwood, and C.N. Stewart, Jr. 2014. "Fukusensor": A genetically engineered plant for reporting DNA damage in response to gamma radiation. *Plant Biotechnology Journal* 12(9):1329–1332.

Perakslis, E.D. 2014. Cybersecurity in health care. *New England Journal of Medicine* 371(5):395–397.

Petsch, B., M. Schnee, A.B. Vogel, E. Lange, B. Hoffman, D. Voss, T. Schlake, A. Thess, K.J. Kallen, L. Stitz, and T. Kramps. 2012. Protective efficacy of *in vitro* synthesized, specific mRNA vaccines against influenza A virus infection. *Nature Biotechnology* 30(12):1210–1218.

Pettitt, J., L. Zeitlin, D.H. Kim, C. Working, J.C. Johnson, O. Bohorov, B. Bratcher, E. Hiatt, S.D. Hume, A.K. Johnson, J. Morton, M.H. Pauly, K.J. Whaley, M.F. Ingram, A. Zovanyi, M. Heinrich, A. Piper, J. Zelko, and G.G. Olinger. 2013. Therapeutic intervention of Ebola virus infection in rhesus macaques with the MB-003 monoclonal antibody cocktail. *Science Translational Medicine* 5(199):199ra113.

Petzold, C.J., L.J.G. Chan, M. Nhan, and P.D. Adams. 2015. Analytics for metabolic engineering. *Frontiers in Bioengineering and Biotechnology* 3:135.

Plesa, C., A.M. Sidore, N.B. Lubock, D. Zhang, and S. Kosuri. 2018. Multiplexed gene synthesis in emulsions for exploring protein functional landscapes. *Science* 359(6373):343–347.

Plowright, R.K., C.R. Parish, H. McCallum, P.J. Hudson, A.I. Ko, A.L. Graham, and J.O. Lloyd-Smith. 2017. Pathways to zoonotic spillover. *Nature Reviews Microbiology* 15(8):502–510.

Polozov, I.V., L. Bezrukov, K. Gawrisch, and J. Zimmerberg. 2008. Progressive ordering with decreasing temperature of the phospholipids of influenza virus. *Nature Chemistry and Biology* 4(4):248–255.

Ponemon Institute. 2013. Cost of Data Breach Study: Global Analysis. May. Available at: https://www.ponemon.org/blog/2013-cost-of-data-breach-global-analysis. Accessed November 2, 2017.

Poptsova, M.S., and J.P. Gogarten. 2010. Using comparative genome analysis to identify problems in annotated microbial genomes. *Microbiology* 156(Pt. 7):1909–1917.

Ptashne, M. 1986. *A Genetic Switch: Gene Control and Phage Lambda*. Oxford: Cell Press & Blackwell Scientific.

Qin, Z., B.G. Compton, J.A. Lewis, and M.J. Buehler. 2015. Structural optimization of 3D-printed synthetic spider webs for high strength. *Nature Communications* 6:Art.7038.

Quenee, L.E., T.M. Hermanas, N. Ciletti, H. Louvel, N.C. Miller, D. Elli, B. Blaylock, A. Mitchell, J. Schroeder, T. Krausz, J. Kanabrocki, and O. Schneewind. 2012. Hereditary hemochromatosis restores the virulence of plague vaccine strains. *Journal of Infectious* Diseases 206(7):1050–1058.

Quick, J., N.J. Loman, S. Duraffour, J.T. Simpson, E. Severi, L. Cowley, J.A. Bore, R. Koundouno, G. Dudas, A. Mikhail, N. Ouédraogo, B. Afrough, A. Bah, J.H. Baum, B. Becker-Ziaja, J.P. Boettcher, M. Cabeza-Cabrerizo, A. Camino-Sánchez, L.L. Carter, J. Doerrbecker, T. Enkirch, I. Carcia-Dorival, N. Hetzelt, J. Hinzmann, T. Holm, L.E. Kafetzopoulou, M. Koropogui, A. Kosgey, E. Kuisma, C.H. Logue, A. Mazzarelli, S. Meisel, M. Mertens, J. Michel, D. Ngabo, K. Nitzsche, E. Pallash, L.V. Patrono, J. Portmann, J.G. Repits, N.Y. Rickett, A. Sachse, K. Singethan, I. Vitoriano, R.L. Yemanaberhan, E.G. Zekeng, R. Trina, A. Bello, A.A. Sall, O. Faye, N. Magassouba, C.V. Williams, V. Amburgey, L. Winona, E. Davis, J. Gerlach, F. Washington, V. Monteil, M. Jourdain, M. Bererd, A. Camara, H. Somlare, M. Gerard, G. Bado, B. Baillet, D. Delaune, K.Y. Nebie, A. Diarra, Y. Savane, R.B. Pallawo, G.J. Gutierrez, N. Milhano, I. Roger, C.J. Williams, F. Yattara, K. Lewandowski, J. Taylor, P. Rachwal, D. Turner, G. Pollakis, J.A. Hiscox, D.A. Matthews, M.K. O'Shea, A.M. Johnson, D. Wilson, E. Hutley, E. Smit, A. Di Caro, R. Woelfel, K. Stoecker, E. Fleischmann, M. Gabriel, S.A. Weller, L. Koivogui, B. Diallo, S. Keita, A. Rambaut, P. Formenty, S. Gunther, and M.W. Carroll. 2016. Real-time portable genome sequencing for Ebola surveillance. *Nature* 530(7589):228–232.

Racaniello, V.R. 2012. Science should be in the public domain. *mBio* 3(1):e0004-12.

Ran, F.A., L. Cong, W.X. Yan, D.A. Scott, J.S. Gootenberg, A.J. Kriz, B. Zetsche, O. Shalem, X. Wu, K.S. Makarova, E. Koonin, P.A. Sharp, and F. Zhang. 2015. *In vivo* genome editing using *Staphylococcus aureus* Cas9. *Nature* 520(7546):186–191.

Repin, V.E., V.G. Pugachev, O.S. Taranov, and E.V. Brenner. 2007. Potential hazard of microorganisms which came from the past [in Russian]. Pp. 183–190 in *Yukagir Mammoth,* G.G. Boeskorov, A.N. Tichonov, and N. Suzuki, eds. Moscow: Saint-Petersburg State University.

Revich, B.A., and M.A. Podolnaya. 2011. Thawing of permafrost may disturb historic cattle burial grounds in East Siberia. *Global Health Action* 4(1):8482.

Revill, J., and C. Jefferson. 2014. Tacit knowledge and the biological weapons regime. *Science and Public Policy* 41(5):597–610.

Ribet, D., and P. Cossart. 2015. How bacterial pathogens colonize their hosts and invade deeper tissues. *Microbes and Infection* 15(3):173–183.

Richards, D.J., Y. Tan, J. Jia, H. Yao, and Y. Mei. 2013. 3D printing for tissue engineering. *Israel Journal of Chemistry* 53(9-10):805–814.

Richardson, S.M., L.A. Mitchell, G. Stracquadanio, K. Yang, J.S. Dymond, J.E. DiCarlo, D. Lee, C.L. Huang, S. Chandrasegaran, Y. Cai, J.D. Boeke, and J.S. Bader. 2017. Design of a synthetic yeast genome. *Science* 355(6329):1040–1044.

Richner, J.M., S. Himansu, K.A. Dowd, S.L. Butler, V. Salazar, J.M. Fox, J.G. Julander, W.W. Tang, S. Shresta, T.C. Pierson, G. Ciaramella, and M.S. Diamond. 2017. Modified mRNA vaccines protect against Zika infection. *Cell* 168(6):1114–1125.

Robbins, J.B., V. Gorgen, P. Min, B.R. Shepherd, and S.C. Presnell. 2013. A novel in vitro three-dimensional bioprinted liver tissue system for drug development. Abstract 7979. Poster presented at Experimental Biology, April 20–24, 2013, Boston, MA. Available at: http://organovo.com/wp-content/uploads/2015/07/04-18-13_EB_Poster_v2.pdf. Accessed November 2, 2017.

Roberts, D.M., A. Nanda, M.J. Havenga, P. Abbink, D.M. Lynch, B.A. Ewald, J. Liu, A.R. Thorner, P.E. Swanson, D.A. Gorgone, M.A. Lifton, A.A. Lemckert, L. Holterman, B. Chen, A. Dilraj, A. Carville, K.G. Mansfield, J. Goudsmit, and D.H. Barouch. 2006. Hexon-chimaeric adenovirus serotype 5 vectors circumvent pre-existing anti-vector immunity. *Nature* 441(7090):239–243.

Robinson, K.M., and J.C. Dunning Hotopp. 2014. Mobile elements and viral integrations prompt considerations for bacteria DNA integration as a novel carcinogen. *Cancer Letters* 352(2):137–144.

Roco, M.C. 2008. Possibilities for global governance of converging technologies. *Journal of Nanoparticle Research* 10(1):11–29.

Rodriguez, P.L., T. Harada, D.A. Christian, D.A. Pantano, R.K. Tsai, and D.E. Discher. 2013. Minimal "self" peptides that inhibit phagocytic clearance and enhance delivery of nanoparticles. *Science* 339(6122):971–975.

Rosen, A., and L. Casciola-Rosen. 2016. Autoantigens as partners in initiation and propagation of autoimmune rheumatic diseases. *Annual Review of Immunology* 34:395–420.

Royal Society. 2015. The Chemical Weapons Convention and Convergent Trends in Science and Technology. Available at: https://royalsociety.org/~/media/policy/projects/brain-waves/2013-08-04-chemical-weapons-convention-and-convergent-trends.pdf. Accessed May 11, 2017.

Runguphan, W., X. Qu, and S.E. O'Connor. 2010. Integrating carbon-halogen bond formation into medicinal plant metabolism. *Nature* 468(7322):461–464.

Sabin, A.B. 1985. Oral poliovirus vaccine: History of its development and use and current challenge to eliminate poliomyelitis from the world. *Journal of Infectious Diseases* 151(3):420–436.

Salis Lab. 2017. RBS Calculator. Penn State University. Available at: https://salislab.net/software. Accessed November 8, 2017.

Sampathkumar, M. 2017. North Korea says it "will take revenge" for US saying it is developing biological weapons. *Independent* News: December 20. Available at: http://www.independent.co.uk/news/world/americas/us-politics/north-korea-biological-weapons-us-revenge-trump-kim-jong-un-pyongyang-a8120376.html. Accessed January 31, 2018.

Saraiva, C., C. Praca, R. Ferreira, T. Santos, L. Ferreira, and L. Bernardino. 2016. Nanoparticle-mediated brain drug delivery: Overcoming blood-brain barrier to treat neurodegenerative diseases. *Journal of Controlled Release* 235:34–47.

Sastalla, I., D.M. Monack, and K.F. Kubatzky. 2016. Bacterial exotoxins: How bacteria fight the immune system [editorial]. *Frontiers in Immunology* 7:Art.300.

Savile, C., J.M. Janey, E.C. Mundorff, J.C. Moore, S. Tam, W.R. Jarvis, J.C. Colbeck, A. Krebber, F.J. Fleitz, J. Brands, P.N. Devine, G.W. Huisman, and G.J. Hughes. 2010. Biocatalytic asymmetric synthesis of chiral amines from ketones applied to sitagliptin manufacture. *Science* 329(5989):305–309.

Schmidt, F., and R.J. Platt. 2017. Applications of CRISPR-Cas for synthetic biology and genetic recording. *Current Opinion in Systems Biology* 5:9–15.

Schwarz, M.C., M. Sourisseau, M.M. Espino, E.S. Gray, M.T. Chambers, D. Tortorella, and M.J. Evans. 2016. Rescue of the 1947 Zika virus prototype strain with a cytomegalovirus promoter-driven cDNA clone. *mSphere* 1(5):e00246-16.

Selle, K., and R. Barrangou. 2015. Harnessing CRISPR-Cas systems for bacterial genome editing. *Trends in Microbiology* 23(4):225–232.

Shen, S.H., C.B. Stauft, O. Gorbatsevych, Y. Song, C.B. Ward, A. Yurovsky, S. Mueller, B. Futcher, and E. Wimmer. 2015. Large scale re-coding of an arbovirus genome to rebalance its insect versus mammalian preference. *Proceedings of the National Academy of Sciences of the United States of America* 112(15):4749–4754.

Shen, Y., Y. Wang, T. Chen, F. Gao, J. Gong, D. Abramczyk, R. Walker, H. Zhao, S. Chen, W. Liu, Y. Luo, C.A. Müller, A. Paul-Dubois-Taine, B. Alver, G. Stracquadanio, L.A. Mitchell, Z. Luo, Y. Fan, B. Zhou, B. Wen, F. Tan, Y. Wang, J. Zi, Z. Xie, B. Li, K. Yang, S.M. Richardson, H. Jiang, C.E. French, C.A. Nieduszynski, R. Koszul, A.L. Marston, Y. Yuan, J. Wang, J.S. Bader, J. Dai, J.D. Boeke, X. Xu, Y. Cai, and H. Yang. 2017. Deep functional analysis of synII, a 770-kilobase synthetic yeast chromosome. *Science* 355(6329):eaaf4791.

Shirakawa, K., L. Chavez, S. Hakre, V. Calvanese, and E. Verdin. 2013. Reactivation of latent HIV by histone deacetylase inhibitors. *Trends in Microbiology* 21(6):277–285.

Sieber, K.B., P. Gajer, and J.C. Dunning Hotopp. 2016. Modeling the integration of bacterial rRNA fragments into the human cancer genome. *BMC Bioinformatics* 17:134.

Siegel, J.B. A. Zanghellini, H.M. Lovick, G. Kiss, A.R. Lambert, J.L. St Clair, J.L. Gallaher, D. Hilvert, M.H. Gelb, B.L. Stoddard, K.N. Houk, F.E. Michael, and D. Baker. 2010. Computational design of an enzyme catalyst for a stereoselective bimolecular Diels-Alder reaction. *Science* 329(5989):309–313.

Sleator, R.D. 2010. The story of *Mycoplasma mycoides* JCVI-syn1.0. *Bioengineered Bugs* 1(4):229–230.

Slomovic, S., K. Pardee, and J.J. Collins. 2015. Synthetic biology devices for in vitro and in vivo diagnostics. *Proceedings of the National Academy of Sciences of the United States of America* 112(47):14429–14435.

Smanski, M.J., S. Bhatia, D. Zhao, Y.J. Park, L.B.A. Woodruff, G. Giannoukos, D. Ciulla, M. Busby, J. Calderon, R. Nicol, D.B. Gordon, D. Densmore, and C.A. Voigt. 2014. Functional optimization of gene clusters by combinatorial design and assembly. *Nature Biotechnology* 32:1241–1249.

Smanski, M.J., H. Zhou, J. Claesen, B. Shen, M.A. Fischbach, and C.A. Voigt. 2016. Synthetic biology to access and expand nature's chemical diversity. *Nature Reviews Microbiology* 14(3):135–149.

Spark Therapeutics. 2017. FDA approves Spark Therapeutics' LUXTURNA™ (voretigene neparvovec-rzyl), a one-time gene therapy for patients with confirmed biallelic RPE65 mutation-associated retinal dystrophy. Press Release: December 19. Available at: http://ir.sparktx.com/news-releases/news-release-details/fda-approves-spark-therapeutics-luxturnatm-voretigene-neparvovec. Accessed January 31, 2017.

Steinle, H., A. Behring, C. Schlensak, H.P. Wendel, and M. Avci-Adali. 2017. Concise review: Application of *in vitro* transcribed messenger RNA for cellular engineering and reprogramming: Progress and challenges. *Stem Cells* 35(1):68–79.

Steinmetz, M., and R. Richter. 1994. Plasmids designed to alter the antibiotic resistance expressed by insertion mutations in *Bacilus subtilis,* through in vivo recombination. *Gene* 142:79–83.

Strauch, E., J.A. Hammer, A. Konietzny, S. Schneiker-Bekel, W. Arnold, A. Goesmann, A. Pühler, and L. Beutin. 2008. Bacteriophage 2851 is a prototype phage for dissemination of the Shiga toxin variant gene 2c in *Escherichia coli* O157:H7. *Infection and Immunity* 76(12):5466–5477.

Suntharalingam, G., M.R. Perry, S. Ward, S.J. Brett, A. Castello-Cortes, M.D. Brunner, and N. Panoskaltsis. 2006. Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412. *New England Journal of Medicine* 355(10):1018–1028.

Swenson, K. 2015. Drugs, viruses, and robots: Weapons on the rise for a better fight against cancer. Autodesk News: March 24. Available at: https://www.autodesk.com/redshift/drugs-viruses-and-robots-weapons-on-the-rise-for-a-better-fight-against-cancer. Accessed January 31, 2018.

Synlogic. 2017. IND-Enabling Studies. Available at: https://www.synlogictx.com/pipeline/pipeline/. Accessed November 9, 2017.

Szybalski, W. 1974. *In vivo* and *in vitro* initiation of transcription. Pp. 23–24 in *Control of Gene Expression,* A. Kohn and A. Shatkay, eds. New York: Plenum Press.

Tanaka, A., P.S. Leung, and M.E. Gershwin. 2018. Environmental basis of primary biliary cholangitis. *Experimental Biology and Medicine* 243(2):184–189.

Tanaka, T., W. Zhang, Y. Sun, Z. Shuai, A.S. Chida, T.P. Kenny, G.X. Yang, I. Sanz, A. Ansari, C.L. Bowlus, G.C. Ippolito, R.L. Coppel, K. Okazaki, X.S. He, P.S.C. Leung, and M.E. Gershwin. 2017. Autoreactive monoclonal antibodies from patients with primary biliary cholangitis recognize environmental xenobiotics. *Hepatology* 66(3):885–895.

Taneja, V., and C.S. David. 2001. Lessons from animal models for human autoimmune diseases. *Nature Immunology* 2(9): 781–784.

Tate, M.D., E.R. Job, Y. Deng, V. Gunalan, S. Maurer-Stroh, and P.C. Reading. 2014. Playing hide and seek: How glycosylation of the influenza virus hemagglutinin can modulate the immune response to infection. *Viruses* 6(3):1294–1316.

The Australia Group. 2007. Fighting the Spread of Chemical and Biological Weapons: Strengthening Global Security. Available at: http://www.australiagroup.net/en/agb_july2007.pdf. Accessed November 29, 2017.

Thissen, J.B., K. McLoughlin, S. Gardner, S. Gu, S. Mabery, T. Slezak, and C. Jaing. 2014. Analysis of sensitivity and rapid hybridization of a multiplexed Microbial Detection Array. *Journal of Virological Methods* 201:73–78.

Tizei, P.A.G., E. Csibra, L. Torres, V.B. Pinheiro. 2016. Selection platforms for directed evolution in synthetic biology. *Biochemical Society Transactions* 44(4):1165–1175.

Toman, Z., C. Dambly-Chaudière, L. Tenenbaum, and M. Radman. 1985. A system for detection of genetic and epigenetic alterations in *Escherichia coli* induced by DNA-damaging agents. *Journal of Molecular Biology* 186(1):97–105.

Tucker, J.B. 2012. *Innovation, Dual Use, and Security: Managing the Risks of Emerging Biological and Chemical Technologies*. Cambridge: MIT Press.

Tulman, E.R., G. Delhon, C.L. Alfonso, Z. Lu, L. Zsak, N.T. Sandybaev, U.Z. Kerembekova, V.L. Zaitsev, G.F. Kutish, and D.L. Rock. 2006. Genome of horsepox virus. *Journal of Virology* 80(18):9244–9258.

UNOG (United Nations Office at Geneva). 2017. The Biological Weapons Convention. . Available at: https://www.unog.ch/80256EE600585943/(httpPages)/04FBBDD6315AC720C1257180004B1B2F?OpenDocument. Accessed November 13, 2017.

UN Security Council. 2004. Resolution 1540 (2004). S/RES/1540 (2004). Available at: http://www.un.org/en/ga/search/view_doc.asp?symbol=S/RES/1540%20(2004). Accessed November 15, 2017.

U.S. Congress. 2000. Senator Kennedy of Massachusetts, speaking on prohibiting genetic discrimination, on June 29, Amendment No. 3681. 106th Cong., 2nd sess. *Congressional Record* 146(85):S6049

U.S. Congress. 2006. Engineering Bio-Terror Agents: Lessons from the Offensive U.S. and Russian Biological Weapons Programs. Hearing Before the Subcommittee on Prevention of Nuclear and Biological Attack of the Committee on Homeland Security, House of Representatives One Hundred Ninth Congress First Session, July 13, 2005. Washington, DC: U.S. Government Printing Office [includes testimony from Roger Brent and Mike Callahan].

U.S. Government. 2012. United States Government Policy for Oversight of Life Sciences Dual Use Research of Concern. March 29. Available at: https://www.phe.gov/s3/dualuse/Documents/us-policy-durc-032812.pdf. Accessed on May 11, 2017.

U.S. Government. 2014. United States Government Policy for Institutional Oversight of Life Sciences Dual-Use Research of Concern. September 24. Available at: https://www.phe.gov/s3/dualuse/Documents/durc-policy.pdf. Accessed May 11, 2017.

