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Review Article

Environment signal dependent biocontainment systems for engineered organisms: Leveraging triggered responses and combinatorial systems

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ABSTRACT

As synthetic biology advances, the necessity for robust biocontainment strategies for genetically engineered organisms (GEOs) grows increasingly critical to mitigate biosafety risks related to their potential environmental release. This paper aims to evaluate environment signal-dependent biocontainment systems for engineered organisms, focusing specifically on leveraging triggered responses and combinatorial systems. There are different types of triggers—chemical, light, temperature, and pH—this review illustrates how these systems can be designed to respond to environmental signals, ensuring a higher safety profile. It also focuses on combinatorial biocontainment to avoid consequences of unintended GEO release into an external environment. Case studies are discussed to demonstrate the practical applications of these systems in real-world scenarios.

1. Introduction

Synthetic biology is a discipline dedicated to engineering novel biological systems and redesigning existing biological pathways using molecular biology and computational modeling [1]. The field of synthetic biology has been shown to grow significantly in the past decades, and along with it, the use of genetically engineered organisms (GEOs) has become more relevant. The development of synthetic biology has accelerated through the development of techniques and assays such as CRISPR-Cas (Clustered Regularly Interspaced Short Palindromic Repeats) gene editing and microfluidics. Synthetic biology is used for applications such as drug production, precise diagnosis, and biofuel synthesis. Through its products, the field of synthetic biology has contributed to chemical engineering and public health [2]. Advanced strategies like multiplex biocontainment are frequently employed in bacteria such as *E. coli* but are less commonly applied to yeast [3]. Xenobiology, on the other hand, adopts a novel approach by engineering biological systems with distinct biochemistries to minimize the likelihood of gene transfer. Despite its promising potential, the development of fully integrated systems remains a challenging endeavor [4].

However, with the sudden growth of the field, concerns have also emerged. Concerns regarding biosafety, biosecurity, and ethics of synthetic biology have also increased. These concerns particularly pertain to the biosafety of synthetic biology, the concern for the escape of GEOs into the environment, unintended harm, and the possibility of horizontal gene-transfer. However, these concerns can be mitigated by implementing biocontainment measures [5]. Such measures are indispensable for safely integrating synthetic biology products and GEOs into the natural environment.

Research in biocontainment was pioneered at the Asilomar conference in 1976, which brought to light the need to incorporate genetic containment, biocontainment in bioremediation, and a multi-vectorial system for viral particle production [6]. However, the first biocontainment in clinical use was only in 2007, after which multilayered biocontainment and programmable biocontainment circuits became relevant.

GEOs, living entities capable of growth and self-replication, necessitate a highly resilient biocontainment system to avoid the inadvertent release of even a minute fraction of organisms that could potentially dominate an ecosystem. As per the National Institute of Health's guideline, a GEO escape rate below 1 in 10^8 cells is deemed to be acceptably safe, a standard that several biocontainment systems currently fulfill [7]. As the scale of GEO deployment continues to expand, the effectiveness of these systems in containment and/or

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extermination may prove inadequate in averting the accumulation of GEOs in the environment, accordingly the robustness and efficiency of these systems must be enhanced to ensure biosafety.

Biocontainment strategies have been meticulously developed to obstruct genetic self-replication, inducing synthetic auxotrophy, activating genetic circuits that culminate in the termination of the organism, and intercepting horizontal gene-transfer mechanisms [8]. There is an urgent need for improved, inducible cell "suicide" systems that encompass a diversity of "keys" and "locks" (induced apoptotic systems) [9]. Currently, only a few unique systems exist. The design for a kill switch in open environments is a challenging problem as there is a need for an absolute ON and OFF state.

"Kill switches" are characterized as synthetic mechanisms that lead to the demise of cells given specific circumstances. Numerous kill switches have been investigated for the confinement of modified microorganisms, albeit they inherently incorporate lethal genes that are activated under specified non-ideal conditions [10].

It is also crucial to highlight the development of artificial cell chassis that mitigate the risk of autonomous replication, thereby improving the controllability of their functions. One example is Cyborg cells, which consist of a synthetic polymer network that restricts natural cell division [11]. Even hydrogel encapsulation systems allow limited GEO escape while permitting intercellular communication inside the hydrogel-alginate system and protecting against unfavorable conditions like low pH or antibiotics [12]. Chromosome-free cells that relied primarily on the glycolysis pathway genes for longevity and inducible genetic circuits for targeted expression also showed promise in the non-replicating cell development studies [13]. However, this research is still in its infancy and the scalability and stability of such systems is uncertain. Thus, it is important to address crucial key aspects like the design of an efficient chassis, optimization of metabolic regulation and improvement in metabolic models to form a strong basis for the active use of artificial cell chassis.