Vabret, N., M. Bailly-Bechet, A. Lepelly, V. Najburg, O. Schwartz, B. Verrier, and F. Tangy. 2014. Large-scale nucleotide optimization of simian immunodeficiency virus reduces its capacity to stimulate type I interferon in vitro. *Journal of Virology* 88(8):4161–4172.

van Courtland Moon, J.E. 2006. The US biological weapons program. Pp. 9–46 in *Deadly Cultures: Biological Weapons since 1945,* M.L. Wheelis, L. Rózsa, and M.R. Dando, eds. Cambridge, MA: Harvard University Press.

van den Broek, M., M.F. Bachmann, G. Köhler, M. Barner, R. Escher, R. Zinkernagel, and M. Kopf. 2000. IL-4 and IL-10 antagonize IL-12-mediated protection against acute vaccina virus infection with a limited role of IFN-γ and nitric oxide synthetase 2. *Journal of Immunology* 164(1):371–378.

Velasco, E., T. Agheneza, K. Denecke, G. Kirchner, and T. Eckmanns. 2014. Social media and Internet-based data in global systems for public health surveillance: A systematic review. *Milbank Quarterly* 92(1):7–33.

Verma, I.M., and N. Somia. 1997. Gene therapy—promises, problems, and prospects. *Nature* 389:239–242.
Vidic, J., M. Manzano, C.M. Chang, and N. Jaffrezic-Renault. 2017. Advanced biosensors for detection of pathogens related to livestock and poultry. *Veterinary Research* 48(1):11.
Vogel, K.M. 2012. *Phantom Menace or Looming Danger? A New Framework for Assessing Bioweapons Threats*. Baltimore, MD: Johns Hopkins University Press.
Vogel, K.M. 2013. The need for greater multidisciplinary, sociotechnical analysis: The bioweapons case. *Studies in Intelligence* 57(3):1–10.
Voigt, C.A. 2012. Synthetic biology [editorial]. *ACS Synthetic Biology* 1:1–2.
Wain-Hobson, S. 2014. An avian H7N1 gain-of-function experiment of great concern. *mBio* 5(5):e01882-14.
Wang, B., C. Yang, G. Tekes, S. Mueller, A. Paul, S.P. Whelan, and E. Wimmer. 2015. Recoding of the vesicular stomatitis virus L gene by computer-aided design provides a live attenuated vaccine candidate. *mBio* 6(2):e00237-15.
Wang, H.H., F.J. Isaacs, P.A. Carr. Z.Z. Sun, G. Xu, C.R. Forest, and G.M. Church. 2009. Programming cells by multiplex genome engineering and accelerated evolution. *Nature* 460(7257):894–898.
Wang, J., Z. Lu, M.G. Wientjes, and J.L.S. Au. 2010. Delivery of siRNA therapeutics: Barriers and carriers. *AAPS Journal* 12(4):492–503.
Wang, Y., Z. Zhang, S. Seo, P. Lynn, T. Lu, Y. Jin, and H. P. Blaschek. 2016. Bacterial genome editing with CRISPR-Cas9: Deletion, integration, single nucleotide modification, and desirable "clean" mutant selection in *Clostridium beijerinckii* as an example. *American Chemical Society Synthetic Biology* 5(7):721–732.
Weber, W., and M. Fussenegger. 2012. Engineering biomedical applications of synthetic biology. *Nature Reviews* 13:21–35.
Weiss, R., S. Basu, S. Hooshangi, A. Kalmbach, D. Karig, R. Mehreja, and I. Netravali. 2003. Genetic circuit building blocks for cellular computation, communication, and signal processing. *Natural Computing* 2:47–84.
Westfall, P.J., D.J. Pitera, J.R. Lenihan, D. Eng, F.X. Woolard, R. Regentin, T. Horning, H. Tsuruta, D.J. Melis, A. Owens, S. Fickes, D. Diola, K.R. Benjamin, J.D. Keasling, M.D. Leavell, D.J. McPhee, N.S. Renninger, J.D. Newman, and C.J. Paddon. 2012. Production of amorphadiene in yeast, and its conversion to dihydroartemisinic acid, precursor to the antimalarial agent artemisinin. *Proceedings of the National Academy of Sciences of the United States of America* 109(3):E111–E118.
Wheelis, M. 2002. Biological warfare at the 1346 siege of Caffa. *Emerging Infectious Diseases* 8(9):971–975.
White House. 2012. National Bioeconomy Blueprint. Available at: https://obamawhitehouse.archives.gov/sites/default/files/microsites/ostp/national_bioeconomy_blueprint_april_2012.pdf. Accessed June 22, 2017.
Willis, H.H., M.L. DeKay, M.G. Morgan, H.K. Florig, and P.S. Fischbeck. 2004. Ecological risk ranking: Development and evaluation of a method for improving public participation in environmental decision making. *Risk Analysis* 24(2):363–378.
Willis, H.H., J.M. Gibson, R.A. Shih, S. Geschwind, S. Olmstead, J. Hu, A.E. Curtright, G. Cecchine, and M. Moore. 2010. Prioritizing environmental health risks in the UAE. *Risk Analysis* 30(12):1842–1856.
Wimmer, E. 2006. The test-tube synthesis of a chemical called poliovirus: The simple synthesis of a virus has far-reaching societal implications. *EMBO Reports* 7(Spec.):S3–S9.
Wimmer, E., and A.V. Paul. 2011. Synthetic poliovirus and other designer viruses: What have we learned from them? *Annual Review of Microbiology* 65:583–609.
Wimmer, E., S. Mueller, T.M. Tumpy, and J.K. Taubenberger. 2009. Synthetic viruses: A new opportunity to understand and prevent viral disease. *Nature Biotechnology* 27(12):1163–1172.
Wolinsky, H. 2016. The FBI and biohackers: An unusual relationship. *EMBO Reports* 17(6):793–796.
WRAIR (Walter Reed Army Institute of Research). 2017. Multidrug-Resistant Organism Repository and Surveillance Network (MRSN). Available at: http://www.wrair.army.mil/OtherServices_MRSN.aspx. Accessed November 14, 2017.
Wu, Y., B.Z. Li, M. Zhao, L.A. Mitchell, Z.X. Xie, Q.H. Lin, X. Wang, W.H. Xiao, Y. Wang, X. Zhou, H. Liu, X. Li, M.Z. Ding, D. Liu, L. Zhang, B.L. Liu, X.L. Wu, F.F. Li, X.T. Dong, B. Jia, W.Z. Zhang, G.Z. Jiang, Y. Liu, X. Bai, T.Q. Song, Y. Chen, S.J. Zhou, R.Y. Zhu, F. Gao, Z. Kuang, X. Wang, M. Shen, K. Yang, G. Stracquadanio, S.M. Richardson, Y. Lin, L. Wang, R. Walker, Y. Luo, P.S. Ma, H. Yang, Y. Cai, J. Dai, J.S. Bader, J.D. Boeke, and Y.J. Yuan. 2017. Bug mapping and fitness testing of chemically synthesized chromosome X. *Science* 355(6329):eaaf4706.
Xie, Z.X., B.Z. Li, L.A. Mitchell, Y. Wu, X. Qi, Z. Jin, B. Jia, X. Wang, B.X. Zeng, H.M. Liu, X.L. Wu, Q. Feng, W.Z. Zhang, W. Liu, M.Z. Ding, X. Li, G.R. Zhao, J.J. Qiao, J.S. Cheng, M. Zhao, Z. Kuang, X. Wang, J.A. Martin, G. Stracquadanio, K. Yang, X. Bai, J. Zhao, M.L. Hu, Q.H. Lin, W.Q. Zhang, M.H. Shen, S. Chen, W. Su, E.X. Wang, R. Guo, F. Zhai, X.J. Guo, H.X. Du, J.Q. Zhu, T.Q. Song, J.J. Dai, F.F. Li, G.Z. Jiang, S.L. Han, S.Y. Liu, Z.C. Yu, X.N. Yang, K. Chen, C. Hu, D.S. Li, N. Jia, Y. Liu, L.T. Wang, S. Wang, X.T. Wei, M.Q. Fu, L.M. Qu, S.Y. Xin, T. Liu, K.R. Tian, X.N. Li, J.H. Zhang, L.X. Song, J.G. Liu, J.F. Lv, H. Xu, R. Tao, Y. Wang, T.T. Zhang, Y.X. Deng, Y.R. Wang, T. Li, G.X. Ye, X.R. Xu, Z.B. Xia, W. Zhang, S.L. Yang, Y.L. Liu, W.Q. Ding, Z.N. Liu, J.Q. Zhu, N.Z. Liu, R. Walker, Y. Luo, Y. Wang, Y. Shen, H. Yang, Y. Cai, P.S. Ma, C.T. Zhang, J.S. Bader, J.D. Boeke, and Y.J. Yuan. 2017. "Perfect" designer chromosome V and behavior of a ring derivative. *Science* 355(6329):eaaf4704.

Xiu-Hua, J., L. Shao-Chun, H. Bing, Z. Xiang, Z. Jing, L. Wei-Hua, L. Qian, L. Ting, X. Xiao-Ping, and C. Xi-Gu. 2010. Tyrosinase small interfering RNA effectively suppresses tyrosinase gene expression *in vitro* and *in vivo*. *Molecular Biology International* 2010:240472.

Yang, C., S. Skiena, B. Futcher, S. Mueller, and E. Wimmer. 2013. Deliberate reduction of hemagglutinin and neuraminidase expression of influenza virus leads to an ultraprotective live vaccine in mice. *Proceedings of the National Academy of Sciences of the United States of America* 110(23):9481–9486.

Yang, J., K.D. Hirschi, and L.M. Farmer. 2015. Dietary RNAs: New stories regarding oral delivery. *Nutrients* 7(5):3184–3199.

Yao, L., J. Ou, C.Y. Cheng, H. Steiner, W. Wang, G. Wang, and H. Ishii. 2015. bioLogic: Natto cells as nanoactuators for shape changing interfaces. Pp. 1–10 in *CHI'15: Proceedings of the 33rd Annual ACM Conference on Human Factors in Computing Systems, April 18-23, 2015, Seoul, Republic of Korea*. New York: ACM.

Yim, H., R. Haselbeck, W. Niu, C. Pujol-Baxley, A. Burgard, J. Boldt, J. Khandurina, J.D. Trawick, R.E. Osterhout, R. Stephen, J. Estadilla, S. Teisan, H.B. Schreyer, S. Andrae, T.H. Yang, S.Y. Lee, M.J. Burk, and S.V. Dien. 2011. Metabolic engineering of *Escherichia coli* for direct production of 1,4-butanediol. *Nature Chemical Biology* 7:445–452.

Young, S.D. 2015. A "big data" approach to HIV. Epidemiology and Prevention. *Preventive Medicine* 70:17–18.

Zarogoulidis, P., K. Darwiche, W. Hohenforst-Schmidt, H. Huang, Q. Li, L. Freitag, and K. Zarogoulidis. 2013. Inhaled gene therapy in lung cancer: Proof-of-concept for nano-oncology and nanobiotechnology in the management of lung cancer. *Future Oncology* 9(8):1171–1194.

Zetsche, B., J.S. Gootenberg, O.O. Abudayyeh, I.M. Slaymaker, K.S. Makarova, P. Essletzbichler, S.E. Volz, J. Joung, J. van der Oost, A. Regev, E.V. Koonin, and F. Zhang. 2015. Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell* 163(3):759–771.

Zhang, B., X. Pan, G.P. Cobb, and T.A. Anderson. 2007. microRNAs as oncogenes and tumor suppressors. *Developmental Biology* 302(1):1–12.

Zhang, H., Q. Cheng, A. Liu, G. Zhao, and J. Wang. 2017. A novel and efficient method for bacteria genome editing employing both CRISPR/Cas9 and an antibiotic resistance cassette. *Frontiers in Microbiology* 8:812.

Zhang, L., D. Hou, Z. Chen, D. Li, L. Zhu, Y. Zhang, J. Li, Z. Bian, X. Liang, X. Cai, Y. Yin, C. Wang, T. Zhang, D. Zhu, D. Zhang, J. Xu, Q. Chen, Y. Ba, J. Liu, Q. Wang, J. Chen, J. Wang, M. Wang, Q. Zhang, J. Zhang, K. Zen, and C. Zhang. 2012. Exogenous plant MIR168a specifically targets mammalian LDLRAP1: Evidence of cross-kingdom regulation by microRNA. *Cell Research* 22(1):107–126.

Zhang, W., G. Zhao, Z. Luo, Y. Lin, L. Wang, Y. Guo, A. Wang, S. Jiang, Q. Jiang, J. Gong, Y. Wang, S. Hou, J. Huang, T. Li, Y. Qin, J. Dong, Q. Qin, J. Zhang, X. Zou, X. He, L. Zhao, Y. Xiao, M. Xu, E. Cheng, N. Huang, T. Zhou, Y. Shen, R. Walker, Y. Luo, Z. Kuang, L.A. Mitchell, K. Yang, S.M. Richardson, Y. Wu, B.Z. Li, Y.J. Yuan, H. Yang, J. Lin, G.Q. Chen, Q. Wu, J.S. Bader, Y. Cai, J.D. Boeke, and J. Dai. 2017. Engineering the ribosomal DNA in a megabase synthetic chromosome. *Science* 355(6329):eaaf3981.

Zhang, Y., B.M. Lamb, A.W. Feldman, A.X. Zhou, T. Lavergne, L. Li, and F.E. Romesberg. 2016. A semisynthetic organism engineered for the stable expansion of the genetic alphabet. *Proceedings of the National Academy of Sciences of the United States of America* 114(6):1317–1322.

Zhang, Y., J.L. Ptacin, E.C. Fischer, H.R. Aerni, C.E. Caffaro, K. San Jose, A.W. Feldman, C.R. Turner, and F.E. Romesberg. 2017. A semi-synthetic organism that stores and retrieves increased genetic information. *Nature* 551(7682):644–647.

Zivot, J.B., and W.D. Hoffman. 1995. Pathogenic effects of endotoxin. *New Horizons* 3(2):267–275.

Zolnik, B.S., A. Gonzalez-Fernandez, N. Sadrieh, and M.A. Dobrovolskaia. 2010. Nanoparticles and the immune system. *Endocrinology* 151(2):458–465.

Appendix A

Specific Synthetic Biology Concepts, Approaches, and Tools

This appendix describes a core set of current synthetic biology concepts, approaches, and tools that enable each step of the Design-Build-Test (DBT) cycle, focusing particularly on areas in which advances in biotechnology may raise the potential for malicious acts that were less feasible before the age of synthetic biology. Although the examples presented are intentionally quite broad and somewhat arbitrary—and do not represent an exhaustive list of all technologies or all possible applications of synthetic biology—they provide useful context for understanding how specific tools or approaches might enable the potential capabilities analyzed in Chapters 4–6. In addition, while the main known concepts, approaches, and tools at the time of writing are captured, this list will need to be updated and modified to stay relevant as the science advances. The relative maturity of the different technologies is described in Table A-1 to give a sense of which technologies are in widespread use, which are just in development, and which are somewhere in between.

DESIGN

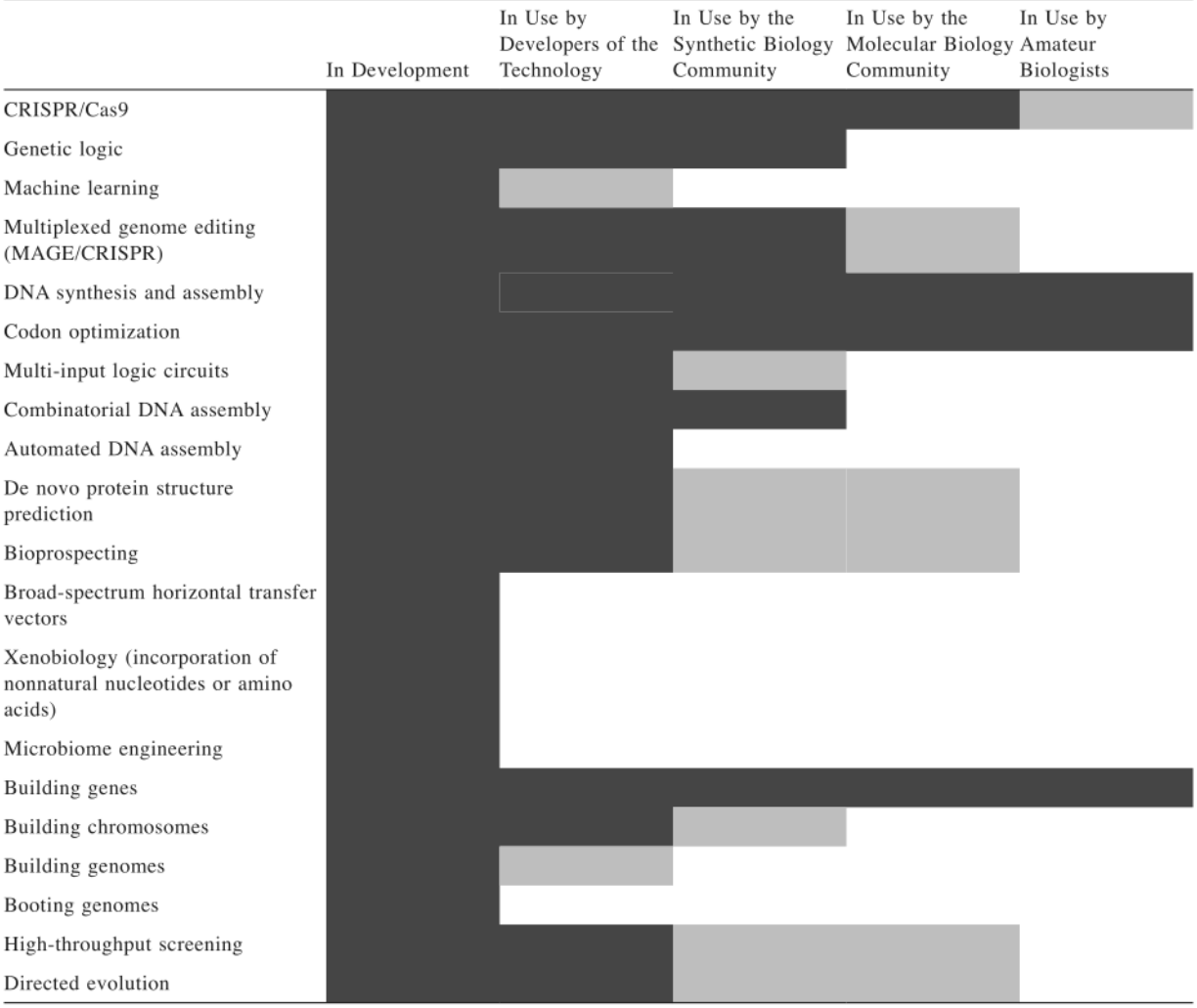
Concepts, approaches, and tools most closely aligned with the Design phase of the DBT cycle are those that enable researchers to envision and plan the engineering of biological components. This report takes a broad view of Design to include both the technologies that enable design and design objectives; as such, this grouping includes both synthetic biology technologies and examples of the types of applications that they might enable.

Automated Biological Design

Engineering biological components can be a challenging proposition; organisms are complex, and scientific understanding of biology remains incomplete. Designers must consider the effects of a large array of potential variables, including DNA bases, codons, amino acids, genes and gene segments, regulatory elements, environmental context, empirical and theoretical design rules, and many other elements. Automated biological design, known in the field as bio-design automation, lowers the barrier to designing genetic constructs by automating some decisions and processes that would otherwise require a high level of expertise or a long time to carry out. This automation is enabled by tools such as computer algorithms, software environments, and machine learning.

Some automated design tools help researchers specify the desired function of the biological construct or how the parts in the construct will be organized. Other tools help to transform these specifications into collections of realizable DNA constructs; many software tools, for example, help manage and visualize synthetic DNA sequences

TABLE A-1 Summary of Relative Maturity of Selected Synthetic Biology Concepts, Approaches, and Tools[a]



[a]For each column, darker shading indicates routine use for that community, lighter shading indicates emerging use, and white background indicates little or no use. Adoption flows from left to right in most cases.

as they are being designed. Computer software can greatly enhance the designer's ability to predict a design's function and performance, making it more feasible to engineer increasingly complex biological functions and potentially reducing the time and resources required to generate and test designs. Some predictive components of these tools are fairly straightforward, such as the virtual translation of a gene's DNA sequence into the corresponding chain of amino acids. Other functions are more complex, such as the predicted cross-interaction of transcription factors in a genetic circuit.[1] There has been significant progress, for example, in the automated compilation of in vitro and in vivo transcription-dependent or translation-dependent genetic circuits starting from high-level functional or performance specifications (Brophy and Voigt, 2014). Software can also allow designers to create

[1] "Genetic circuits" in synthetic biology are analogous to electronic circuits. Just as electronic circuits are comprised of individual electronic components (e.g., resistors, transistors) assembled together to perform a desired function (e.g., sensing, actuation), genetic circuits are constructed from the assembly of biological components. These components are encoded in the DNA and may include, for example, DNA binding sites, promoters, or transcription factors. As an example, a genetic circuit could be constructed to detect (sense) a particular metabolite and to initiate expression of a protein once the metabolite concentration crosses a certain threshold (actuate).

large libraries of combinatorial variants quickly and use machine learning to converge on optimal solutions. This allows for higher levels of design abstraction and the use of standards to exchange information globally between software frameworks.