This review paper aims to examine synthetic biology-based biocontainment strategies, focusing on kill switch mechanisms and combinatorial systems. It comprehensively overviews current methods, evaluates their efficacy and constraints, discusses the rationale for integrating combinatorial approaches, and analyzes real-world case studies. The paper also discusses various types of kill-switch mechanisms and their triggers, highlighting the advantages of combinatorial systems and presenting pertinent experimental approaches and case studies. Through this thorough analysis, we aim to shed light on the current state-of-the-art in biocontainment and inspire further innovation in this critical field.

2. Types of kill switches based on the trigger

Advances in synthetic biology have enabled the practical application of genetically engineered organisms (GEOs) in real-world scenarios. Biocontainment strategies have become increasingly important with these applications. Microorganisms evolve to remove genetic elements that hinder their growth. Kill switches are artificial systems that are activated under specific environmental conditions, leading to the expression of lethal genes, which eventually lead to the death of the cell. Kill switches that have a low expression of toxins in the required conditions are terminated by the growth of the microorganisms. Unlike multi-layered kill switches, which remain stable for several generations with the supply of survival factors from the external environment, most kill switches lose their functionality within a few days [14]. Different kill switch types have been developed based on the trigger mechanism.

2.1. Chemical induction

Chemically induced kill switch mechanisms for biocontainment include using CRISPR-based circuits and modular, reprogrammable genetic circuits. In one study, two CRISPR-based kill switches were engineered in *Escherichia coli* Nissle 1917, including a single-input chemical-responsive switch and a dual-input chemical and temperature-responsive switch [14]. Another study developed two safeguard systems, the "Deadman" and "Passcode" kill switches, which use unbalanced reciprocal transcriptional repression and hybrid LacI-GalR family transcription factors to control cell viability (Fig. 1a) [15]. These synthetic gene circuits efficiently kill *E. coli* and can be reprogrammed to change their environmental inputs and killing mechanism.

Toxin-antitoxin (TA) systems consist of two genes that encode a stable toxic protein targeting an essential cellular process and a labile antitoxin that prevents the activity of the toxin [16]. When the toxin is exhibited, it induces a bactericidal effect that eliminates the host cells (Fig. 1b). Type II systems can be prominently used as a target for kill switch mechanisms due to their ability to promote the stability of mobile genetic elements and their "selfishness" [17]. Toxins are proteinaceous in all cases, while antitoxins could be RNA or proteins. If the plasmid encoding the TA system is not inherited by a daughter cell, the antitoxin is degraded by cellular proteases and not replenished, liberating the latent toxin to kill the cell, and thereby diminishing the population of plasmid-free cells [18,19]. TA systems have no human analog and serve as ideal targets for antibacterial drugs by directly targeting the TA system's protein to prevent antitoxin inhibition of the toxin by disrupting or preventing complex formation. The bacteria can also be targeted by modulating the response of the TA expression or activation of proteases responsible for antitoxin degradation. Studying specific TA systems, identifying their respective cell stress responses, and activating programmed cell death can help us design the chemicals and small molecule inhibitors.

Promoter engineering techniques (Fig. 1c) can be utilized by involving specific genes sensitive to various chemical compounds to create a chemical-sensitive promoter [20,21]. Promoter engineering in prokaryotic organisms has been effectively demonstrated through the construction of synthetic promoters, particularly in *Lactococcus lactis* and *E. coli*. In *E. coli*, promoters are often regulated by multiple transcription factors, forming complex networks that enhance the versatility of promoter engineering. This multifactorial control allows for sophisticated regulation of gene expression, akin to eukaryotic systems [22]. The strength of these engineered promoters varies significantly, with some achieving over 2000 relative units [23]. Induction times can be rapid, as seen with the r1t system, which responds within hours to mitomycin C [24].

Chemically induced dimerization (CID) (Fig. 1d) creates artificial protein complexes by introducing a chemical inducer that promotes the binding of two protein domains. This technique allows for the controlled activation or inhibition of specific protein functions [25,26]. The method uses small molecule inducers that bind to specific protein domains and bring them together to form a functional complex. The inducer molecule bridges the two protein domains and promotes their interaction and, thus, their future dimerization. This method is used as a kill switch to activate or inhibit protein functions by bringing them together or separating protein domains depending on the use case. Protein engineering approaches can be used to create an inducer molecule as well. To ensure the safety and effectiveness of this method, the inducer molecule must be non-immunogenic and non-toxic.

CID can be achieved using various inducer molecules such as rapamycin, fluorogenic inducers of proximity, and small bi-functional molecules like chemical dimerization [27,28]. These molecules enable the controlled proximity of proteins, facilitating studying biological processes with high spatiotemporal resolution. Additionally, novel approaches like host-guest systems and metal ion chemistry have been proposed to modulate protein dimerization. This method can be exemplified by the dimerization of the Locus for X-ray sensitivity A; LexA repressor in *E. coli*, which binds cooperatively to DNA, enhancing its regulatory function [29]. Another example is the biotin repressor; BirA, which dimerizes upon binding to its corepressor, bio-5′-AMP, with an equilibrium constant of 11 μM [30]. The dimerization process can be rapid, with significant effects observed within minutes to hours,

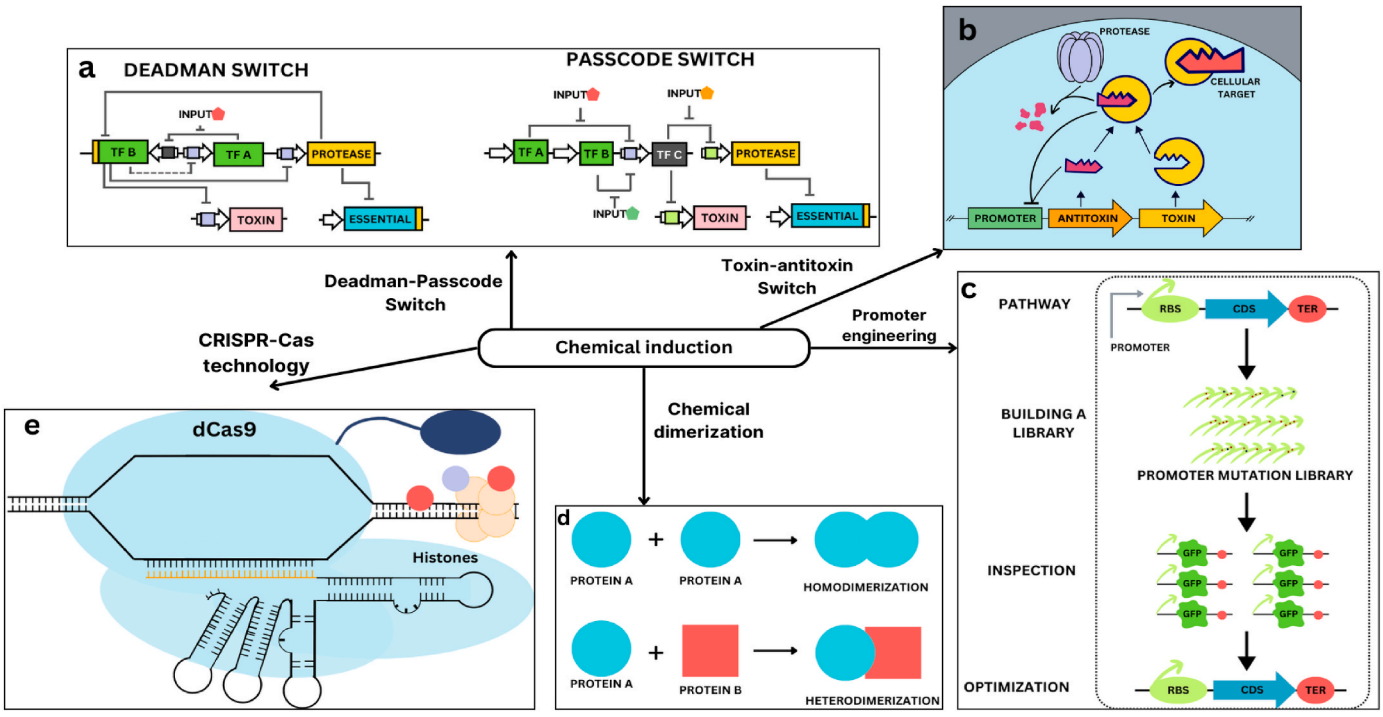

Fig. 1. Methods of chemically induced Kill Switches. a. Deadman and Passcode kill switches. b. Toxin-Antitoxin system. c. Promoter engineering techniques. d. Chemically induced dimerization to create an artificial non function hetero dimer protein that is nonfunctional. e. Chemically inducible CRISPR defense system to prevent CRISPR genome editing.; TF, Transcription factor; RBS, Ribosome binding site; CDS, coding DNA sequence; TER, Terminator; GFP, Green fluorescent protein.

depending on the specific system and conditions. The strength of dimerization varies; for instance, the dissociation constant for the Fur protein dimer is in the millimolar range, indicating moderate affinity. This method allows for versatile applications as small molecules can be synthesized easily. However potential instability of dimer forms under varying conditions such as pH and ionic strength can limit effectiveness [29]. CID modules also effectively act as molecular switches and thus control biological processes. The molecule simeprevir is one of the many novel CID modules identified to regulate the action of various microbes and induce apoptosis [31].