In addition to aiding biological design, automation tools are used in other phases of the DBT cycle, as well. For example, researchers can use automated assembly tools to plan how to physically create their designed constructs most efficiently or to send designs created in silico directly to remote manufacturing facilities. These designs can be distributed across locations to massively parallelize the construction process. Once a construct is assembled, automated testing tools can be used to verify that it functions as designed. Taken together, a greater predictive capacity, automated assembly, and rapid testing can be expected to facilitate the engineering of increasingly difficult biological functions. Some example applications of automated biological design that are useful to consider in the context of biodefense include design of genes and proteins and bioprospecting and pathway design.

Design of Genes and Proteins

Automated design programs can create thousands of genetic design variants by combining libraries of genetic "parts" in various ways, an approach known as combinatorial library design. The developers of such programs typically build certain design rules into the algorithm to increase the chances that the designs created will be functional from a biological standpoint. Once the program is in use, the variants it creates can be used to improve design rules via machine learning or statistical analysis. Through this learning process the programs are able to refine subsequent designs; the process also could ultimately remove human designers from the design process, allowing DNA design, assembly, and verification equipment to explore large genetic design spaces automatically. The results of combinatorial library design programs can be stored and shared electronically for researchers to validate each other's designs, merge multiple designs, or otherwise manipulate the outputs.

Computer-aided design is also being applied to engineer protein structures, which are crucial to many biological processes. Examples of key protein functions being pursued include folding into a desired structure, binding to another protein or to a small molecule, and catalyzing a chemical reaction. Researchers have already made significant progress toward the predictive design of protein structures and engineering existing peptides and proteins for new functionalities. Automated design tools could facilitate the pursuit of more complex protein engineering, such as designing a new protein or enzyme capable of functioning with a level of specificity similar to that of natural proteins.

Bioprospecting and Pathway Design

Software can also enable designers to search for existing enzymes or biochemical pathways that could be incorporated into genetic designs to produce chemicals of interest. This type of searching is known as in silico bioprospecting. Using this approach, researchers systematically screen a large body of DNA sequence data to identify genes or protein domains that encode enzymes capable of performing a desired chemical reaction. After identifying hundreds of candidate genes, researchers produce selected genes synthetically and test their functions in vitro or in vivo. Additional software tools can be used to engineer more complex biochemical pathways by helping the user visualize those pathways, including their connections to the larger metabolic network of the cell, and estimate how different factors affect the levels of the various compounds produced. In this way, simulation and modeling tools can help to identify where adjustments might be most impactful, such as by increasing the expression of one gene product or by deactivating or downregulating a gene involved in a competing pathway.

Metabolic Engineering

Metabolic engineering involves the manipulation of biochemical pathways within a cell, frequently with the objective of producing a desired chemical. The desired chemical may be new or one that the cell already makes, and it may be simple (e.g., ethanol) or more complex (e.g., polypeptide or polyketide antibiotics). Based on a detailed understanding of the network of biochemical reactions within the cell, researchers can identify the

genes involved in crucial steps in the network of biosynthetic pathways and then adjust them to improve yields. This process is rarely as simple as increasing the expression of all enzymes in the pathway, which can lead to overconsumption of cellular resources and harm the cell's ability to grow and produce effectively. In addition, some intermediate chemical products of the pathway may be toxic to the cell, in which case it can be important to carefully regulate how rapidly such compounds are produced and consumed. Other pathways that compete with production of the final product may also need to be adjusted. Because biochemical pathways are often complex, engineering them frequently involves the use of sophisticated computer software. Metabolic engineering could potentially be used to produce toxins, narcotics, or other products relevant to biodefense. For example, yeast has already been engineered to produce opioids in minute quantities (Thodey et al., 2014). It is also conceivable that these techniques could be used to engineer organisms in the human microbiota to produce compounds that alter human health, perception, or behavior.

Phenotype Engineering

The phenotype of an organism can be affected by multiple genetic components. While there are some phenotypes for which it is possible to identify specific genes or circuits that would need to be added or altered in order to achieve a particular outcome, such as the capability for horizontal transfer (the movement of genes from one organism to another, as opposed to the vertical transfer of genes from parent to offspring) and transmissibility (the ability to pass from one organism to another), in many other cases it is difficult to determine the multiple genetic components that may impact phenotype. In the past, an organism's phenotypes were manipulated largely by the accumulation of sequential mutations, which in many cases led to local rather than global optimizations of function. More recently, the explosion of sequence information and accompanying systems biology characterizations of multiple organisms have provided a cornucopia of possibilities for engineering phenotypes that involve much more complex networks of genetic components. In parallel, the rise of DNA construction and genome editing technologies could facilitate the construction of multiple variants that involve alterations to multiple genes in an organism. By applying high-throughput screening or selection to these variant libraries, it may be possible to isolate pathogens with dramatically modified phenotypes relevant to their potential weaponization, such as environmental stability, resistance to desiccation, and ability to be mass produced and dispersed.

Horizontal Transfer and Transmissibility

The spread and impacts of a given pathogen are closely tied to its ability to replicate and be transmitted to naïve hosts. Synthetic biology technologies could potentially be applied to make a pathogen's genes more easily transmitted, such as by enabling or enhancing the horizontal transfer of genes. Genes, circuits, or episomes (pieces of genetic information that can replicate independently of the host) can already be engineered to be horizontally transferred by exploiting commonalities in replication and transformation machinery; for example, the introduction of invasin genes has been used to alter the host ranges of bacteria (Palumbo and Wang, 2006; Wollert et al., 2007). New research aims to combine multiple such techniques to create near-universal horizontal transfer vectors with expanded functionality; if successful, this work could broaden the potential areas of concern (Fischbach and Voigt, 2010; Yaung et al., 2014). Combinatorial methods that are available via library synthesis and either high-throughput screening or directed evolution may also potentially be used to alter or expand horizontal transfer and transmissibility. Past research has demonstrated that even low-throughput directed evolution of functions can be used to enhance airborne transmission of H5N1 influenza virus between mammals (Herfst et al., 2012; Imai et al., 2012).

Xenobiology

Xenobiology refers to the study or use of biological components not found naturally on Earth (Schmidt, 2010). A simple example is the engineered incorporation of a new amino acid (one not typically found in living cells) into a cell's proteins. Recent research has demonstrated that it is possible to engineer cells to employ a genetic code different from that shared by most life on Earth, or to incorporate nonnatural DNA bases (beyond adenine,

thymine, cytosine, and guanine) into a cell's DNA (Chen et al., 2016; Feldman et al., 2017). Such approaches could potentially be used to block infection by viruses or prevent undesired horizontal transfer of gene function. Cells with alternative DNA bases, codons, amino acids, or genetic codes may also be able to evade detection based on standard methods such as polymerase chain reaction (PCR), DNA sequencing, or antibody-based assays.

Human Modulation

While past considerations of biodefense concerns have largely been focused on pathogens, synthetic biology raises new possibilities for modifying a person's physiology or environment in ways that may lead to dysfunction, disease, or increased susceptibility to disease. For example, altering the makeup or functions of the gut microbiome could either enhance a person's health or cause dysfunction. Modulation of the immune system—the body's defense against pathogens—is another hypothetical possibility worthy of consideration, as is epigenetic modification (changes in how cells express genes but not changes to the DNA sequence itself). In short, there is now a large amount of information available about the human form that could potentially inform phenotype modulation in different ways.

BUILD

Technologies and applications most closely aligned with the Build phase of the DBT cycle are those that are used to physically create actual biological components. Synthetic biology is often pursued in an iterative fashion, blurring the lines among the Design, Build, and Test phases, and some technologies can play a role in multiple phases. Considered here are technological capabilities and advances related to specified changes and to the construction of libraries for high-throughput screening or directed evolution.

Factors that may impact the level of concern related to Build capabilities include cost, time, and ease of access for DNA construction; the complexity of libraries that can be generated for directed evolution; and the difficulties inherent in rendering the DNA "operable" (i.e., the ability to create a synthetic DNA sequence that actually functions within a living system).

DNA Construction

DNA construction refers to technologies that can be used to produce a desired DNA molecule de novo. The general and overlapping terms "DNA synthesis" and "DNA assembly" are included in this category. Much of modern biotechnology depends on having DNA molecules of defined sequence; synthetic DNA has been used, for example, to advance understanding of the basic workings of the genetic code, to enable modern DNA sequencing, and to develop and enable common use of PCR. In addition, gene editing technologies such as zinc finger nucleases, TALENs, and CRISPR/Cas9 each depend on some amount of synthetic DNA. Decreasing costs and increased production scales have made it far more feasible to use synthetic DNA for a variety of purposes. Before DNA construction technologies became available, the only way to obtain a particular DNA segment of interest was to find it in an organism. Now, nearly any DNA—whether natural or designed—can be obtained by simply ordering the sequence to be synthesized from one of many commercial suppliers or by making it on a laboratory DNA synthesizer. While DNA is the most common product of DNA construction technologies, these technologies can also be used to create synthetic RNA molecules and chemical modifications to DNA or RNA.

This access is tremendously enabling for the many beneficial uses of biotechnology, but also has ramifications for potential malicious use. For example, DNA construction could conceivably be leveraged to make toxins, enhance a pathogen, re-create a known pathogen, or even create an entirely new pathogen. Generally speaking, ready access to synthetic DNA allows designers to construct, test, and revise their designs more easily. Many DNA synthesis companies have agreed to screen orders in accordance with guidelines from the U.S. Department of Health and Human Services (HHS, 2015), although limitations of these guidelines have been described (Carter and Friedman, 2015).

Factors that may impact the level of concern related to DNA construction capabilities include cost, time,

ease of access, and difficulty of rendering the DNA "operable." The size of a segment of synthetic DNA (a DNA construct) is typically described in base pairs for double-stranded DNA and nucleotides for single-stranded DNA. DNA constructs can range from a few nucleotides to several thousand base pairs to entire genomes. Generally speaking, longer DNA constructs are more difficult to produce (or assemble) and using them requires additional laboratory skills compared to shorter constructs. The following examples describe potential uses of DNA construction in ascending order of length and complexity.

Oligonucleotides (Several to Hundreds of Nucleotides)

In its most basic form, DNA construction produces oligonucleotides (oligos), single strands of user-defined sequence that can range in length from a few nucleotides to a few hundred. Oligos can be combined to construct longer DNA sequences. Oligos are extremely useful for a wide variety of research tasks that involve manipulating and analyzing DNA, including sequencing and PCR, as well as site-directed mutagenesis and genome-scale gene editing (e.g., using multiplexed automated genome engineering, or MAGE; Gallagher et al., 2014). Although oligos are typically too short to form the types of protein-encoding genes necessary to support more complex biological functions, they can be used to encode regulatory regions (such as promoters or enhancers), certain short polypeptide-based toxins, transfer RNA, and guide RNA molecules such as those employed for gene editing.

Genes (Hundreds to Thousands of Base Pairs)

Most genes range from a few hundred to a few thousand base pairs in length. Synthetic genes are available commercially as either cloned DNA (in which the product is verified as correct and pure, and typically delivered as part of a general circular plasmid DNA vector) or uncloned linear fragments of DNA (which typically contain some amount of undesired mutations). Potential uses for synthetic genes are at least as diverse as the range of genetic functions found in nature. Genes could be used for a wide variety of malicious purposes, for example, to enhance the pathogenicity of an organism or to produce a toxin.

Genetic Systems (Thousands to Hundreds of Thousands of Base Pairs)

Genetic systems are groups of genes that work together to achieve a more complex function but fall short of supporting an entire cell. For example, genetic systems could be used to encode a biosynthetic pathway or to form engineered genetic circuits that combine operations such as sensing, computing, and actuation. Viral genomes can also be considered as genetic systems, and the genomes for several viruses have already been synthesized and used to produce fully infectious virions (Blight et al., 2000; Cello et al., 2002; Tumpey et al., 2005). Viral genomes can vary from thousands to hundreds of thousands of base pairs in length; large viral genomes (e.g., orthopox viruses) are currently more challenging to synthesize than small ones (e.g., polio).

Cellular Genomes (Millions of Base Pairs)

DNA construction can also be used to assemble the genome for an entire single-celled organism. In 2010, researchers synthesized and assembled the DNA genome of the bacterium *Mycoplasma mycoides* and used that genome to produce a self-replicating cell (Gibson et al., 2010). This was a difficult, time-consuming, and costly process. At about one million base pairs, the synthetic genome was also one of the smallest known in the microbial world. Nevertheless, this feat demonstrated that it is possible to re-create a living, reproducing organism based on its genetic data. In this case, researchers "booted" their synthetic genome by inserting it into the cell body of a closely related organism, leading to complete replacement of its natural genome with the synthetic one. It remains to be seen how generalizable this approach can be for larger microbial genomes and other types of cells. Other researchers are currently pursuing the construction of bacterial and yeast genomes ranging from 4 to 11 megabase pairs in length; these efforts also use an existing close relative, replacing or "patching" the natural genome with large fragments of the synthetic genome (Richardson et al., 2017). Concerns have been raised about the possibility

of using whole-genome construction to generate dangerous organisms that otherwise could not be obtained without attracting attention (or might not be obtainable at all).

Editing of Genes or Genomes

A variety of technologies allows the modification of specified bases or genes within a pathogen, vector, or host. Such technologies could potentially be utilized to imbue pathogens with new functions; for example, site-directed mutagenesis capabilities could allow the construction of viral variants with novel properties such as altered immunogenicity or species range. Examples include oligonucleotide-meditated mutagenesis, recombination-mediated genetic engineering ("recombineering") and related techniques (Murphy and Campellone, 2003; Ejsmont et al., 2011), CRISPR/Cas9-based genome editing approaches, and MAGE. Most significantly, newer gene editing platforms such as CRISPR/Cas9 enable the modification of a wide range of organisms. Both the ease with which pathogens can be modified and the types of possible phenotypes that could arise from such modifications would be relevant to an assessment of vulnerabilities related to gene or genome editing.

In the past, genome engineering was a painstaking process that required individual genes to be modified serially. Now, however, multiple genes can potentially be modified in parallel and iteratively. For example, with MAGE, multiple synthetic oligos are created that differ from the existing host genome in at least one base pair. These synthetic oligos are then inserted into a population of cells, where they essentially overwrite the targeted portion of DNA in the cells. MAGE has been used to optimize metabolic pathways, turn off sets of genes, tune gene activity up or down, and engineer a microbial genome with an altered genetic code.

While the biochemical mechanisms MAGE relies on are common throughout both simple and complex organisms, MAGE has primarily been demonstrated in *Escherichia coli*, and the work required to adapt MAGE to a new species may prove cumbersome. In contrast, genetic engineering and CRISPR/Cas9-based technologies may allow engineering in many new species, providing convenient paths to the further identification of altered phenotypes via either high-throughput screening or directed evolution of organisms with radically new phenotypes and genome-wide sequence changes.

Library Construction

One of the watershed differences that has been enabled by improvements in DNA construction is the ability to generate large libraries of genetic variants. Such libraries can be sieved for improved phenotypes without knowing precisely what variants will arise. This contrasts with the more deliberate process of gene and genome engineering described above (Editing of Genes or Genomes), but there are overlaps between the two approaches because an increased knowledge of how genotype relates to phenotype can guide library design and thereby improve the probability that a given phenotype will be achieved. As an analogy, library construction techniques allow the construction of many more "darts," and knowledge of genotype-to-phenotype relationships, gained through experiments with gene and genome editing, provides an increasingly larger "target" at which to throw those darts. In particular, the ability to construct degenerate oligonucleotides in a wide variety of ways, including by codon mutagenesis or with nucleotides that are inherently mutagenic, provides a means to construct both large and relatively targeted libraries.

Because DNA can span thousands or even millions of base pairs, designers typically prioritize which parts to vary based on analyses and educated guesses about which changes are most likely to yield the desired results. For example, a designer may use protein structure analysis and visualization software to identify specific parts of a protein that might affect the desired function, such as its enzymatic specificity, build proteins with random variation in those specific parts, and then test how each random variation affects enzymatic specificity.

Booting of Engineered Constructs

With some exceptions, synthesized DNA (or RNA) does not perform biological functions on its own. The process of inducing raw genetic material to perform biological functions is known as "booting," a term borrowed

from computer technology, where booting refers to the ability to execute functions on digital information by taking it out of storage and putting it into an active state. Booting a synthetic construct is most relevant to the Build and Test phases of the DBT cycle. In the context of biodefense, booting may also be important for a malicious actor's ability to deliver a bioagent to a target.

Booting in biological systems can take many forms. In the context of viruses, booting may be broadly considered to mean that viral nucleic acids are delivered to cells, where the viral nucleic acids are subsequently able to replicate. A few viruses have been booted by merely delivering their genetic material into host cells, whereas others require additional genetic components expressed separately in host cells in order to produce infectious viral particles. In the context of bacteria, researchers have successfully booted synthetic bacterial genomes by replacing part or all of the genetic contents of natural or synthesized cells with a partial or full synthetic genome. Booting a fully functioning, self-replicating bacterium is significantly more complex than booting a virus.

Perhaps the simplest example of booting engineered constructs is through the use of episomes, pieces of genetic information that can autonomously replicate but typically cannot be readily transferred between cells. Plasmids (typically found in prokaryotes) and extrachromosomal linear arrays of DNA (typically found in eukaryotes) are examples of episomes. Episomes are the most common vector that synthetic biologists use to boot engineered constructs, and there are many available techniques to boot episomes. Although episomes in general are not as complex as full viral or bacterial genomes, they can be used to, for example, introduce a viral genome into a cell and then use the host cell's transcription, translation, and replication machinery to boot the virus. It may even be possible to use a similar approach to boot a free-living organism. It is also possible for some episomes to spread through a microbial population and between individuals, albeit in general more slowly than a viral infection would.

TEST

Testing is used to determine whether a design or biological product created with synthetic biology tools has the desired properties. Tests are typically performed at many stages of a project; for example, a researcher might use computer models to determine if a design is likely to work, then perform tests to validate that the correct DNA construct has been synthesized, then boot the construct to verify that it is capable of performing the intended biological functions. Testing might involve the use of cell cultures, model organisms in laboratory conditions, organisms in the wild, or even potentially human populations.

Test results can be used to further refine a design based on information gained from experimental measurements and observations, and the DBT cycle begins again. In general, state-of-the-art synthetic biology efforts require a great deal of testing in order to yield organisms with the desired properties, making Test both a crucial step and a substantial bottleneck in the DBT cycle. It is a matter of debate whether malicious actors could skip the Test phase and still successfully carry out a biological attack. While a test can be applied to a single variant, in practice it is often more desirable to carry out multiple tests in parallel (high-throughput screening) or to have organisms "test" themselves (directed evolution).

High-Throughput Screening

Automation provides the means to screen thousands to billions of individual variants of an organism for function or phenotype. High-throughput testing in cell cultures is a type of screening test commonly used in synthetic biology. Such tests can be used to answer more specific questions (e.g., did this precise genomic change yield the desired phenotypic alteration?) or more exploratory questions (e.g., did any of these 100,000 combinatorial variants in one viral protein yield the desired phenotypic alteration?). Technologies for cheaper and faster screening are in high demand across the biological and biomedical communities, in particular for "-omics" approaches that are agnostic to the type of organism being tested, such as genomics, transcriptomics, metabolomics, and proteomics.

Screening-based tests are performed serially, evaluating different designs or biological products one at a time. Using multiplexing and automation, researchers have developed high-throughput screening–based tests capable of screening tens to thousands of prototypes. On the other hand, selection-based tests (see below, Directed Evolution) are more difficult to design than screening-based tests, but allow much higher throughput.

Directed Evolution

In nature, the process of evolution selects the best performers from a genetic pool that includes some degree of random variation. Researchers can use a similar process to create prototype biological components representing multiple competing variations and then select among them for the phenotypes that best match the desired outcomes. Prototypes can vary based on smaller changes—different DNA bases, codons, or amino acids, for example—or based on larger-scale differences such as the configuration of multiple genes within a genetic circuit. Like automated biological design, directed evolution is a synthetic biology technique that spans all three phases of the DBT cycle. By building and evolving constructs with random variations, researchers use directed evolution to refine new designs through an iterative approach. The primary difference between high-throughput screening and directed evolution is that in directed evolution, individual organisms compete for the ability to replicate. For example, genomic variations could be introduced into a modified pathogen to produce a large library of variant organisms, which could then be tested for the ability to grow in the presence of an antibiotic. Directed evolution can thus be used to evaluate millions of prototype biological components in parallel, though typically, only one or a few variants would ultimately emerge as successful.

This approach can allow a researcher to sidestep the need for predictive design by creating libraries of millions or more variants and then selecting or screening them to find those few that have a desired set of properties. For example, a researcher could randomly alter residues within specific genes or across an entire genome and then select for a desired phenotype, such as growth, tropism, or lysis. Importantly, the selection can be carried out directly in a host organism, thus allowing for the selection of host-related phenotypes, such as transmissibility (ability to move from an infected to an uninfected host) or pathogenicity (e.g., necrosis within particular tissues). The most promising variants that emerge can be refined further through additional iterations of rational design or selection, following the DBT cycle. Many of the same methods used for library construction and high-throughput screening can also be used for directed evolution, and these different approaches can be combined. For example, a researcher could conduct a high-throughput screen of variants created by a CRISPR/Cas9 library, MAGE, or DNA shuffling (a technique whereby a set of related genes or genomes is broken down into smaller pieces that are randomly reassembled). The variants selected by the screen could then be selected for growth on a novel substrate, potentially identifying both a gene and an organism whose sequence was not fully included in any of the original precursor genes.

REFERENCES

Blight, K.J., A.A. Kolykhalov, and C.M. Rice. 2000. Efficient initiation of HCV RNA replication in cell culture. *Science* 290(5498):1972–1974.

Brophy, J.A., and C.A. Voigt. 2014. Principles of genetic circuit design. *Nature Methods* 11(5):508–520.

Carter, S.R., and R.M. Friedman. 2015. DNA Synthesis and Biosecurity: Lessons Learned and Options for the Future. J. Craig Venter Institute. Available at: http://www.jcvi.org/cms/fileadmin/site/research/projects/dna-synthesis-biosecurity-report/report-complete.pdf. Accessed January 31, 2018.

Cello, J., A.V. Paul, and E. Wimmer. 2002. Chemical synthesis of poliovirus cDNA: Generation of infectious virus in the absence of natural template. *Science* 297(5583):1016–1018.

Chen, T., N. Hongdilokkul, Z. Liu, D. Thirunavukarasu, and F.E. Romesberg. 2016. The expanding world of DNA and RNA. *Current Opinion in Chemical Biology* 34:80–87.

Ejsmont, R.K., P. Ahlfeld, A. Pozniakovsky, A.F. Stewart, P. Tomancak, and M. Sarov. 2011. Recombination-mediated genetic engineering of large genomic DNA transgenes. *Methods in Molecular Biology* 772:445–458.

Feldman, A.W., V.T. Dien, and F.E. Romesberg. 2017. Chemical stabilization of unnatural nucleotide triphosphates for the in vivo expansion of the genetic alphabet. *Journal of the American Chemical Society* 139(6):2464–2467.

Fischbach, M., and C.A. Voigt. 2010. Prokaryotic gene clusters: A rich toolbox for synthetic biology. *Biotechnology Journal* 5(12):1277–1296.

Gallagher, R.R., Z. Li, A.O. Lewis, and F.J. Isaacs. 2014. Rapid editing and evolution of bacterial genomes using libraries of synthetic DNA. *Nature Protocols* 9:2301–2316.

Gibson, D.G., J.I. Glass, C. Lartigue, V.N. Noskov, R.Y. Chuang, M.A. Algire, G.A. Benders, M.G. Montague, L. Ma, M.M. Moodie, C. Merryman, S. Vashee, R. Krishnakumar, N. Assad-Garcia, C. Andrews-Pfannkoch, E.A. Denisova, L. Young, Z.Q. Qi, T.H. Segall-Shapiro, C.H. Calvey, P.P. Parmar, C.A. Hutchison III, H.O. Smith, and J.C. Venter. 2010. Creation of a bacterial cell controlled by a chemically synthesized genome. *Science* 329(5987):52–56.

Herfst, S., E.J. Schrauwen, M. Linster, S. Chatinimitkul, E. de Wit, V.J. Munster, E.M. Sorrell, T.M. Bestebroer, D.F. Burke, D.J. Smith, G.F. Rimmelzwaan, A.D. Osterhaus, and R.A. Fouchier. 2012. Airborne transmission of influenza A/H5N1 virus between ferrets. *Science* 336(6088):1534–1541.

HHS (U.S. Department of Health and human Services). 2015. Screening Framework Guidance for Providers of Synthetic Double-Stranded DNA. Available at: https://www.phe.gov/Preparedness/legal/guidance/syndna/Pages/default.aspx. Accessed May 22, 2017.

Imai, M., T. Watanabe, M. Hatta, S.C. Das, M. Ozawa, K. Shinya, G. Zhong, A. Hanson, H. Katsura, S. Watanabe, C. Li, E. Kawakami, S. Yamada, M. Kiso, Y. Suzuki, E.A. Maher, G. Neumann, and Y. Kawaoka. 2012. Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets. *Nature* 486(7403):420–428.

Murphy, K.C., and K.G. Campellone. 2003. Lambda Red-mediated recombinogenic engineering of enterohemorrhagic and enteropathogenic *E. coli*. *BMC Molecular Biology* 4:11.

Palumbo, R.N., and C. Wang. 2006. Bacterial invasin: Structure, function, and implication for targeted oral gene delivery. *Current Drug Delivery* 3(1):47–53.

Richardson, S.M., L.A. Mitchell, G. Stracquadanio, K. Yang, J.S. Dymond, J.E. DiCarlo, D. Lee, C.L.V. Huang, S. Chandrasegaran, Y. Cai, J.D. Boeke, and J.S. Bader. 2017. Design of a synthetic yeast genome. *Science* 355(6329):1040–1044.

Schmidt, M. 2010. Xenobiology: A new form of life as the ultimate biosafety tool. *Bioessays* 32(4):322–331.

Thodey, K., S. Galanie, and C.D. Smolke. 2014. A microbial biomanufacturing platform for natural and semisynthetic opioids. *Nature Chemical Biology* 10:837–844.

Tumpey, T.M., C.F. Basler, P.V. Aguilar, H. Zeng, A. Solórzano, D.E. Swayne, N.J. Cox, J.M. Katz, J.K. Taubenberger, P. Palese, and A. Garcia-Sastre. 2005. Characterization of the reconstructed 1918 Spanish influenza pandemic virus. *Science* 310(5745):77–80.

Wollert, T., B. Pasche, M. Rochon, S. Deppenmeier, J. van den Heuvel, A.D. Gruber, D.W. Heinz, A. Lengeling, and W. Schubert. 2007. Extending the host range of *Listeria monocytogenes* by rational protein design. *Cell* 129(5):891–902.

Yaung, S.J., C.M. Church, and H.H. Wang. 2014. Recent progress in engineering human-associated microbiomes. *Methods in Molecular Biology* 1151:3–25.

Appendix B

Selected Prior Analyses Used to Inform the Framework

Prior biodefense analyses and other sources were reviewed in developing the factors and elements that form the framework presented in this report. This appendix provides further summary information about several of these sources to illustrate different approaches to assessing potential synthetic biology concerns. It is not intended to be a comprehensive compendium of all prior risk governance and biotechnology assessment approaches.

CONSIDERATIONS FROM *GLOBALIZATION, BIOSECURITY, AND THE FUTURE OF THE LIFE SCIENCES*

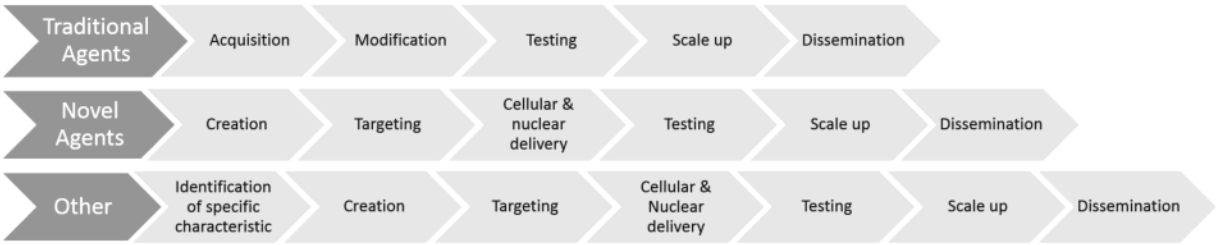
The report *Globalization, Biosecurity, and the Future of the Life Sciences* (also sometimes referred to as the "Lemon-Relman" report from the names of its committee co-chairs) classified emerging technologies into categories based on their characteristics as concerning and warranting particular attention for further risk assessment (IOM and NRC, 2006). These four groupings were:

> (1) technologies that seek to acquire novel biological or molecular diversity; (2) technologies that seek to generate novel but pre-determined and specific biological or molecular entities through directed design; (3) technologies that seek to understand and manipulate biological systems in a more comprehensive and effective manner; and (4) technologies that seek to enhance production, delivery, and 'packaging' of biologically active materials. (IOM and NRC, 2006, p. 4)

This categorization is wholly focused on features of the technology itself in terms of capabilities it might generate.

CAPABILITIES-BASED WEAPON DEVELOPMENT FRAMEWORK FROM NATIONAL DEFENSE UNIVERSITY

This approach, developed at National Defense University (2016) indicates the points at which potential impacts in the age of synthetic biology could be achieved. Beginning at the far left and working across each step of the bioweapon development pathway, one may determine the steps at which synthetic biology could have an impact on the development pathway (see Figure B-1).



Acquisition = theft from lab or transport, harvest from nature, synthetic recreation
Creation = wet-bench laboratory work and genetic design, synthetic creation
Targeting = target to specific genome elements
Cellular/Nuclear delivery = capability which allows viable delivery to specific cells and the nucleus
Testing = animal models, field testing
Scale up = mass production, freeze drying, encapsulation, storage/stockpiling
Dissemination = sprayer, point delivery mechanism, filling

For each capability listed, address the following questions

How do advances in synthetic biology enable this capability?
What barriers must be overcome?
How soon will this capability emerge? (current, near, mid, long)
What actors could develop this capability?
What are the consequences of the emergence of this capability?

FIGURE B-1 Approach to considering steps where synthetic biology could impact bioweapon development. Developed by National Defense University. SOURCE: National Defense University, 2016.

This model was used by National Defense University at a tabletop exercise to assess where gene editing technology (such as CRISPR/Cas) provides heightened capability for creating bioweapons. The approach provides insight into *where* synthetic biology may have an impact, rather than defining specific characteristics of the technologies themselves.

DECISION FRAMEWORK FROM *INNOVATION, DUAL USE, AND SECURITY*

Jonathan Tucker's "Decision Framework" published in *Innovation, Dual Use, and Security* (Tucker, 2012) suggests a number of attributes that are relevant to the study charge, as restated below:

(1) Characteristics of the technology:
   a. Accessibility
   b. Ease of misuse
(2) Characteristics of governability:
   a. Embodiment (material "tangibility" of technologies)
   b. Maturity
   c. Convergence (number of technologies that come together to create new technology)
   d. Rate of advance
   e. International diffusion
(3) Level(s) amenable to mitigation
   a. State
   b. Institution
   c. Individual
   d. Product
   e. Knowledge

This framework encompasses a variety of features that touch on features of the technology (level of difficulty, maturity, speed of advance, and convergence with other technologies), who has access, and the severity of the outcome if it is misused. This framework also considers options for mitigation, as well as how the cost compares to the benefit of the technology. It is used primarily to assess technology in terms of relative risk on these levels.

EXPERIMENTAL AIMS FROM *BIOTECHNOLOGY RESEARCH IN AN AGE OF TERRORISM*

In 2004, the National Academies produced the report *Biotechnology Research in an Age of Terrorism* (NRC, 2004), known as the "Fink report" after its chairman, geneticist Gerald R. Fink, which made the case that scientists have an "affirmative moral duty to avoid contributing to the advancement of biowarfare or bioterrorism." The Fink report highlights a list of specific experimental aims that that should trigger additional safety and security examination, even if performed for valid scientific reasons. These include experiments that would

(1) Render a vaccine ineffective,
(2) Confer resistance to antibiotics or antivirals (countermeasures),
(3) Enhance virulence of a pathogen or make a nonpathogen virulent,
(4) Increase transmissibility of a pathogen,
(5) Alter the host range of a pathogen,
(6) Enable evasion of detection or diagnostic, or
(7) Enable weaponization of an agent or toxin.

The report features broad recommendations for mitigation of negative outcomes, to include community outreach, research review (including creation and use of a review board), focused research on mitigation, and international cooperation and outreach. This framework primarily focused on the creation of mitigation tools, but also the creation of a core backbone for biosecurity policy development. The Fink report also led to the creation of the National Science Advisory Board for Biosecurity, a federal advisory committee administered by the U.S. Department of Health and Human Services, which has produced a number of influential reports on dual-use research.

NATIONAL INSTITUTES OF HEALTH CONTAINMENT GUIDELINES

The National Institutes of Health Guidelines (NIH, 2016), conceived initially with the advent of recombinant DNA, provide risk assessment frameworks that enable decision making about the level of biocontainment that can best protect laboratory workers, along with suggestions for mitigation plans. Formal risk groups were developed with respect to particular pathogens.

These guidelines focus on capabilities of particular agents, potential adverse outcomes (accidental infection of laboratory workers or the public), and mitigation strategies. Perhaps most relevant to this study are the characteristics identified for consideration with respect to containment, which include

Virulence;
Pathogenicity;
Potency;
Environmental stability;
Route of spread/communicability;
Availability of vaccine or treatment;
Gene product effects such as toxicity, physiological activity, and allergenicity; and
Any strain that is known to be more hazardous than the parent (wild-type) strain.

CATEGORIES OF EXPERIMENTS HIGHLIGHTED BY THE DURC PROCESS

The Dual Use Research of Concern (DURC) process was initially triggered by concerns over the publication of sequence manipulation information that could map out the creation of a potentially dangerous virus; however, the DURC policies that resulted are more focused on experiments of concern rather than control of information per se. The DURC policies for government and institutions (U.S. Government, 2012, 2014) utilize the Federal Select Agent Program Select Agents and Toxins list and highlight categories of experiments similar to those in the Fink report. These categories include experiments that

(1) Enhance the harmful consequences of the agent or toxin;
(2) Disrupt immunity or the effectiveness of an immunization against the agent or toxin without clinical and/or agricultural justification;
(3) Confer to the agent or toxin resistance to clinically and/or agriculturally useful prophylactic or therapeutic interventions against that agent or toxin or facilitates their ability to evade detection methodologies;
(4) Increase the stability, transmissibility, or the ability to disseminate the agent or toxin;
(5) Alter the host range or tropism of the agent or toxin;
(6) Enhance the susceptibility of a host population to the agent or toxin; or
(7) Generate or reconstitute an eradicated or extinct agent or toxin listed.

Similar to the Fink report, this list is focused on capabilities that the technology provides to produce a harmful biological entity. The DURC policy is intended to be used to make decisions about funding dual-use experiments.

SOCIETAL RISK EVALUATION SCHEME (SRES)

The SRES approach developed by Cummings and Kuzma (2017) was applied to a set of four case studies of synthetic biology applications. The suggested characteristics for assessing risks of synthetic biology applications are based primarily on outcomes of an adverse event and whether or not mitigation exists. It also includes a novel consideration of society's attitude toward a potentially adverse outcome, which include considerations such as

(1) Human health risks,
(2) Environmental health risks,
(3) Unmanageability,
(4) Irreversibility,
(5) Likelihood that a technology will enter the marketplace,
(6) Lack of human health benefits,
(7) Lack of environmental benefits, and
(8) Anticipated level of public concern.

Since this approach was a risk-benefit framework, it goes beyond the scope of the study charge for this committee, which did not attempt to address the benefits of synthetic biology capabilities.

GRYPHON ANALYSES

In a presentation to the committee, a representative from Gryphon Scientific described an approach for considering how advances in synthetic biology may change the landscape for acquisition of biological threat agents. For example, synthetic biology advances might enable particular threat agents to be synthesized or for a less pathogenic microorganism to be modified into a threat agent, in comparison to alternative acquisition routes such as culturing from clinical or environmental samples or theft. The approach taken by the analysis was comparative and was motivated by the guiding question, "What advantages (or disadvantages) do synthetic biology acquisition routes provide to a malicious actor, relative to alternative acquisition routes?" (Casagrande et al., 2017). The

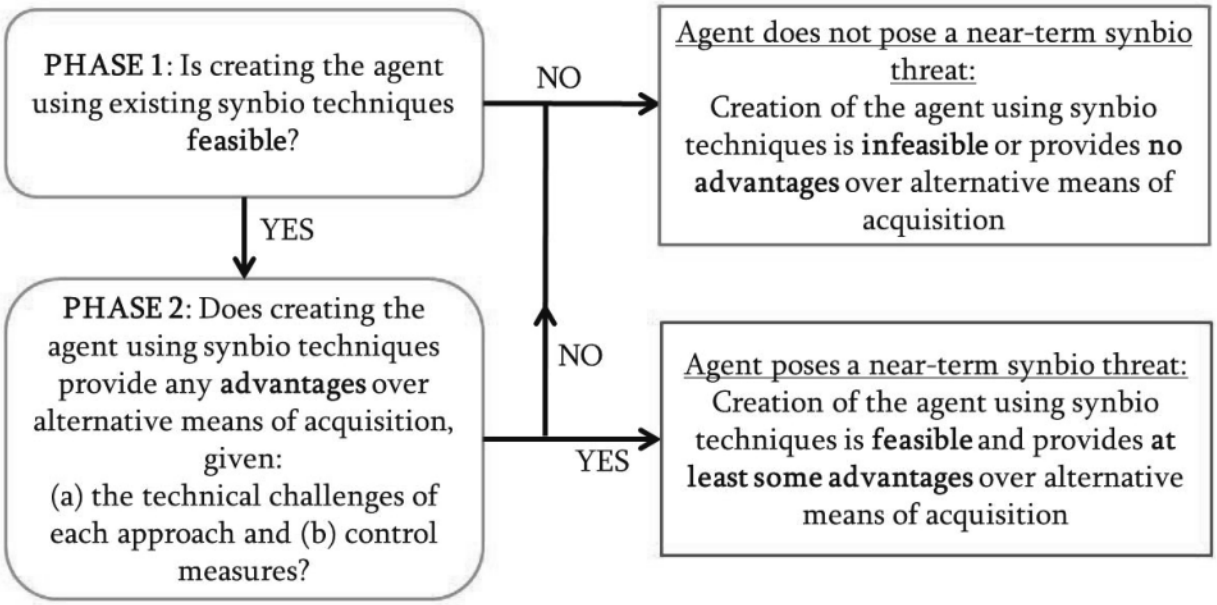


FIGURE B-2 Approach to conducting an assessment of how synthetic biology changes the threat agent landscape. SOURCE: Modified from Casagrande et al., 2017.

framework used in the analysis, depicted in Figure B-2, included two phases. The first phase asked whether creating a particular biological threat agent was possible using synthetic biology. If so, the second phase asked whether the use of synthetic biology provided acquisition advantages over alternative approaches to obtaining that agent. The results of these two phases informed the determination of whether the agent did or did not pose a near-term threat.

Prior work by Gryphon Scientific, described in the presentation, also considered whether novel biotechnologies, including synthetic biology, have the potential to influence and streamline classical weaponization steps for biological agents. For example, the presenter noted that agents developed using synthetic biology might be developed with increased potency, increased ability to grow to larger numbers, enhanced environmental persistence, increased transmissibility, and the ability to overcome host resistance. However, the use of synthetic biology tools might not be the most effective means to achieve these objectives because of intrinsic factors (such as a lack of knowledge) as well as extrinsic factors such as the need for continual testing of weapons products along a development pathway.

REFERENCES

Casagrande, R., C. Meyer, and K. Berger. 2017. Assessing Biodefense Vulnerabilities Posed by Synthetic Biology: Insights from Related Gryphon Studies. Presentation to the Committee by Gryphon Scientific, January 26.

Cummings, C.L., and J. Kuzma. 2017. Societal Risk Evaluation Scheme (SRES): Scenario-based multi-criteria evaluation of synthetic biology applications. *PLoS ONE* 12(1):e0168564.

IOM and NRC (Institute of Medicine and National Research Council). 2006. *Globalization, Biosecurity, and the Future of the Life Sciences*. Washington, DC: The National Academies Press.

National Defense University. 2016. *Challenge or Crisis: Security Risk Posed by Gene Editing and Synthesis*. Workshop Report, September 14.

NIH (National Institutes of Health). 2016. NIH Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules. April. Available at: https://osp.od.nih.gov/wp-content/uploads/2013/06/NIH_Guidelines.pdf. Accessed November 8, 2017.

NRC (National Research Council). 2004. *Biotechnology Research in an Age of Terrorism*. Washington, DC: The National Academies Press.

Tucker, J.B. 2012. Decision framework. Pp. 67–84 in *Innovation, Dual Use, and Security: Managing the Risks of Emerging Biological and Chemical Technologies*. Cambridge, MA: MIT Press.

U.S. Government. 2012. United States Government Policy for Oversight of Life Sciences Dual Use Research of Concern. March 29. Available at: https://www.phe.gov/s3/dualuse/Documents/us-policy-durc-032812.pdf. Accessed May 11, 2017.

U.S. Government. 2014. United States Government Policy for Institutional Oversight of Life Sciences Dual-Use Research of Concern. September 24. Available at: https://www.phe.gov/s3/dualuse/Documents/durc-policy.pdf. Accessed May 11, 2017.

Appendix C

Questions to Stimulate Consideration of Framework Factors

The following illustrative questions were developed to stimulate consideration of the framework factors and facilitate use of the framework to assess specific potential capabilities. These are not intended to represent every question that can be posed, and some questions can be applicable to assessing more than one factor.

Usability of the Technology—Ease of Use

- How long is the oligonucleotide, gene, or genome involved?
- If an entire genome is being created, how easy is it to assemble?
- For an entire genome, how easy is it to "boot"?
- What is the scale and complexity of modification or synthesis involved? For example, is the target a virus, bacterium, fungus, or a larger organism, and how does this affect the ease of use?
- Can the desired construct be ordered commercially, or would regulatory oversight (e.g., Select Agent rules) or construct length make this unlikely?
- Are reagent kits available to make the process easier?
- Are genomic design tools and relevant "parts" databases available to help achieve desired goals?
- How reliable is the available genomic sequence information?
- How reliable is the available genotype-to-phenotype information, and how does this affect the ease of use for the intended purpose?
- Is there a recipe or standard operating procedure available for the intended use, and if so, has it been demonstrated to work previously?
- Is specialized equipment required, and if so, is it readily available for purchase or via contract?
- What level of specialized knowledge, hands-on training, and tacit knowledge is required?
- Are suitable test conditions (e.g., cell cultures, model organisms) available?