An alternative method for chemically induced kill switches involves metabolic poisoning, which uses a chemical absent from the target environment to inhibit unwanted growth [32]. The selected chemical can function in conjunction with a riboswitch to establish a synthetic addiction system dependent on the chemical for regulating the expression of a key transcription factor. In the presence of the chemical, the riboswitch undergoes a conformational change, enhancing the expression of the transcription factor and generating a positive feedback loop. If the chemical is removed, the transcription factor is altered to downregulate the initially upregulated protein expression [33]. Metabolic poisoning can selectively inhibit specific microbial strains, allowing for precise biocontainment. Metabolite toxicity varies based on concentration and chemical structure, complicating the predictability of outcomes [34].

If the genetically engineered organism (GEO) or kill switch gene is CRISPR/Cas-based, the enzymatic activity of the Cas9 nuclease can be precisely regulated using anti-CRISPR proteins. These proteins, naturally evolved in bacteriophages, offer a mechanism to control genome editing in GEOs. Furthermore, they hold significant promise for containing gene drives [35]. Non-proteinaceous anti-CRISPR small molecules, such as nucleic acid inhibitors of Cas9, effectively inhibit Cas9's DNA cleavage activity. These inhibitors work in conjunction with anti-PAM and anti-tracer molecules, providing a highly efficient mechanism for controlling Cas9 function [36]. CRISPR systems can also be engineered to respond to chemical signals, such as 4-hydroxytamoxifen (4-OHT), allowing for rapid and reversible activation of gene editing functions (Fig. 1e) [37]. The engineered kill switches exhibit a quick response, with activation occurring within hours of chemical induction, demonstrating effective control over microbial populations. Some of these designs incorporate multiple inputs, such as temperature and chemical signals, to further refine control over the kill switch activation [14].

In a previous study, an RNA-only delivery kill switch was exploited to eliminate specific tumor cells in a complex environment using L7Ae (a member of a superfamily of proteins that bind k-turns in RNA) which can detect the required mutation and perform logic computation [38]. However, this approach can be challenging due to difficulties in designing an RNA system that responds solely to a small molecule, which can lead to off-target effects, especially in complex tissues. However, a more promising approach is a similar method using RNA aptamers that bind to the small molecule aptamer and induce a downstream killing mechanism [39].

2.2. *Light induction*

Optical dimers serve as robust tools for light-inducible regulation of protein-protein interactions, enabling precise spatial, temporal, and dose-dependent control of biological processes. In these systems, one of the interacting proteins is photosensory, remaining in an unexcited state until activated by light, at which point it undergoes a conformational change. Without photoexcitation, the protein reverts to its original state. Various optical dimer systems utilize distinct light-sensing domains, such as phytochromes, cryptochromes, Light-oxygen-voltage-sensing domain (LOV domains), and UVR8, to develop photoactivatable actuators (Fig. 2a). Table 1 highlights a range of photosensitive promoters. The COMBINES-LID (combinatorial binders-enabled selection of LID) technique offers a highly specific method for designing genetically encoded actuators to manipulate biological processes optically [40,41]. In experimental nude mice injected with actuator cells, activation of the kill switch gene increased by 25-fold following 24 h of illumination [40].