Usability of the Technology—Rate of Development

- Are significant improvements to the technology being published on at least an annual basis?
- What aspects are improving? (Examples of aspects to consider include total processing time, cost, laboratory space footprint, level of automation, accuracy, throughput, user interface, and output reporting.)

- What types of uses are driving commercial development and market adoption?
- Is there competition spurring the rate of the technology's development, or does one company have a monopoly?
- Are there multiple different markets for the technology, spurring technological development and innovation, or is it tightly focused on one specific market?
- Is there an open-source user community helping to drive the technology forward by sharing new developments?

Usability of the Technology—Barriers to Use

- Are there critical bottlenecks that, once overcome, will significantly improve ease of use (e.g., CRISPR/Cas9 for gene editing, photolithography for oligonucleotide synthesis)?
- What barriers may hinder wider market adoption and penetration of the technology involved, and how might these be overcome?
- Would significant improvements in Build capabilities (e.g., capacity for increased construct length or reduced cost of synthesis) be accompanied by corresponding improvements in capabilities for Design and Testing relevant to the intended application, or would those aspects remain as barriers?
- Are there gaps in fundamental knowledge about pathways and genotype-to-phenotype relationships that may hamper the use of genomic design tools for the intended use?

Usability as a Weapon—Production and Delivery

- Could synthetic biology (or its use in combination with other biotechnology advances) be used to enhance replication or growth characteristics of an agent in order to support scale-up?
- Could synthetic biology (or its use in combination with other biotechnology advances) help to scale up production of the agent without its losing infectivity or other key features?
- Could synthetic biology be used to make an agent "hardier" in the varied environments it may encounter during storage and delivery (e.g., could it survive the adverse conditions that might be expected in the context of dispersal)?
- Could synthetic biology be used to stabilize the agent or facilitate dispersal and survival?
- How might the agent be delivered to those targeted (e.g., mass dispersal, contamination of food or water, a needlestick), and how might this delivery mechanism affect requirements for production, stabilization, or testing?
- Could synthetic biology (or its use in combination with other biotechnology advances) facilitate novel or enhanced forms of delivery?
- Is large-scale production of the agent needed to have an impact?
- Could synthetic biology help to reduce the organizational footprint, expertise, or equipment required for production?

Usability as a Weapon—Scope of Casualty

- Could synthetic biology be used to enhance host susceptibility to a given agent in a way that would worsen the severity of an attack or increase the number of casualties?
- How many individuals could be targeted for harm using this capability (ranging from a single assassination to thousands of people, or more)?
- Is the agent highly transmissible, thus allowing it to spread beyond those affected by the initial attack?
- Would an attack based on this capability be expected to be lethal or incapacitating?
- Could an attack based on this capability have psychological effects or affect the functioning of the targeted group? For example, could it incite fear, create panic, and/or allow the takeover of a particular region or infrastructure?
- What might the duration of the impact be?

- In what environment(s) might the agent be used?
- Could the agent become established in domestic animals or agricultural livestock (e.g., plague in cats) or wildlife, causing longer-term effects on humans and requiring difficult and costly eradication?

Usability as a Weapon—Predictability of Results

- Does the agent need to be tested extensively to confirm that it is efficacious?
- Is there a relevant animal model for the agent? How predictable is that model for human infection by the same agent?
- What is the fidelity of the technology? How reproducibly can a particular result be obtained?
- Are there known engineering strategies or preexisting research outlining methods to predictably produce the desired result? Can the properties of a bioagent be modeled with computational tools?
- Is there knowledge regarding the evolutionary stability of an engineered pathogen or pathway? For example, is it likely a synthetic construct will mutate to increase or decrease functionality or activity? Or can slow-evolving pathogens be generated to avoid attenuation?

Requirements of Actors—Access to Expertise

- How common and widespread is the technical expertise needed to exploit the necessary technology, and could expertise in another, related area suffice?
- Would expertise in more than one area be required to pursue the capability, and would the range of technological expertise likely require a group of people to provide the expertise?
- Would developing this capability require or be enhanced by interaction with the legitimate research community, or could it be performed autonomously?

Requirements of Actors—Access to Resources

- What are the equipment costs, and how quickly are equipment costs decreasing?
- Are cheaper versions of the necessary technology becoming available, and are they robust enough to raise concerns?
- Can reagents be acquired from multiple vendors, or is there a secondary market (e.g., eBay) where the equipment can be acquired at a lower cost?
- What are the material or reagent costs?
- What is the shelf life of the required reagents?
- What are the labor costs? Is specialized training required, and if so, what are the costs involved in that training?
- What are the maintenance or service costs, and how frequently is maintenance or service needed?
- What facility costs are associated with the necessary technology (e.g., special plumbing, cooling, airflow, filtration, vibration isolation)?
- What is the biosafety risk to the actor, and what costs might the actor incur to protect the safety of those doing the work?
- What would it cost to conceal the pursuit of this capability from authorities (or other nations)?

Requirements of Actors—Organizational Footprint Requirements

- What is the organizational footprint (e.g., equipment and other laboratory infrastructure, personnel) needed to utilize the necessary technology?
- Is the infrastructure required to use this technology widespread or rare?
- Could existing organizations or infrastructure be leveraged to develop this capability (e.g., dual use of legitimate biotechnology infrastructure), or would the work require a secret facility with a particular set of infrastructure requirements?

- If additional infrastructure would be required for malicious use, would it require an incremental increase in capacity or major additions?

Potential for Mitigation—Deterrence and Prevention Capabilities

- Can the development of this capability be controlled or prevented through regulation or other means, either in the United States or internationally? Do nations have agreements relevant to applicable regulations?
- Is the necessary technology geographically centralized or widely distributed?

Potential for Mitigation—Capability to Recognize an Attack

- To what degree can beneficial and malicious use of the technology involved in this capability be distinguished?
- Are there particular activities or equipment associated with this technology that may indicate when it is being used to prepare for an attack?
- Could the capability be used to engineer an agent that evades typical disease surveillance methodologies (e.g., to cause an unusual constellation of symptoms)?
- Could the capability be used to engineer an agent that evades typical identification and characterization methodologies (e.g., to create an agent that lacks the phenotypes or DNA sequence used for laboratory identification)?
- Would it be possible to assess whether the agent was created synthetically, as opposed to emerging naturally?
- Could the capability enable targeting of particular subpopulations, and if so, could this targeting be detected with available disease surveillance mechanisms?
- Could environmental surveillance (e.g., direct sensing via BioWatch or similar approaches, animal sentinels, sensing without direct contact [standoff detection]) provide earlier warning of a bioweapon attack than waiting for ill individuals to present in the public health system?
- Can mining social media in real time provide indications of when and where an attack or outbreak based on this capability might take place, compared to traditional public health surveillance mechanisms?

Potential for Mitigation—Attribution Capabilities

- How feasible would it be to use DNA sequencing to compare samples of the agent with samples from recovered evidence?
- Would the technique used to construct or modify the agent leave a genomic "scar" that could potentially be used as evidence?
- Would it be possible to identify a design "signature" linking the use of this technology with a given group or laboratory?
- Would the development of this capability be associated with certain physical properties that could be used to compare samples of the agent with samples from recovered evidence?

Potential for Mitigation—Consequence Management Capabilities

- Will existing civilian and military public health infrastructure and mitigation approaches to minimize morbidity and mortality be effective against an attack using this capability?
- Are there currently effective medical countermeasures available for an attack using this capability, or would it be possible to quickly develop vaccines, drugs, or antitoxins to mitigate the spread and impact of the agent over the longer term?
- Would the effectiveness of those mitigation approaches rely on knowing how an agent was created?
- Would it be possible to understand the genotype, phenotype, or chemical composition of the agent to inform how its effect can be mitigated?

Appendix D

Committee Biographies

Michael Imperiale, (*Chair*), Ph.D., is the Arthur F. Thurnau Professor and Associate Chair of Microbiology and Immunology at the University of Michigan Medical School. Dr. Imperiale's research focuses on the molecular biology of the small DNA tumor virus BK polyomavirus and specifically on how the virus traffics through the cell and interacts with the host intrinsic immune functions. Dr. Imperiale is a previous member of the National Science Advisory Board for Biosecurity and has been deeply involved in the policy discussion regarding the potential risks and benefits of gain-of-function research. In 2010, he was elected as a Fellow of the American Academy of Microbiology and was named a Fellow of the American Association for the Advancement of Science in 2011. He is the founding editor-in-chief of *mSphere* and also serves as an editor for *mBio*. In addition to his laboratory research, Dr. Imperiale is involved in science policy. He serves on the Committee on Science, Technology, and Law at the National Academies of Sciences, Engineering, and Medicine and previously served on the Planetary Protection Subcommittee at NASA. Dr. Imperiale received his B.A., M.A., and Ph.D. from Columbia University, all in biological sciences.

Patrick Boyle, Ph.D., is the head of design at Ginkgo Bioworks, a Boston-based synthetic biology company that makes and sells engineered organisms. Dr. Boyle's team provides design tools and synthetic biology expertise to Ginkgo's organism engineers and is an integral part of Ginkgo's Design, Build, Test, and Ferment strategy for organism engineering. Dr. Boyle has extensive hands-on experience with the day-to-day applications of synthetic biology, as well as with working within the existing regulatory structure surrounding synthetic biology. Dr. Boyle received his Ph.D. in biological and biomedical sciences from Harvard Medical School.

Peter A. Carr, Ph.D., is a senior scientist at the Massachusetts Institute of Technology's Lincoln Laboratory, where he leads the synthetic biology research program. His research interests span genome engineering, rapid prototyping of both hardware and wetware, DNA synthesis and error correction, risk evaluation, and biodefense. Dr. Carr is the director of judging for the International Genetically Engineered Machine (iGEM) competition and is deeply knowledgeable about both the practice and potential implications of synthetic biology, with a special focus on the potential impacts on biodefense. Dr. Carr received his bachelor's degree in biochemistry from Harvard and his Ph.D. in biochemistry and molecular biophysics from Columbia University.

Douglas Densmore, Ph.D., is associate professor in the Department of Electrical and Computer Engineering and a Hariri Institute for Computing and Computational Science and Engineering Faculty Fellow, both at Boston University. His research focuses on the development of tools for the specification, design, and assembly of synthetic biological systems, drawing upon his experience with embedded system-level design and electronic design automation. He is the director of the Cross-disciplinary Integration of Design Automation Research group at Boston University, where his team of staff and postdoctoral researchers, undergraduate interns, and graduate students develops computational and experimental tools for synthetic biology. He is the lead investigator for the National Science Foundation Expeditions "Living Computing Project" and a senior member of the Institute of Electrical and Electronics Engineers and the Association for Computing Machinery. Dr. Densmore received his Ph.D. in electrical engineering from the University of California, Berkeley.

Diane DiEuliis, Ph.D., is a senior research fellow at National Defense University (NDU). Her research areas focus on emerging biological technologies, biodefense, and preparedness for biological threats. Dr. DiEuliis also studies issues related to dual-use research, disaster recovery research, and behavioral, cognitive, and social science as it relates to important aspects of deterrence and preparedness. Prior to joining NDU, Dr. DiEuliis was the deputy director for policy in the Office of the Assistant Secretary for Preparedness and Response, U.S. Department of Health and Human Services. Dr. DiEuliis also previously served in the Office of Science and Technology Policy at the White House and was a program director at the National Institutes of Health. Dr. DiEuliis has broad knowledge about the policy implications of emerging technologies, as well as the intricacies that accompany instituting new policies to regulate such emerging technologies. Dr. DiEuliis received her Ph.D. in biological sciences from the University of Delaware.

Andrew Ellington, Ph.D., is the Fraser Professor of Biochemistry at the University of Texas at Austin. Dr. Ellington's research focuses on the development and evolution of artificial life, including nucleic acid operating systems that can function both in vitro and in vivo. His laboratory aims to "[reduce] synthetic biology . . . to an engineering discipline rather than a buzzword." Dr. Ellington has received the Office of Naval Research Young Investigator Award, Cottrell Award, and Pew Scholar Award. He has advised numerous government agencies on biodefense and biotechnology issues and was recently named a National Security Science and Engineering Faculty Fellow. He was also recently named a Fellow of the American Academy of Microbiology and of the American Association for the Advancement of Science. Dr. Ellington has also helped found the aptamer companies Archemix and b3 Biosciences, and has an intimate understanding of both the academic and commercial sides of synthetic biology, as well as the challenges to both. Dr. Ellington earned his Ph.D. in biochemistry and molecular biology from Harvard.

Gigi Kwik Gronvall, Ph.D., is a senior associate at the Johns Hopkins Center for Health Security and visiting faculty at the Johns Hopkins Bloomberg School of Public Health. An immunologist by training, Dr. Gronvall's work addresses how scientists can diminish the threat of biological weapons and how they can contribute to an effective response against a biological weapon or a natural epidemic. Dr. Gronvall is the author of the 2016 book *Synthetic Biology: Safety, Security, and Promise* (Health Security Press). She is a member of the Threat Reduction Advisory Committee, which provides the Secretary of Defense with independent advice and recommendations on reducing the risk to the United States, its military forces, and its allies and partners posed by nuclear, biological, chemical, and conventional threats. Dr. Gronvall has testified before Congress on topics relating to biosafety and biosecurity and is widely regarded as an expert on the role of scientists in health and national security matters. Dr. Gronvall earned her Ph.D. from Johns Hopkins University.

Charles Haas, Ph.D., is the L.D. Betz Professor of Environmental Engineering and head of the Department of Civil, Architectural, and Environmental Engineering at Drexel University. His broad research interests include the estimation of human health risks from environmental exposures to pathogens and their control using engineering interventions and drinking water treatment. Dr. Haas is broadly knowledgeable in the field of risk assessment, particularly in the context of complex and interdependent systems. Dr. Haas previously served as co-director of the Center for Advancing Microbial Risk Assessment, which was jointly funded by the U.S. Department of

Homeland Security and the U.S. Environmental Protection Agency. Dr. Haas has served on a number of National Academies committees, including serving as chair of the Committee to Review Risk Assessment Approaches for the Medical Countermeasures Test and Evaluation Facility at Fort Detrick, Maryland. Dr. Haas received his Ph.D. in environmental engineering from the University of Illinois at Urbana-Champaign.

Joseph Kanabrocki, Ph.D., is the associate vice president for research safety and professor of microbiology in the Biological Sciences Division of the University of Chicago. Dr. Kanabrocki is tasked with instilling a culture that focuses on the health and well-being of all university personnel engaged in research activities. Dr. Kanabrocki is an expert in biosafety and biosecurity issues, especially practical ones arising from day-to-day laboratory work due to his appointment as biological safety officer and select agent responsible official for the University of Chicago. Dr. Kanabrocki is a member of the National Institutes of Health Recombinant DNA Advisory Committee and currently a member of the National Science Advisory Board for Biosecurity (NSABB). Dr. Kanabrocki served as co-chair of the NSABB Working Group that produced the 2016 report *Recommendations for the Evaluation and Oversight of Proposed Gain-of-Function Research*. Dr. Kanabrocki received his Ph.D. in microbiology from the University of South Dakota School of Medicine.

Kara Morgan, Ph.D., is a principal at Quant Policy Strategies, LLC. Her work in public health policy analysis includes developing and evaluating data-driven decision support tools to support effective risk management decision making. She has worked extensively on risk assessment and, in particular, on how results from risk assessments can be effectively integrated into decision-making processes. Prior to founding Quant Policy Strategies, Dr. Morgan was a research leader at Battelle Memorial Institute. Prior to that position, Dr. Morgan worked at the U.S. Food and Drug Administration (FDA) in several advisory and leadership positions for 10 years. Through her work supporting the National Nanotechnology Initiative during her time at FDA, in 2005 she published one of the first articles to establish a framework for informing risk analysis about nanoparticles. Her research in expert elicitation, decision analysis, and risk analysis has led to numerous publications developing and applying risk frameworks to decision making about microbial food safety and the pharmaceutical manufacturing quality. She is an adjunct professor at the John Glenn College for Public Affairs at Ohio State University and serves as an appointed member of the State Board of Education in Ohio. Dr. Morgan received her Ph.D. in engineering and public policy from Carnegie Mellon University.

Kristala Jones Prather, Ph.D., is the Arthur D. Little Professor of Chemical Engineering at the Massachusetts Institute of Technology (MIT). Her research interests are centered on the engineering of recombinant microorganisms for the production of small molecules, especially focusing on the design and assembly of biological pathways to target compounds and the incorporation of novel control strategies for regulation of metabolism. Prior to joining MIT's faculty, Dr. Prather worked in Bioprocess Research and Development at Merck Research Laboratories. She has received numerous awards, including a position on the MIT Technology Review's TR35, a list of innovators under the age of 35; the National Science Foundation's Faculty Early Career Development (CAREER) award; and the *Biochemical Engineering Journal* Young Investigator Award. Dr. Prather has been recognized for excellence in teaching at MIT with several awards, including the School of Engineering's Junior Bose Award for Excellence in Teaching, and through appointment as a MacVicar Faculty Fellow, the highest honor given for undergraduate teaching at MIT. Dr. Prather received her Ph.D. from the University of California, Berkeley.

Thomas Slezak, M.S., is an associate program leader at Lawrence Livermore National Laboratory. Mr. Slezak is a computer scientist and manages a team of biologists and software engineers to find innovative solutions for diagnosing and characterizing dangerous pathogens. Mr. Slezak's team has developed PCR assays, pan-microbial microarrays (recently commercialized by Affymetrix), and DNA sequence analysis software to support a broad range of pathogen detection and forensic programs in biodefense and human and animal health. Mr. Slezak co-chaired a Blue Ribbon Panel on bioinformatics for the U.S. Centers for Disease Control and Prevention that led to new funding for the Advanced Molecular Detection program, and was a developer of the nationwide BioWatch system. Mr. Slezak has served on three National Academies' panels on biodefense topics, as well as on the National

Academies' Standing Committee on Biodefense Programs to Advise the Department of Defense. Mr. Slezak received his M.S. in computer science at the University of California, Davis.

Jill Taylor, Ph.D., is the director of the New York State Department of Health Wadsworth Center and a faculty member of the Wadsworth School of Laboratory Sciences. The Wadsworth Center is the only research-intensive public health laboratory in the nation, and Dr. Taylor has served as its director, deputy director, and interim director for the past 12 years. Dr. Taylor previously served as the director of the Wadsworth Center's Clinical Virology Program, which focused on introducing molecular technologies to ensure responsiveness to the state's changing public health needs, with particular emphasis on influenza virus. She also contributes to policy discussions at the national level as a member of the Board of Scientific Counselors of the U.S. Centers for Disease Control's Office of Infectious Diseases and as a member of the Board of Regents of the National Library of Medicine. Dr. Taylor is well versed in developing future research agendas and analysis of new policy proposals and their implications. Dr. Taylor received her Ph.D. from the University of Queensland, Australia.

Appendix E

Disclosure of Conflict of Interest

The conflict-of-interest policy of the National Academies of Sciences, Engineering, and Medicine (www.nationalacademies.org/coi) prohibits the appointment of an individual to a committee such as the one that authored this Consensus Study Report if the individual has a conflict of interest that is relevant to the task to be performed. An exception to this prohibition is permitted only if the National Academies determine that the conflict is unavoidable and the conflict is promptly and publicly disclosed.

When the committee that authored this report was established, a determination of whether there was a conflict of interest was made for each committee member given the individual's circumstances and the task being undertaken by the committee. A determination that an individual has a conflict of interest is not an assessment of that individual's actual behavior or character or ability to act objectively despite the conflicting interest.

Dr. Patrick Boyle was determined to have a conflict of interest because he is an employee of Ginkgo Bioworks.

The National Academies determined that the experience and expertise of Dr. Boyle was needed for the committee to accomplish the task for which it was established. The National Academies could not find another available individual with the equivalent experience and expertise who did not have a conflict of interest. Therefore, the National Academies concluded that the conflict was unavoidable and publicly disclosed it through the National Academies Current Projects System (www8.nationalacademies.org/cp).

Appendix F

Study Methods

COMMITTEE COMPOSITION

The National Academies of Sciences, Engineering, and Medicine (the National Academies) appointed a committee of 13 experts to undertake the statement of task. Members provide the perspectives of academia, industry, government, and the nonprofit sector and have experience in synthetic biology, biosafety, microbiology, public health, bioinformatics, and risk assessment. Appendix D provides the biographical information for each committee member.

MEETINGS AND INFORMATION GATHERING

The committee deliberated from approximately January 2017 to February 2018. To respond to its charge, the committee gathered information and data relevant to its statement of task by conducting a review of available literature and other publicly available resources, inviting experts to share perspectives at public meetings, and soliciting public comments online and in person. The study was conducted in two phases. In Phase 1 of the study, the committee met several times in person and held webinars to gather information, understand the needs of the relevant federal agencies, and develop a tool for assessing the biodefense threat to guide the study's second phase. During this phase, the committee defined the type of framework that would guide the assessment of concerns, identified major categories of relevant technologies and applications to assess, and discussed the factors to include in the assessment. In Phase 2, the committee met additional times and incorporated further input and data gathering to refine the framework for assessing potential biodefense concerns. It applied this framework to analyze specific potential applications of synthetic biology and to identify current areas of concern created by synthetic biology.

Over the course of the study, the committee held seven meetings in Washington, D.C., and Irvine, California. Three of these seven meetings included an open information-gathering component. During these open meetings, the committee heard from a variety of academic and private-sector researchers, as well as federal government officials. These meetings focused on understanding the current and near-term research being conducted in the field of synthetic biology and relevant adjacent scientific fields, understanding the current operations and research occurring within the federal government, understanding the existing concerns of biodefense and biosecurity professionals, and enlisting the assistance of these academics and professionals to scan the horizon for potential future technol-

ogy developments and emerging threats. The remaining four meetings were closed to the public and served as time for the committee members to deliberate and write their report. The three open meetings are detailed below.

The first open meeting, held January 26–27, 2017, in Washington, D.C., provided an opportunity for the committee to discuss the study charge with the sponsor, as well as relevant needs of nonsponsor government agencies. The committee also heard a general overview of synthetic biology, a report out on previous work that had been performed by the President's Council of Advisors on Science and Technology and the JASON advisory group relevant to this study, and a presentation from another group that had done risk analyses and framework development for the U.S. Department of Defense.

The second meeting, held May 24–25, 2017, in Washington, D.C., included speakers who reviewed relevant aspects and current research on DNA synthesis, assembly, and engineering; on virus engineering, transmissibility, and zoonosis; on the idea of "ease of use" and its applicability to potential risks arising from synthetic biology; and an exercise in horizon-scanning and looking to the future.

The third meeting, held July 6–7, 2017, in Washington, D.C., included speakers who presented on the current state of public health and military preparedness; on efficacy of design in synthetic biology, focusing on what is truly possible and what is still not possible; on the current state of human modulation; and on emerging technologies that might assist or abet overcoming existing technical barriers.

The committee also held two public webinars. The first was held March 10, 2017, and included talks on how to approach creating a strategic framework to assess the potential risks of synthetic biology, as well as a review of some of the objectives and accomplishments of the biological weapons program of the Soviet Union.

The second webinar was held March 23, 2017, and included a talk on a review of prior attempts at frameworks and strategies to assess potential risks of synthetic biology. Both of these webinars were advertised and open to the public, although the committee did not accept questions or comments from the public during these webinars because their primary purpose was to serve as information-gathering activities for the committee.

PUBLIC COMMUNICATION

The committee's two largest data-gathering meetings, in May and July 2017, provided opportunities to interact with additional stakeholders, including interested researchers and other parties. These participants contributed their views during open discussions following speaker presentations. The committee also worked to make its activities as transparent and accessible as possible for those who may not have been able to attend in person. The study website, http://nas-sites.org/dels/studies/strategies-for-identifying-and-addressing-vulnerabilities-posed-by-synthetic-biology, was updated regularly to reflect the recent and planned activities of the committee. Study outreach included a study-specific e-mail address for submitting comments and questions to the committee.

Following the release of the study's interim report in August 2017, the study committee requested input from the public via an online survey. The survey was distributed widely through existing National Academies mailing lists, through the social and professional networks of the study committee, and through the Engineering Biology Research Consortium's mailing list. Public comments were collected, and the committee members reviewed all comments and incorporated relevant and applicable commentary into their work on the final report.

Any information provided to the committee from outside sources or through the online comment tool is available by request through the National Academies' Public Access Records Office.

Invited Speakers

The following individuals were invited speakers at meetings and data-gathering sessions of the committee:

Chris Anderson
University of California, Berkeley

Polina Anikeeva
Massachusetts Institute of Technology

Ralph Baric
University of North Carolina

Kavita Berger
Gryphon Scientific

Ronald Breaker
Yale University

Roger Brent
Fred Hutchinson Cancer Research Center

Tom Burkett
Baltimore Underground Science Space

Sarah Carter
Science Policy Consulting

Rocco Casagrande
Gryphon Scientific

Christophoer Chyba
Princeton University

Susan Coller-Monarez
Department of Homeland Security

Patrik D'haeseleer
Lawrence Livermore National Laboratory

Drew Endy
Stanford University

Gerald L. Epstein
Office of Science and Technology Policy

Aaron P. Esser-Kahn
University of California, Irvine

Carolyn M. Floyd
Office of the Director of National Intelligence

John Glass
J. Craig Venter Institute

D. Christian Hassell
U.S. Department of Defense

Michael Jewett
Northwestern University

CDR Franca Jones
Armed Forces Health Surveillance Center

Lawrence Kerr
U.S. Department of Health and Human Services

Gregory Koblentz
George Mason University

George Korch
U.S. Department of Health and Human Services

Sriram Kosuri
University of California, Los Angeles

Jens H. Kuhn
NIH/NIAID Integrated Research Facility at Fort Detrick

Todd Kuiken
North Carolina State University

Devin Leake
Ginkgo Bioworks

Monique Mansoura
Massachusetts Institute of Technology

Corey Meyer
Gryphon Scientific

Paul Miller
Synlogic

Piers Millett
Biosecure, Ltd.

Steve Monroe
U.S. Centers for Disease Control and Prevention

Richard Murray
California Institute of Technology

Megan Palmer
Stanford University

Colin Parrish
Cornell University

Amy Rasley
Lawrence Livermore National Laboratory

Ryan Ritterson
Gryphon Scientific

Howard Salis
Pennsylvania State University

Dan Tawfik
Weizmann Institute of Science, Israel

Luke Vandenberghe
Harvard University

Harry Yim
Genomatica

Received: 2 September 2020 | Revised: 16 October 2020

DOI: 10.1002/bies.202000240

BioEssays WILEY

PROBLEMS & PARADIGMS

Prospects & Overviews

The genetic structure of SARS-CoV-2 does not rule out a laboratory origin

SARS-COV-2 chimeric structure and furin cleavage site might be the result of genetic manipulation

Rossana Segreto[1] | **Yuri Deigin**[2]

[1] Department of Microbiology, University of Innsbruck, Innsbruck, Austria

[2] Youthereum Genetics Inc., Toronto, Ontario, Canada

Correspondence
Rossana Segreto, Department of Microbiology, University of Innsbruck, Technikerstraße 25, 6020 Innsbruck, Austria.
Email: Rossana.Segreto@uibk.ac.at

No external funding was received for this work.

Rossana Segreto and Yuri Deigin contributed equally to this study.

Abstract

Severe acute respiratory syndrome-coronavirus (SARS-CoV)-2′s origin is still controversial. Genomic analyses show SARS-CoV-2 likely to be chimeric, most of its sequence closest to bat CoV RaTG13, whereas its receptor binding domain (RBD) is almost identical to that of a pangolin CoV. Chimeric viruses can arise *via* natural recombination or human intervention. The furin cleavage site in the spike protein of SARS-CoV-2 confers to the virus the ability to cross species and tissue barriers, but was previously unseen in other SARS-like CoVs. Might genetic manipulations have been performed in order to evaluate pangolins as possible intermediate hosts for bat-derived CoVs that were originally unable to bind to human receptors? Both cleavage site and specific RBD could result from site-directed mutagenesis, a procedure that does not leave a trace. Considering the devastating impact of SARS-CoV-2 and importance of preventing future pandemics, researchers have a responsibility to carry out a thorough analysis of all possible SARS-CoV-2 origins.

KEYWORDS

BtCov/4991, furin cleavage site, Gain-of-function studies, pangolin CoV, RaTG13, receptor binding domain, SARS-CoV-2

INTRODUCTION

Nearly a year has passed since the outbreak of severe acute respiratory syndrome-coronavirus 2 (SARS-CoV-2) in Wuhan, China, and its origin is still controversial. Despite the international research effort conducted, a natural host, either direct or intermediate, has not yet been identified. The hypothesis that the Wuhan Huanan Seafood Wholesale Market was the first source for animal–human virus transmission has now been conclusively dismissed[i] and the few market samples that were collected showed only human-adapted SARS-CoV-2, with no traces of zoonotic predecessor strains[ii]. Almost all scientific papers published to date purport that SARS-CoV-2 has a natural origin, and the only published paper considering possible a lab origin[1] focuses on serial passage as the technique that could justify SARS-CoV-2 special

[i] Areddy, J. T. (**2020**). China rules out animal market and lab as coronavirus origin. *The Wall Street Journal*. https://www.wsj.com/articles/china-rules-out-animal-market-and-lab-as-coronavirus-origin-11590517508 (last accessed on Oct 15, 2020).

[ii] Zhan, S. H., Deverman, B. E., Chan, Y. A. (**2020**). SARS-CoV-2 is well adapted for humans. What does this mean for re-emergence? *BioRxiv*. https://doi.org/10.1101/2020.05.01.073262 (last accessed on Oct 15, 2020).

adaptation to human cells. We here describe how the two main SARS-CoV-2 features, (1) the presence of a furin cleavage site missing in other CoVs of the same group and (2) an receptor binding domain (RBD) optimized to bind to human cells[2] might be the result of lab manipulation techniques such as site-directed mutagenesis. The acquisition of both unique features by SARS-CoV-2 more or less simultaneously is less likely to be natural or caused only by cell/animal serial passage.

SARS-COV-2'S CLOSEST RELATIVES ARE BAT AND PANGOLIN CORONAVIRUSES

Zhou et al.[3] from the Wuhan Institute of Virology (WIV) were the first to identify and characterize a new coronavirus (CoV), SARS-CoV-2. The genomic sequences obtained from early cases shared 79% sequence identity to the CoVs that caused severe acute respiratory syndrome (SARS-CoV) in 2002–2003 and 96.2% sequence identity to RaTG13 (MN996532), a CoV sequence detected from a *Rhinolophus affinis* bat. RaTG13 is currently the closest phylogenetic relative for SARS-CoV-2 found,[4] but its complete genomic sequence was not published before the outbreak of SARS-CoV-2 and the original sample was collected in the Yunnan province (China) by the same group of WIV researchers in 2013. Zhou et al.[3] stated to have found a match between SARS-CoV-2 and a short region of RNA-dependent RNA polymerase (RdRp) of a CoV in their database and then fully sequenced the original sample collected in 2013, which they called RaTG13.

We discovered that the RdRp of RaTG13 has 100% nucleotide identity with the sequence BtCoV/4991 (KP876546), which was identified by Ge et al.[5] in a *Rhinolophus affinis* bat in the Yunnan province in 2013, same location and year as RaTG13. BtCoV/4991 was collected in a mine colonized by bats near Tongguanzhen, Mojiang, Yunnan. The WIV researchers were invited to investigate the mine after six miners there had contracted severe pneumonia in 2012[iii], and three of the miners have died.[6] The miners have been tasked with clearing out bat droppings in the mine, and the severity of their pneumonia correlated with the duration of exposure to the mine.[7] Four miners' samples subsequently underwent testing at WIV, where Immunoglobulin G (IgG) antibodies against SARS were identified in all samples.[8] Considering that only about 5300 people were infected in mainland China during the SARS outbreak of 2002–2004, most of whom resided in Guandong, the odds of four miners in Yunnan retaining antibodies from the 2002–2004 SARS outbreak are negligible. On the other hand, it is possible that the SARS antibody test administered to the miners cross-reacted with a novel SARS-like bat virus that the miners had acquired at the mine. Ge et al.[5] have identified a number of CoVs in the mine, but based on the phylogenetic analysis, BtCoV/4991 was the only SARS-related strain, clearly separated from all known alpha- and beta-CoVs at that time. BtCoV/4991 was also different from other bat CoVs in the phylogenetic analysis carried out by Wang et al. in 2019.[9] Chen et al.[10] identified BtCoV/4991 as the closest sequence to SARS-CoV-2 because RaTG13 had not yet been published at that time. BtCoV/4991 and RaTG13 have been later asserted to be two different coding names of the same strain, as their original authors at WIV registered the two strains as one entry in the Database of Bat-associated Viruses (DBatVir).[iv]

In late July 2020, Zhengli Shi, the leading CoV researcher from WIV, in an email interview [11] asserted the renaming of the RaTG13 sample and unexpectedly declared that the full sequencing of RaTG13 has been carried out as far back as in 2018 and not after the SARS-CoV-2 outbreak, as stated in Zhou et al.[3] The reversal in WIV's stance on when exactly RaTG13 was fully sequenced could have been due to the discovery by independent researchers into the origins of SARS-CoV-2 that the filenames of the raw sequencing reads deposited by WIV on May 19, 2020[v] seem to indicate that sequencing for RaTG13 was done in 2017 and 2018.[vi] However, no formal erratum about year of sequencing and sample renaming from the authors of Zhou et al. [3] has yet appeared, or as far as is currently known, has been submitted.

The second non-human RdRp sequence closest to BtCoV/4991 (91.89% nucleotide identity) is the CoV sequence MP789 (MT084071) isolated in 2019 in a Malaysian pangolin (*Manis javanica*) from the Guangdong province (GD), China.[12] The envelope protein of MP789 shows surprisingly 100% aminoacidic identity with the corresponding protein in RaTG13, in bat-SL-CoVZXC21 (MG772934.1), in bat-SL-CoVZC45 (MG772933.1) and in some early SARS-CoV-2 isolates (e.g. YP_009724392).[13] The envelope protein of CoVs is involved in critical aspects of the viral lifecycle, such as viral entry, replication and pathogenesis.[14]

BAT COVS HAVE BEEN THOROUGHLY STUDIED AND GENETICALLY MANIPULATED

Many studies have reported that bats are natural reservoirs for a broad diversity of potentially pathogenic SARS-like CoVs.[15,16] Some of these viruses can potentially directly infect humans[17], whereas others need to mutate their spike protein in order to effectively bind to the human angiotensin 1-converting enzyme 2 (hACE2) receptor and mediate virus entry.[18] In order to evaluate the emergence potential of novel CoVs, researchers have created a number of chimeric CoVs, consisting of bat CoV backbones, normally unable to infect human cells, whose spike proteins were replaced by those from CoVs compatible with human ACE2. These chimeras were meant to simulate recombination events that might occur in nature.[19,20] Such gain-of-function experiments have raised a number of biosafety concerns and stirred controversy among researchers and the general public. One of the main arguments in favor of gain-of-function studies is the need to be prepared with an arsenal of drugs and vaccines for the next pandemic.[21]

[iii] Qiu, J. (**2020**). How China's 'Bat Woman' hunted down viruses from SARS to the new coronavirus. *Sci. Am.* https://www.scientificamerican.com/article/how-chinas-bat-woman-hunted-down-viruses-from-sars-to-the-new-coronavirus1/ (last accessed on Oct 15, 2020).

[iv] DBatVir – *The Database of Bat-Associated Viruses*. http://www.mgc.ac.cn/cgi-bin/DBatVir/main.cgi?func=accession&acc=MN996532 (last accessed on Oct 15, 2020).

[v] SRX8357956: amplicon sequences of RaTG13. https://www.ncbi.nlm.nih.gov/sra/SRX8357956 (last accessed on Oct 15, 2020).

[vi] Anon. (**2020**). Names of the RaTG13 amplicon sequences. https://web.archive.org/web/20200918174030/https://graph.org/RaTG13-Amplicon-Names-07-03 (last accessed on Oct 15, 2020).

By contrast, one of the main arguments against them is that the next pandemic itself could be caused by those experiments, due to the risk of lab escape.[22,23]

In recent years, the field of corona-virology had been focused on pan-CoV therapies and vaccines, as evident from research conducted in the past 5 years,[24–27] as well as from media reports.[vii] Synthetically generating diverse panels of potential pre-emergent CoVs was declared a goal of active grants for the EcoHealth Alliance, which funded some of such research at WIV, in collaboration with laboratories in the USA and other international partners.[viii]

CREATING CHIMERIC COVS WITH NOVEL RBDS HAS GONE ON FOR DECADES

Researchers have been generating chimeric CoVs for over two decades, long before the advent of modern sequencing or genetic engineering techniques. For example, in 1999, a group from Utrecht University used targeted RNA recombination to create a "cat-and-mouse" CoV chimera: the RBDs of a feline and murine CoV were swapped, demonstrating that this exchange swapped also species tropism during *in vitro* experiments.[28]

In 2007, the Shi group at WIV created a series of "bat-man" CoV chimeric spike proteins while trying to determine what exactly confers CoVs the ability to jump from one species to another. The researchers used different segments of the spike protein of the human SARS virus to replace corresponding segments in the spike protein of a bat viral backbone. It was concluded that a relatively short region (aa 310 to 518) of the spike protein "was necessary and sufficient to convert Rp3-S into a huACE2-binding molecule,"[29] that is to provide the bat CoV spike protein with a novel ability of binding to a human ACE2 receptor.

In 2008, the Baric group at the University of North Carolina (UNC) took the WIV research one step further: instead of using human immunodeficiency viruses (HIV) pseudo-viruses with bat CoV spike proteins, a live chimeric CoV was created. Following the experiments of their 2007 WIV colleagues, the Baric group used a bat SARS-like CoV as a backbone and replaced its RBD with the RBD from human SARS.[30]

In 2015, the Shi and Baric groups joined forces and published probably the most famous gain-of-function virology paper, which described the creation of another synthetic chimeric virus.[19] This time the RBD of a mouse-adapted SARS backbone (SARS-MA15) was replaced by the RBD of RsSHC014, a bat strain previously isolated from Yunnan bats in 2011 by the Shi group. In 2016, the Baric group repeated their 2015 experiment using the same SARS-MA15 backbone and the RBD from Rs3367,[31] a close relative of RsSHC014 also previously found in Yunnan by WIV and renamed "WIV1" after live culturing.[17]

Probably the largest reported number of novel chimeric viruses created was described in a 2017 paper from the Shi group at WIV,[15] in which the authors reported creating eight chimeric viruses using WIV1 as a backbone and transplanting into it various RBDs from bat SARS-like viruses. These viruses were collected over a span of 5 years from the same cave near Kunming, Yunnan Province, where the Shi group originally found Rs3367 and RsSHC014. Only two of the eight live chimeric viruses were successfully rescued, and those two strains were found to possess the ability to bind to the human ACE2 receptor, as confirmed by experiments in hACE2-expressing HeLa cells and RT-PCR quantification of viral RNA.

SARS-COV-2 SHARES ITS RBD WITH A PANGOLIN COV

The possibility that pangolins could be the intermediate host for SARS-CoV-2 has long been under discussion. [32–34] The biggest divergence between SARS-CoV-2 and RaTG13 is observed in the RBD of their spike proteins.[4] Although its overall genome similarity is lower to SARS-CoV-2 than that of RaTG13, the MP789 pangolin strain isolated from GD pangolins has an almost identical RBD to that of SARS-CoV-2. Indeed, pangolin CoVs and SARS-CoV-2 possess identical amino acids at the five critical residues of the RBD, whereas RaTG13 only shares one amino acid with SARS-CoV-2.[35] ACE2 sequence similarity is higher between humans and pangolins than between humans and bats. Intriguingly, the spike protein of SARS-CoV-2 has a higher predicted binding affinity to human ACE2 receptor than to that of pangolins and bats.[ix] Before the SARS-CoV-2 outbreak, pangolins were the only mammals other than bats documented to carry and be infected by SARS-CoV-2 related CoV.[12] Recombination events between the RBD of CoV from pangolins and RaTG13-like backbone could have produced SARS-CoV-2 as chimeric strain. For such recombination to occur naturally, the two viruses must have infected the same cell in the same organism simultaneously, a rather improbable event considering the low population density of pangolins and the scarce presence of CoVs in their natural populations.[x] Moreover, receptor binding studies of reconstituted RaTG13 showed that it does not bind to pangolin ACE2.[xi]

THE FURIN CLEAVAGE SITE: THE KEY DIFFERENCE BETWEEN SARS-COV-2 AND ITS CLOSEST RELATIVE RATG13

SARS-CoV-2 differs from its closest relative RaTG13 by a few key characteristics. The most striking difference is the acquisition in the

[vii] Kahn, J. (**2020**). How scientists could stop the next pandemic before it starts. *NYT* Magazine. https://www.nytimes.com/2020/04/21/magazine/pandemic-vaccine.html (last accessed on Oct 15, 2020).

[viii] Project Number 2R01AI110964-06, *ECOHEALTH ALLIANCE, INC*., https://projectreporter.nih.gov/project_info_description.cfm?aid=9819304&icde=49645421&ddparam=&ddvalue=&ddsub=&cr=1&csb=default&cs=ASC&pball= (last accessed on Oct 15, 2020).

[ix] Piplani, S., Singh, P. K., Winkler, D. A., Petrovsky, N. (**2020**). In silico comparison of spike protein-ACE2 binding affinities across species; significance for the possible origin of the SARS-CoV-2 virus.*arXiv*. http://arxiv.org/abs/2005.06199 (last accessed on Oct 15, 2020).

[x] Lee, J., Hughes, T., Lee, M.-H., Field, H., Rovie-Ryan, J. J., Sitam, F. T., … Daszak, P. (**2020**). No evidence of coronaviruses or other potentially zoonotic viruses in Sunda pangolins (*Manis javanica*) entering the wildlife trade *via* Malaysia. *BioRxiv*. https://doi.org/10.1101/2020.06.19.158717 (last accessed on Oct 15, 2020).

[xi] Mou, H., Quinlan, B. D., Peng, H., Guo, Y., Peng, S., Zhang, L., … Farzan, M. (**2020**). Mutations from bat ACE2 orthologs markedly enhance ACE2-Fc neutralization of SARS-CoV-2. *BioRxiv*. https://doi.org/10.1101/2020.06.29.178459 (last accessed on Oct 15, 2020).

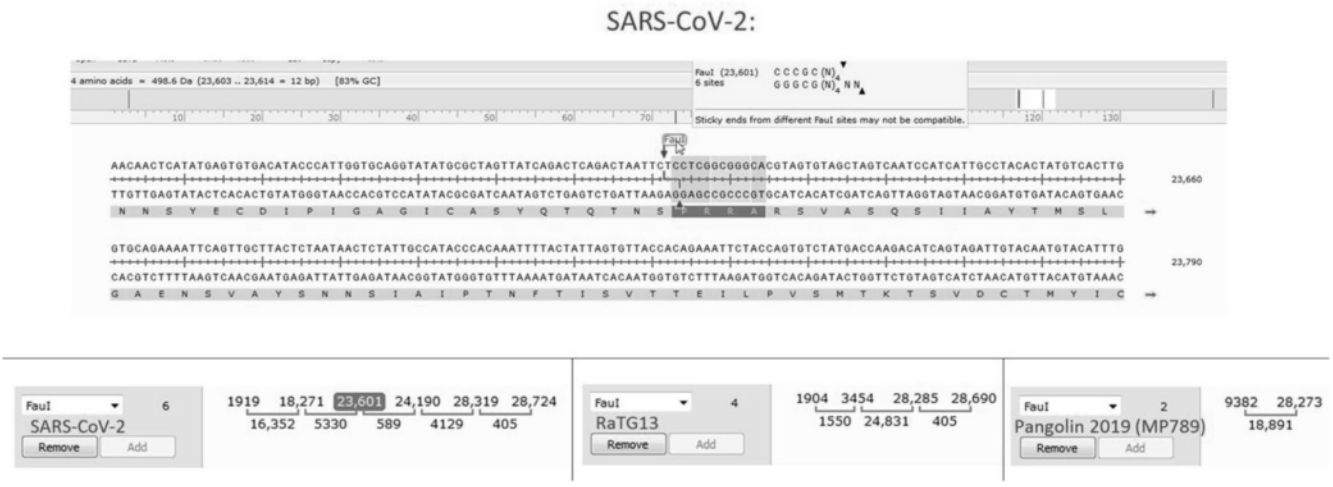


FIGURE 1 Nucleotide sequence of the S protein at the S1/S2 junction in SARS-CoV-2 (NC045512.2) showing the furin cleavage site (in blue) that includes a *FauI* enzyme restriction site

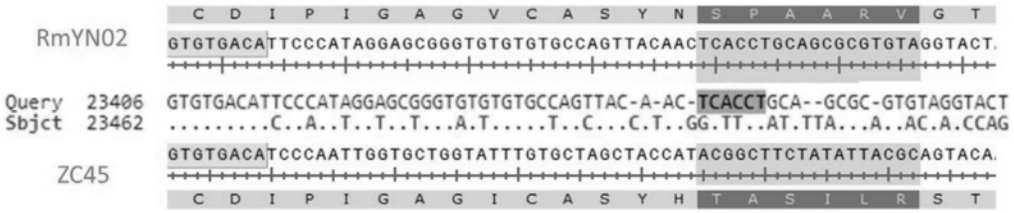


FIGURE 2 Alignment of nucleotide and amino acid sequences of the S protein from bat-SL-CoVZC45 (MG772933.1) and RmYN02 at the S1/S2 junction site. No insertions of nucleotides possibly evolving in a furin cleavage site can be observed (in blue)

spike protein of SARS-CoV-2 of a cleavage site activated by a host-cell enzyme furin, previously not identified in other beta-CoVs of lineage b[36] and similar to that of Middle East respiratory syndrome (MERS) coronavirus.[35] Host protease processing plays a pivotal role as a species and tissue barrier and engineering of the cleavage sites of CoV spike proteins modifies virus tropism and virulence.[37] The ubiquitous expression of furin in different organs and tissues have conferred to SARS-CoV-2 the ability to infect organs usually invulnerable to other CoVs, leading to systemic infection in the body.[38] Cell-cultured SARS-CoV-2 that was missing the above-mentioned cleavage site caused attenuated symptoms in infected hamsters,[39] and mutagenesis studies have confirmed that the polybasic furin site is essential for SARS-CoV-2′s ability to infect human lung cells.[40]

The polybasic furin site in SARS-CoV-2 was created by a 12-nucleotide insert TCCTCGGCGGGC coding for a PRRA amino acid sequence at the S1/S2 junction (Figure 1). Interestingly, the two joint arginines are coded by two CGGCGG codons, which are rare for these viruses: only 5% of arginines are coded by CGG in SARS-CoV-2 or RaTG13, and CGGCGG in the new insert is the only doubled instance of this codon in SARS-CoV-2. The CGGCGG insert includes a *FauI* restriction site, of which there are six instances in SARS-CoV-2 and four instances in RaTG13 (and two in MP789). The serendipitous location of the *FauI* site could allow using restriction fragment length polymorphism (RFLP) techniques[41] for cloning[42] or screening for mutations,[43] as the new furin site is prone to deletions *in vitro*.[39,44]

A study by Zhou et al.[45] reported the discovery of a novel CoV strain RmYN02, which the authors claim exhibits natural PAA amino acid insertions at the S1/S2 cleavage site where SARS-CoV-2 has the PRRA insertion. However, upon close examination of the underlying nucleotide sequence of RmYN02 in comparison with its closest ancestors bat-SL-CoVZC45 and bat-SL-CoVZXC21, no insertions are apparent, just nucleotide mutations (Figure 2).

Therefore, SARS-CoV-2 remains unique among its beta CoV relatives not only due to a polybasic furin site at the S1/S2 junction, but also due to the four amino acid insert PRRA that had created it. The insertion causes a split in the original codon for serine (TCA) in MP789 or RaTG13 to give part of a new codon for serine (TCT) and part of the amino acid alanine (GCA) in SARS-CoV-2 (Figure 3).

The insertion of the furin cleavage site in SARS-CoV-2 is not in frame with the rest of the sequence, when compared with the MP789 and the RaTG13 sequences (Figure 3). Therefore, it is possible to exclude that such insertion could have originated by polymerase slippage or by releasing and repriming, because insertion mutations generated by these mechanisms have been postulated to maintain the reading frame of the viral sequence.[46] The possibility that the furin cleavage site could have been acquired by recombination has been recently

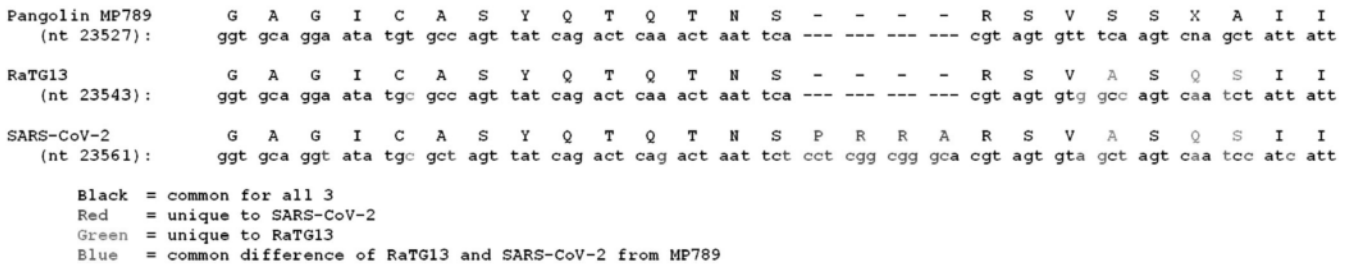


FIGURE 3 Alignment of nucleotide and amino acid sequences of the S protein from RaTG13 (MN996532), MP789 (MT084071) and SARS-CoV-2 (NC045512.2) at the S1/S2 site. The common nucleotides and amino acids are given in black, SARS-CoV-2 unique nucleotides and amino acids in red, RaTG13 unique nucleotides and amino acids in green and common nucleotides and amino acids in SARS-CoV-2 and RaTG13 that differ in MP789 in blue. The codon forserine (TCA) in RaTG13 and MP789 is split in SARS-CoV-2 to give part of a new codon forserine (TCT) and part of the amino acidalanine (GCA)

questioned by Seyran et al.,[47] because the SARS-CoV-2 spike protein seems to lack any further recombination event in contrast with the recombination model of other CoVs.

CRITIQUE OF "THE PROXIMAL ORIGIN OF SARS-COV-2"

Due to the broad-spectrum of research conducted over almost 20 years on bat SARS-CoVs justified by their potential to spill over from animal to human,[48] a possible synthetic origin by laboratory engineering of SARS-CoV-2 cannot be excluded. The widely cited article of Andersen et al.[2] stated that SARS-CoV-2 has most likely a natural origin. The main argument brought by the authors is that the high-affinity binding of the SARS-CoV-2 spike protein to hACE2 could not have been predicted by models based on the RBD of SARS-CoV. Based on the structural analysis conducted by Wan et al.,[49] SARS-CoV-2 has the potential to recognize hACE2 more efficiently than the SARS-CoV, which emerged in 2002. Moreover, generation of CoV chimeric strains has recently demonstrated that bat CoV spikes can bind to the hACE2 receptor with more plasticity than previously predicted.[15] All amino acids in the RBD have been extensively analyzed and new models to predict ACE2 affinity are available.[50] In this regard, BatCoV Rs3367 (99.9% identity to WIV1) has been shown to share with SARS-CoV-2 four out of six critical residues in the RBD. Considering that WIV1 was shown to directly bind to hACE2, the same assumption could easily have been made about SARS-CoV-2 RBD.[51]

As described above, creation of chimeric viruses has been carried out over the years with the purpose of studying the potential pathogenicity of bat CoVs for humans. In this context, SARS-CoV-2 could have been synthesized by combining a backbone similar to RaTG13 with the RBD of CoV similar to the one recently isolated from pangolins[12], because the latter is characterized by a higher affinity with the hACE2 receptor. Such research could have aimed to identify pangolins as possible intermediate hosts for bat-CoV potentially pathogenic for humans. Subsequent serial cell or animal passage, as described by Sirotkin & Sirotkin [1] could have provided the perfect adaptation of the RBD to the hACE2.

Regarding the furin cleavage site, Andersen et al.[2] state that "the functional consequence of the polybasic cleavage site in SARS-CoV-2 is unknown." New studies from several groups have lately identified this activation site as possibly enabling the virus to spread efficiently between humans and attack multiple organs.[52] Experiments on proteolytic cleavage of CoV spike proteins have been recently suggested as future key studies to understand virus transmissibility in different hosts.[50]

Andersen et al.[2] also state, based on the work of Almazan et al.[53] that "the genetic data irrefutably show that SARS-CoV-2 is not derived from any previously used virus backbone." In the last 6 years before the outbreak of SARS-CoV-2 the number of potential bat backbones has been undeniably increased by several bat CoV screenings, last but not least bringing RaTG13 to scientific attention in January 2020. Other possible backbones could, as well, still wait for publication.

Andersen et al.[2] affirm that "the acquisition of both the polybasic cleavage site and predicted O-linked glycans also argues against culture-based scenarios." Methods for insertion of a polybasic cleavage site in infectious bronchitis CoV are given in Cheng et al.[54] and resulted in increased pathogenicity. Concerning the predicted O-linked glycans around the newly inserted polybasic site, it should be noted that this prediction was not confirmed by Cryo-EM inquiry into the SARS-CoV-2 spike glycoprotein.[55] Nevertheless, while it is true that O-linked glycans are much more likely to arise under immune selection, they could be added in the lab through site-directed mutagenesis[56] or arise in the course of *in vivo* experiments, for example, in BLT-L mice with human lung implants and autologous human immune system[57] or in mice expressing the hACE2 receptor.[31] To overcome problems of bat CoV isolation, experiments based on direct inoculation of bat CoV in suckling rats have been carried out.[58] Humanized mice, ferrets, primates and/or other animals with similar ACE2 conformation could have all been used for serial passage experiments, as described in detail by Sirotkin and Sirotkin.[1]

Andersen et al.[2] also state that "subsequent generation of a polybasic cleavage site would have then required repeated passage in cell culture or animals with ACE2 receptors similar to those of humans, but such work has also not previously been described." It should not be excluded that such experiments could have been aborted due to the

SARS-CoV-2 outbreak, before a possible publication of the results or that the results were never intended to be published.

It is important to mention that RaTG13 and the pangolin CoV sequences from smuggled pangolins confiscated in the GD province in March 2019, and to which most of published papers supporting a natural origin of SARS-CoV-2 refer,[2] have recently been questioned as to the accuracy of their assembly data[xii] and require further analyses to prove their correctness.[xiii,xiv] It should also be noted that *in vitro* receptor binding studies of reconstituted RaTG13 yielded some peculiar results.[xi] The most surprising observation was that RaTG13, unlike SARS-CoV-2, is unable to bind ACE2 in *R. macrotis* bats, a close relative of RaTG13's purported host, *R. affinis*[59] (whose ACE2 receptor has not yet been tested). At the same time, RaTG13 was observed to bind hACE2[60], but not as well as ACE2 of rats and mice, to which SARS-CoV-2 did not bind at all. Is it possible that just as SARS-MA15 was a mouse-adapted strain of SARS, RaTG13 is actually a mouse-adapted version of a CoV extracted from the Mojiang cave, rather than a strain obtained from a bat fecal swab? Unfortunately, the RaTG13 sample has been exhausted and it is no longer available for external examination,[11] which is unfortunate given a number of inconsistencies in its sequencing raw data. Also, the status and availability of the Mojiang miners' samples remain as well an open and highly relevant question. Several samples from the miners have been collected[7,8] and likely stored, and it would be of great value to test them for the presence of SARS-CoV-2-like CoVs.

Another open question is the reason for modification and subsequent deletion of WIV's own viral database. In May 2020, several media outlets have reported that the change tracking system of WIV's internal database showed that the database was renamed from "Wildlife-borne viral pathogen database" to "Bat and rodent-borne viral pathogen database," and its description was edited to replace instances of "wild animal" by "bat and rodent"; in addition, mention of "arthropod vectors" was deleted.[xv] The database description reported that it contained over 60 Mb of data in structured query language (SQL) format, but at as of early May 2020 the download link no longer worked.[xvi] Subsequently, the database page was taken down in its entirety but its snapshot is still available on Web Archive.[xvii] It is possible that other international CoV labs might have downloaded the SQL archive of the WIV database before it was taken down, in which case such groups should make those data publicly available.

HOW COULD THE VIRUS HAVE ESCAPED FROM A LAB?

The leak of highly dangerous pathogens from laboratories is not a rare event and occurrences have been documented in several countries. The most notable lab leak known is the 1977 H1N1 lab escape from China that caused a worldwide pandemic.[61] The most recent one is the November 2019 outbreak of brucellosis that occurred in two research centers in Lanzhou, China, infecting over 100 students and staff members.[62] Several lab escapes of the first SARS virus have been reported as well: in the summer of 2003 in Singapore,[63] then in December 2003 in Taiwan,[xviii] and in the spring of 2004 twice in China.[xix]

Concerns about WIV's lab safety were raised in 2018 by U.S. Embassy officials after visiting the Institute and having an interview with Zhengli Shi. The lab auditors summarized their worries in subsequent diplomatic cables to Washington.[xx] Chinese experts have also raised concerns about lab safety in their own country, lamenting that "lab trash can contain man-made viruses, bacteria or microbes" and that "some researchers discharge laboratory materials into the sewer after experiments without a specific biological disposal mechanism."[xxi]

American labs have also had their share of safety issues. Recently, research operations in the Biosafety level (BSL)-4 United States Army Medical Research Institute of Infectious Diseases (USAMRIID) facility in Fort Detrick were interrupted in August 2019 following safety violations, in particular, relating to the disposal of infective materials.[xxii] Other US labs have been cited for safety issues as well.[22]

A number of scenarios causing SARS-CoV-2 to leak from a lab can be hypothesized. For example, an infected animal could have escaped from a lab or it could have scratched or bitten a worker (a concern raised in 2017 about the establishment of a BSL-4 primate vaccine testing facility in Kunming, Yunnan[64]), or a researcher could have accidentally stuck themselves with inoculate (as happened in two cases in Russia[xxiii]). Until 2020, CoVs were not considered particularly deadly or virulent. SARS-like CoVs did not require BSL-4 and could be manipulated under BSL-2 and BSL-3[42] conditions, making an accidental leak more likely. Aerosol experiments with CoVs[65] could result in lab leak as well, because a failure in the equipment used could go unnoticed for a long time before infection of lab workers is detected. Finally, the virus

[xii] Zhang, D. (2020). Anomalies in BatCoV/RaTG13 sequencing and provenance. *Zenodo*. https://zenodo.org/record/3969272 (last accessed on Oct 15, 2020).

[xiii] Singla, M., Ahmad, S., Gupta, C., Sethi, T. (**2020**). De novo assembly of RaTG13 genome reveals inconsistencies further obscuring SARS-CoV-2 origins. *Preprints*. https://doi.org/10.20944/preprints202008.0595.v1 (last accessed on Oct 12, 2020).

[xiv] Chan, Y. A., Zhan, S. H. (**2020**). Single source of pangolin CoVs with a near identical spike RBD to SARS-CoV-2. *BioRxiv*. https://doi.org/10.1101/2020.07.07.184374 (last accessed on Oct 15, 2020).

[xv] Devine, M. (**2020**). What is China covering up about the coronavirus? *NYT* Magazine. https://nypost.com/2020/05/06/what-is-china-covering-up-about-the-coronavirus-devine/ (last accessed on Oct 12, 2020).

[xvi] https://twitter.com/ydeigin/status/1259891518468427776 (last accessed on Oct 15, 2020).

[xvii] Bat and rodent-borne viral pathogen database. https://web.archive.org/web/20200529174243/http://csdata.org/p/308/ (last accessed on Oct 15, 2020).

[xviii] Reuters (**2003**). SARS case confirmed in Taiwan. *Wired*. https://www.wired.com/2003/12/sars-case-confirmed-in-taiwan/ (last accessed on Oct 13, 2020).

[xix] Walgate, R. (**2004**). SARS escaped Beijing lab twice. *The Scientist Magazine*. https://www.the-scientist.com/news-analysis/sars-escaped-beijing-lab-twice-50137 (last accessed on Oct 15, 2020).

[xx] Rogin, J. (**2020**). State Department cables warned of safety issues at Wuhan lab studying bat coronaviruses. *The Washington Post*. https://www.washingtonpost.com/opinions/2020/04/14/state-department-cables-warned-safety-issues-wuhan-lab-studying-bat-coronaviruses/ (last accessed on Oct 15, 2020).

[xxi] Caiyu, L., Shumei, L. (**2020**). Biosafety guideline issued to fix chronic management loopholes at virus labs. *Global Times*. https://www.globaltimes.cn/content/1179747.shtml (last accessed on Oct 15, 2020).

[xxii] Grady, D. (**2020**). Deadly germ research is shut down at army lab over safety concerns. *NYT* Magazine. https://www.nytimes.com/2019/08/05/health/germs-fort-detrick-biohazard.html (last accessed on Oct 15, 2020).

[xxiii] Miller, J. (**2004**). Russian scientist dies in Ebola accident at former weapons Lab. *NYT* Magazine. https://www.nytimes.com/2004/05/25/world/russian-scientist-dies-in-ebola-accident-at-former-weapons-lab.html (last accessed on Oct 15, 2020).

could potentially have leaked through the sewage system if proper waste disposal and/or decontamination procedures were not followed.

CONCLUSIONS AND OUTLOOK

On the basis of our analysis, an artificial origin of SARS-CoV-2 is not a baseless conspiracy theory that is to be condemned[66] and researchers have the responsibility to consider all possible causes for SARS-CoV-2 emergence. The insertion of human-adapted pangolin CoV RBD obtained by cell/animal serial passage and furin cleavage site could arise from site-directed mutagenesis experiments, in a context of evolutionary studies or development of pan-CoV vaccines or drugs. A recent article in Nature[67] affirms that a laboratory origin for SARS-CoV-2 cannot be ruled out, as researchers could have been infected accidentally, and that gain-of-function experiments resulting in SARS-CoV-2 could have been performed at WIV. Genetic manipulation of SARS-CoV-2 may have been carried out in any laboratory in the world with access to the backbone sequence and the necessary equipment and it would not leave any trace. Modern technologies based on synthetic genetics platforms allow the reconstruction of viruses based on their genomic sequence, without the need of a natural isolate.[68]

A thorough investigation on strain collections and research records in all laboratories involved in CoV research before SARS-CoV-2 outbreak is urgently needed. Special attention should be paid to strains of CoVs that were generated in virology laboratories but have not yet been published, as those possibly described in the deleted WIV database. Because finding a possible natural host could take years, as with the first SARS,[67] or never succeed, equal priority should be given to investigating natural and laboratory origins of SARS-CoV-2.

Xiao Qiang, a research scientist at Berkeley, recently stated: "To understand exactly how this virus has originated is critical knowledge for preventing this from happening in the future."[xxi]

ACKNOWLEDGMENTS

We are very grateful to Prof. Allan Krill (NTNU) for proof reading the manuscript, all the valuable comments and being open-minded about controversial hypotheses; Prof. Heribert Insam (Head of the Department of Microbiology; University of Innsbruck) for his support and Dr. Lawrence Sellin for all the useful information. A special thanks goes to Dr. Fernando Castro-Chavez (former Post-Doc at the New York Medical College) for his support with Research Gate. We are very thankful to René Bergelt, for having discovered the database that confirmed our finding that BtCoV4991 and RaTG13 refer to the same sample. Finally, we are extremely grateful to members of the D.R.A.S.T.I.C. (Decentralised Radical Autonomous Search Team Investigating COVID-19) Twitter group for all their work in uncovering many previously unpublished facts about SARS-CoV-2 and its relative strains. In particular, we are grateful to Luigi Warren for continuously probing the possible connection of the 2012 Mojiang pneumonia outbreak to WIV and SARS-CoV-2, to @TheSeeker268 for finding Chinese-language 2013 Xu MSc and 2016 Huang PhD theses, which have confirmed the SARS-like viral nature of the 2012 Mojiang pneumonia outbreak and have elucidated WIV's role in investigating that outbreak,[xxiv] including WIV's collection of the 4991/RaTG13 strain from the Mojiang mine, and to Francisco de Asis de Ribera Martin for providing us the English translation of the two theses, and also discovering the RaTG13 amplicon dates.

CONFLICT OF INTEREST

Rossana Segreto and Yuri Deigin do not have any conflicts of interest.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

ORCID

Rossana Segreto https://orcid.org/0000-0002-2566-7042

REFERENCES

1. Sirotkin, K., & Sirotkin, D. (2020). Might SARS-CoV-2 have arisen *via* serial passage through an animal host or cell culture? A potential explanation for much of the novel coronavirus' distinctive genome. *BioEssays*, *42*, 1-7. https://doi.org/10.1002/bies.202000091
2. Andersen, K. G., Rambaut, A., Lipkin, W. I., Holmes, E. C., & Garry, R. F. (2020). The proximal origin of SARS-CoV-2. *Nat. Med.*, *26*, 450-452. https://doi.org/10.1038/s41591-020-0820-9
3. Zhou, P., Yang, X. L., Wang, X. G., Hu, B., Zhang, L., Zhang, W., … Shi, Z. L. (2020). A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature*, *579*, 270-273. https://doi.org/10.1038/s41586-020-2012-7
4. Cagliani, R., Forni, D., Clerici, M., & Sironi, M. (2020). Computational inference of selection underlying the evolution of the novel coronavirus, severe acute respiratory syndrome coronavirus 2. *J. Virol.*, *94*, 1-11. https://doi.org/10.1128/jvi.00411-20
5. Ge, X. Y., Wang, N., Zhang, W., Hu, B., Li, B., Zhang, Y. Z., … Shi, Z. L. (2016). Coexistence of multiple coronaviruses in several bat colonies in an abandoned mineshaft. *Virol. Sin.*, *31*, 31-40. https://doi.org/10.1007/s12250-016-3713-9
6. Wu, Z., Yang, L., Yang, F., Ren, X., Jiang, J., Dong, J., … Jin, Q. (2014). Novel henipa-like virus, mojiang paramyxovirus, in rats, China, 2012. *Emerg. Infect. Dis.*, *20*, 1064-1066. https://doi.org/10.3201/eid2006.131022
7. Xu, L. (2013). The analysis of 6 patients with severe pneumonia caused by unknown viruses (Master's Thesis). Kunming Medical University, Emergency Medicine (professional degree). http://eng.oversea.cnki.net/Kcms/detail/detail.aspx?filename=1013327523.nh&dbcode=CMFD&dbname=CMFD2014
8. Huang, C. (2016). Novel virus discovery in bat and the exploration of receptor of bat coronavirus HKU9 (PhD Thesis). Chinese Center for Disease Control and Prevention. http://eng.oversea.cnki.net/kcms/detail/detail.aspx?dbcode=CDFD&dbname=CDFDLAST2018&filename=1017118517.nh
9. Wang, N., Luo, C., Liu, H., Yang, X., Hu, B., Zhang, W., … Shi, Z. (2019). Characterization of a new member of alphacoronavirus with unique genomic features in *Rhinolophus* bats. *Viruses*, *11*(4), 379. https://doi.org/10.3390/v11040379
10. Chen, L., Liu, W., Zhang, Q., Xu, K., Ye, G., Wu, W., … Liu, Y. (2020). RNA based mNGS approach identifies a novel human coronavirus from two individual pneumonia cases in 2019 Wuhan outbreak. *Emerg. Microbes Infect.*, *9*, 313-319. https://doi.org/10.1080/22221751.2020.1725399

[xxiv] https://twitter.com/ydeigin/status/1262686286898397189 (last accessed on Oct 15, 2020).

11. Cohen, J. (2020). Wuhan coronavirus hunter Shi Zhengli speaks out. *Science*, *369*, 487-488. https://doi.org/10.1126/science.369.6503.487
12. Liu, P., Chen, W., & Chen, J. P. (2019). Viral metagenomics revealed sendai virus and coronavirus infection of malayan pangolins (*Manis javanica*). *Viruses*, *11*(11), 979. https://doi.org/10.3390/v11110979
13. Bianchi, M., Benvenuto, D., Giovanetti, M., Angeletti, S., Ciccozzi, M., & Pascarella, S. (2020). Sars-CoV-2 envelope and membrane proteins: Structural differences linked to virus characteristics? *Biomed. Res. Int.*, *2020*. https://doi.org/10.1155/2020/4389089
14. Schoeman, D., & Fielding, B. C. (2019). Coronavirus envelope protein: Current knowledge. *Virol. J.*, *16*, 1-22. https://doi.org/10.1186/s12985-019-1182-0
15. Hu, B., Zeng, L. P., Yang, X. Lou, Ge, X. Y., Zhang, W., Li, B., … Shi, Z. L. (2017). Discovery of a rich gene pool of bat SARS-related coronaviruses provides new insights into the origin of SARS coronavirus. *PLoS Pathog.*, *13*, 1-27. https://doi.org/10.1371/journal.ppat.1006698
16. Fan, Y., Zhao, K., Shi, Z. L., & Zhou, P. (2019). Bat coronaviruses in China. *Viruses*, *11*(3), 210-. https://doi.org/10.3390/v11030210
17. Ge, X. Y., Li, J. L., Yang, X. Lou, Chmura, A. A., Zhu, G., Epstein, J. H., … Shi, Z. L. (2013). Isolation and characterization of a bat SARS-like coronavirus that uses the ACE2 receptor. *Nature*, *503*, 535-538. https://doi.org/10.1038/nature12711
18. Graham, R. L., & Baric, R. S. (2010). Recombination, reservoirs, and the modular spike: Mechanisms of coronavirus cross-species transmission. *J. Virol.*, *84*, 3134-3146. https://doi.org/10.1128/jvi.01394-09
19. Menachery, V. D., Yount, B. L., Debbink, K., Agnihothram, S., Gralinski, L. E., Plante, J. A., … Baric, R. S. (2015). A SARS-like cluster of circulating bat coronaviruses shows potential for human emergence. *Nat. Med.*, *21*, 1508-1513. https://doi.org/10.1038/nm.3985
20. Johnson, B. A., Graham, R. L., & Menachery, V. D. (2018). Viral metagenomics, protein structure, and reverse genetics: Key strategies for investigating coronaviruses. *Virology*, *517*, 30-37. https://doi.org/10.1016/j.virol.2017.12.009
21. Racaniello, V. (2016). Moving beyond metagenomics to find the next pandemic virus. *PNAS*, *113*, 2812-2814. https://doi.org/10.1073/pnas.1601512113.
22. Weiss, S., Yitzhaki, S., & Shapira, S. C. (2015). Lessons to be learned from recent biosafety incidents in the United States. *Isr. Med. Assoc. J.*, *17*, 269-273. https://doi.org/10.1073/pnas.1601512113
23. Casadevall, A., & Imperiale, M. J. (2014). Risks and benefits of gain-of-function experiments with pathogens of pandemic potential, such as influenza virus: A call for a science-based discussion. *MBio*, *5*, 1-5. https://doi.org/10.1128/mBio.01730-14
24. Agostini, M. L., Andres, E. L., Sims, A. C., Graham, R. L., Sheahan, T. P., Lu, X., … Denison, M. R. (2018). Coronavirus susceptibility to the antiviral remdesivir (GS-5734) is mediated by the viral polymerase and the proofreading exoribonuclease. *MBio*, *9*, 1-15. https://doi.org/10.1128/mBio.00221-18
25. Xia, S., Liu, M., Wang, C., Xu, W., Lan, Q., Feng, S., … Lu, L. (2020). Inhibition of SARS-CoV-2 (previously 2019-nCoV) infection by a highly potent pan-coronavirus fusion inhibitor targeting its spike protein that harbors a high capacity to mediate membrane fusion. *Cell Res.*, *30*, 343-355. https://doi.org/10.1038/s41422-020-0305-x
26. Totura, A. L., & Bavari, S. (2019). Broad-spectrum coronavirus antiviral drug discovery. *Expert Opin. Drug Discov.*, *14*, 397-412. https://doi.org/10.1080/17460441.2019.1581171
27. Wang, Y., Sun, Y., Wu, A., Xu, S., Pan, R., Zeng, C., … Guo, D. (2015). Coronavirus nsp10/nsp16 Methyltransferase can be targeted by nsp10-derived peptide in vitro and in vivo to reduce replication and pathogenesis. *J. Virol.*, *89*, 8416-8427. https://doi.org/10.1128/jvi.00948-15
28. Kuo, L., Godeke, G. J., Raamsman, M. J. B., Masters, P. S., & Rottier, P. J. M. (2000). Retargeting of coronavirus by substitution of the spike glycoprotein ectodomain: Crossing the host cell species barrier. *J. Virol.*, *74*, 1393-1406. https://doi.org/10.1128/jvi.74.3.1393-1406.2000
29. Maier, H. J., Bickerton, E., & Britton, P. (2015). *Coronaviruses – Methods and protocols*. London: Humana Press.
30. Becker, M. M., Graham, R. L., Donaldson, E. F., Rockx, B., Sims, A. C., Sheahan, T., … Denison, M. R. (2008). Synthetic recombinant bat SARS-like coronavirus is infectious in cultured cells and in mice. *PNAS*, *105*, 19944-19949. https://doi.org/10.1073/pnas.0808116105
31. Menachery, V. D., Yount, B. L., Sims, A. C., Debbink, K., Agnihothram, S. S., Gralinski, L. E., … Baric, R. S. (2016). SARS-like WIV1-CoV poised for human emergence. *PNAS*, *113*, 3048-3053. https://doi.org/10.1073/pnas.1517719113
32. Li, X., Zai, J., Zhao, Q., Nie, Q., Li, Y., Foley, B. T., & Chaillon, A. (2020). Evolutionary history, potential intermediate animal host, and cross-species analyses of SARS-CoV-2. *J. Med. Virol.*, *92*, 602-611. https://doi.org/10.1002/jmv.25731
33. Lam, T. T. Y., Jia, N., Zhang, Y. W., Shum, M. H. H., Jiang, J. F., Zhu, H. C., … Cao, W. C. (2020). Identifying SARS-CoV-2-related coronaviruses in Malayan pangolins. *Nature*, *583*, 282-285. https://doi.org/10.1038/s41586-020-2169-0
34. Xiao, K., Zhai, J., Feng, Y., Zhou, N., Zhang, X., Zou, J. J., … Shen, Y. (2020). Isolation of SARS-CoV-2-related coronavirus from Malayan pangolins. *Nature*, *583*, 286-289. https://doi.org/10.1038/s41586-020-2313-x
35. Zhang, T., Wu, Q., & Zhang, Z. (2020). Probable pangolin origin of SARS-CoV-2 associated with the COVID-19 outbreak. *Curr. Biol.*, *30*, 1346-1351.E2. https://doi.org/10.1016/j.cub.2020.03.022
36. Coutard, B., Valle, C., de Lamballerie, X., Canard, B., Seidah, N. G., & Decroly, E. (2020). The spike glycoprotein of the new coronavirus 2019-nCoV contains a furin- like cleavage site absent in CoV of the same clade. *Antivir. Res.*, *176*, 104742. https://doi.org/10.1016/j.antiviral.2020.104742
37. Letko, M., Marzi, A., & Munster, V. (2020). Functional assessment of cell entry and receptor usage for SARS-CoV-2 and other lineage B betacoronaviruses. *Nat. Microbiol.*, *5*, 562-569. https://doi.org/10.1038/s41564-020-0688-y
38. Wang, Q., Qiu, Y., Li, J. Y., Zhou, Z. J., Liao, C. H., & Ge, X. Y. (2020). A unique protease cleavage site predicted in the spike protein of the novel pneumonia coronavirus (2019-nCoV) potentially related to viral transmissibility. *Virol. Sin.*, *35*, 337-339. https://doi.org/10.1007/s12250-020-00212-7
39. Lau, S., Wang, P., Mok, B. W., Zhang, A. J., Chu, H., Lee, A. C., … Chen, H. (2020). Attenuated SARS-CoV-2 variants with deletions at the S1 / S2 junction. *Emerg. Microbes Infect.*, *9*, 837-842. https://doi.org/10.1080/22221751.2020.1756700
40. Hoffmann, M., & Kleine-Weber, H. (2020). A multibasic cleavage site in the spike protein of SARS-CoV-2 is essential for infection of human lung cells. *Mol. Cell.*, *78*, 779-784.E5. https://doi.org/10.1016/j.molcel.2020.04.022
41. Kaundun, S. S., Marchegiani, E., Hutchings, S. J., & Baker, K. (2019). Derived polymorphic amplified cleaved sequence (dPACS): A novel PCR-RFLP procedure for detecting known single nucleotide and deletion – insertion polymorphisms. *Int. J. Mol. Sci.*, *20*(13), 3193. https://doi.org/10.3390/ijms20133193
42. Zeng, L. P., Gao, Y. T., Ge, X. Y., Zhang, Q., Peng, C., Yang, X. L., … Shi, Z. L. (2016). Bat severe acute respiratory syndrome-like coronavirus WIV1 encodes an extra accessory protein, ORFX, involved in modulation. *J. Virol.*, *90*, 6573-6582. https://doi.org/10.1128/JVI.03079-15
43. Khan, S. G., Muniz-Medina, V., Shahlavi, T., Baker, C. C., Inui, H., Ueda, T., … Kraemer, K. H. (2002). The human XPC DNA repair gene: Arrangement, splice site information content and influence of a single nucleotide polymorphism in a splice acceptor site on alternative splicing and function. *Nucleic Acids Res.*, *30*, 3624-3631. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC134237/.
44. Liu, Z., Zheng, H., Lin, H., Li, M., Yuan, R., Peng, J., … Lu, J. (2020). Identification of common deletions in the spike protein of severe acute

respiratory syndrome coronavirus 2. *J. Virol.*, *94*, e00790-20. https://doi.org/10.1128/JVI.00790-20
45. Zhou, H., Chen, X., Hu, T., Li, J., Song, H., Liu, Y., … Shi, W. (2020). A Novel bat coronavirus closely related to SARS-CoV-2 contains natural insertions at the S1/S2 cleavage site of the spike protein. *Curr. Biol.*, *30*, 2196-2203.E3. https://doi.org/10.1016/j.cub.2020.05.023
46. Steinhauer, D. A. (1999). Role of hemagglutinin cleavage for the pathogenicity of influenza virus. *Virology*, *258*, 1-20. https://doi.org/10.1006/viro.1999.9716
47. Seyran, M., Pizzol, D., Adadi, P., El-Aziz, T. M. A., Hassan, S. S., Soares, A., … Brufsky, A. M. (2020). Questions concerning the proximal origin of SARS-CoV-2. *J. Med. Virol.*, 03. https://doi.org/10.1002/jmv.26478
48. Wang, L. F., & Anderson, D. E. (2019). Viruses in bats and potential spillover to animals and humans. *Curr. Opin. Virol.*, *34*, 79-89. https://doi.org/10.1016/j.coviro.2018.12.007
49. Wan, Y., Shang, J., Graham, R., Baric, R. S., & Li, F. (2020). Receptor recognition by the novel coronavirus from Wuhan: An analysis based on decade-long structural studies of SARS coronavirus. *J. Virol.*, *94*(7), 1-9. https://doi.org/10.1128/jvi.00127-20
50. Cui, J., Li, F., & Shi, Z. L. (2019). Origin and evolution of pathogenic coronaviruses. *Nat. Rev. Microbiol.*, *17*, 181-192. https://doi.org/10.1038/s41579-018-0118-9
51. Fraguas Bringas, C., & Booth, D. (2020). Identification of a SARS-like bat coronavirus that shares structural features with the spike glycoprotein receptor-binding domain of SARS-CoV-2. *Access Microbiol.*, 10-17. https://doi.org/10.1099/acmi.0.000166.
52. Mallapati, S. (2020). Why does the coronavirus spread so easily between people? *Nature*, *579*, 183. https://www.nature.com/articles/d41586-020-00660-x.
53. Almazán, F., Sola, I., Zuñiga, S., Marquez-Jurado, S., Morales, L., Becares, M., & Enjuanes, L. (2014). Coronavirus reverse genetic systems: Infectious clones and replicons. *Virus Res.*, *189*, 262-270. https://doi.org/10.1016/j.virusres.2014.05.026
54. Cheng, J., Zhao, Y., Xu, G., Zhang, K., Jia, W., Sun, Y., … Zhang, G. (2019). The S2 subunit of QX-type infectious bronchitis coronavirus spike protein is an essential determinant of neurotropism. *Viruses*, *11*(10), 972. https://doi.org/10.3390/v11100972
55. Wrapp, D., Wang, N., Corbett, K. S., Goldsmith, J. A., Hsieh, C. L., Abiona, O., … McLellan, J. S. (2020). Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation. *Science*, *367*, 1260-1263. https://doi.org/10.1126/science.abb2507
56. Du, L., Tai, W., Yang, Y., Zhao, G., Zhu, Q., Sun, S., … Li, F. (2016). Introduction of neutralizing immunogenicity index to the rational design of MERS coronavirus subunit vaccines. *Nat. Commun.*, *7*, 1-9. https://doi.org/10.1038/ncomms13473
57. Wahl, A., De, C., Abad Fernandez, M., Lenarcic, E. M., Xu, Y., Cockrell, A. S., … Garcia, J. V. (2019). Precision mouse models with expanded tropism for human pathogens. *Nat. Biotechnol.*, *37*, 1163-1173. https://doi.org/10.1038/s41587-019-0225-9
58. Hu, D., Zhu, C., Ai, L., He, T., Wang, Y., Ye, F., … Wang, C. (2018). Genomic characterization and infectivity of a novel SARS-like coronavirus in Chinese bats. *Emerg. Microbes Infect.*, *7*, 1-10. https://doi.org/10.1038/s41426-018-0155-5
59. Hron, T., Farkašová, H., Gifford, R. J., Benda, P., Hulva, P., Görföl, T., … Elleder, D. (2018). Remnants of an ancient deltaretrovirus in the genomes of horseshoe bats (Rhinolophidae). *Viruses*, *10*(4), 185-. https://doi.org/10.3390/v10040185
60. Shang, J., Ye, G., Shi, K., Wan, Y., Luo, C., Aihara, H., … Li, F. (2020). Structural basis of receptor recognition by SARS-CoV-2. *Nature*, *581*, 221-224. https://doi.org/10.1038/s41586-020-2179-y
61. Wertheim, J. O. (2010). The re-emergence of H1N1 influenza virus in 1977: A cautionary tale for estimating divergence times using biologically unrealistic sampling dates. *PLoS ONE*, *5*, 2-5. https://doi.org/10.1371/journal.pone.0011184
62. Cyranoski, D. (2019). Chinese institutes investigate pathogen outbreaks in lab workers. *Nature*, https://www.nature.com/articles/d41586-019-03863-z
63. Lim, P. L., Kurup, A., Gopalakrishna, G., Chan, K. P., Wong, C. W., & Leo, Y. S. (2004). Laboratory-acquired severe acute respiratory syndrome. *N. Engl. J. Med.*, *350*, 1740-1745. https://doi.org/10.1056/NEJMoa032565
64. Cyranoski D. (2017). Inside the Chinese lab poised to study world's most dangerous pathogens. *Nature*, *542*, 399-401 https://doi.org/10.1038/nature.2017.21487
65. Totura, A., Livingston, V., Frick, O., Dyer, D., Nichols, D., & Nalca, A. (2020). Small particle aerosol exposure of African Green Monkeys to MERS-CoV as a model for highly pathogenic coronavirus infection. *Emerg. Infect. Dis.*, *26*. https://doi.org/10.3201/eid2612.201664.
66. Calisher, C., Carroll, D., Colwell, R., Corley, R. B., Daszak, P., Drosten, C., … Turner, M. (2019). Statement in support of the scientists, public health professionals, and medical professionals of China combatting COVID-19. *The Lancet*, *395*, E42-E43. https://doi.org/10.1016/S0140-6736(20)30418-9
67. Cyranoski, D. (2020). The biggest mystery: What it will take to trace the coronavirus source. *Nature*, https://doi.org/10.1038/d41586-020-01541-z.
68. Thao, T. T. N., Labroussaa, F., Ebert, N., V'kovski, P., Stalder, H., Portmann, J., … Thiel, V. (2020). Rapid reconstruction of SARS-CoV-2 using a synthetic genomics platform. *Nature*, *582*, 561-565. https://doi.org/10.1038/s41586-020-2294-9

How to cite this article: Segreto, R., & Deigin, Y. (2020). The genetic structure of SARS-CoV-2 does not rule out a laboratory origin. *BioEssays*, e2000240. https://doi.org/10.1002/bies.202000240.