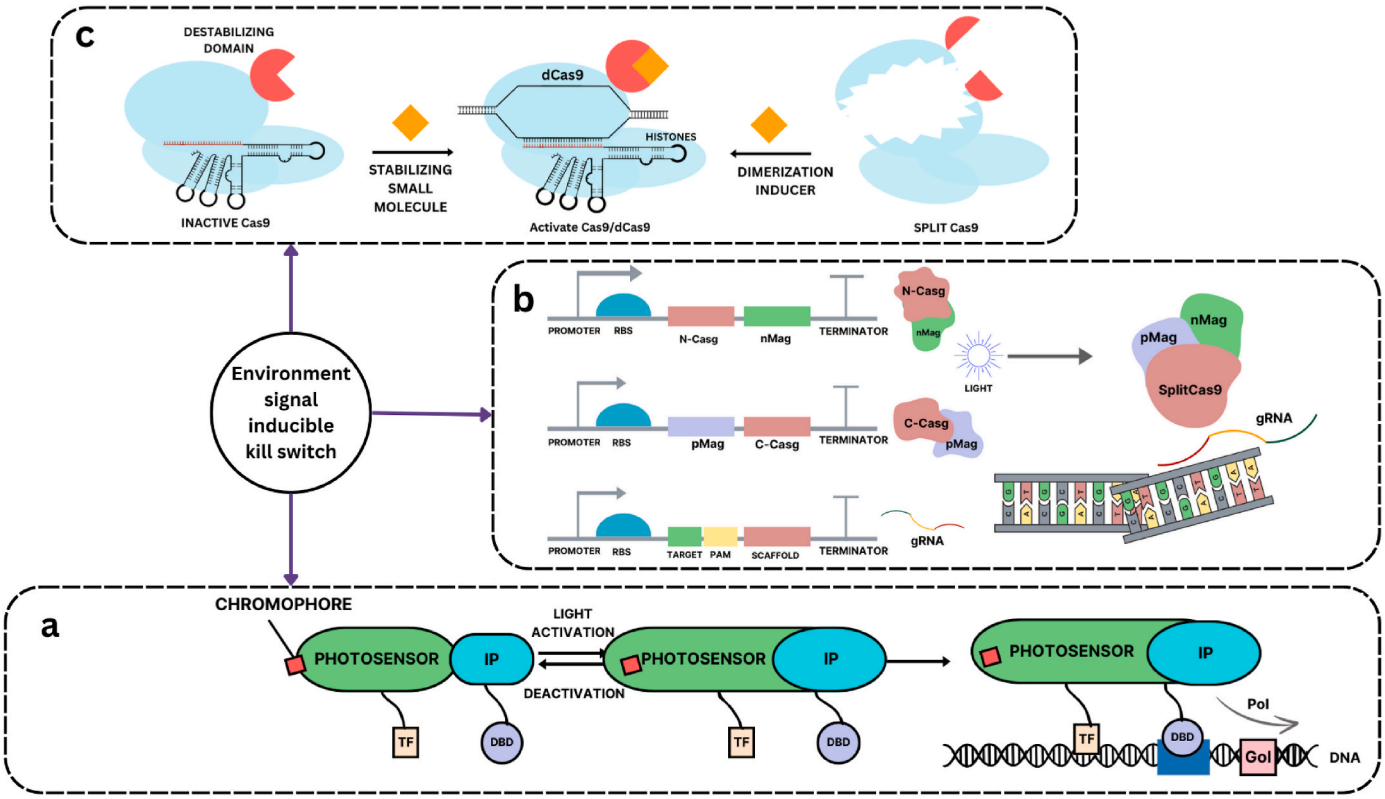

Fig. 2. Key innovations in light-responsive gene-editing systems include: a) photoactivable actuator, This is a schematic overview of a Two-hybrid light-inducible gene expression systems is presented. These systems depend on light-triggered interactions between protein 1 (IP) and protein 2 (Photosensor). The IP is fused to a DNA-binding domain (DBD) that specifically binds to its corresponding DNA sequence. The photosensor is attached to a transcriptional activation domain (TF). When light activates the interaction between P1 and P2, TF is recruited to the promoter, leading to the expression of the gene of interest (GOI). b). Engineered photoswitches called Magnets in a Cas9 system, Methods for conditional control of Cas9 activity include various strategies. (A) One approach involves inactivating Cas9 by fusing it to a small molecule- or light-responsive domain, or by splitting Cas9 into N- and C-terminal fragments that can be reassembled in response to light or a small molecule, restoring its activity. In this system, the N- and C-terminal fragments of Cas9 are linked to engineered light-responsive domains, termed positive magnet (pMag) and negative magnet (nMag). When exposed to blue light, pMag and nMag dimerize, creating a split system that results in lower background activity and a greater fold induction of Cas9 activity. and C. The engineering of single-chain photoswitchable Cas9 (ps-Cas9) proteins these can be implemented individually or together as a biocontainment method. A strategy for degrading Cas9 involves using a heterobifunctional small molecule, where one end binds to a small-molecule binding domain attached to Cas9, while the other end targets CRBN. This interaction promotes ubiquitination and subsequent proteasomal degradation of Cas9.

Table 1
Photosensitive Promoters and their origin for the creation of a photoactivatable actuator.

Name	Origin	Citation
CRY2/CIB1	*Arabidopsis thaliana*	[42]
UVR8/UVR8	*Arabidopsis thaliana*	[43]
UVR8/COP1	*Arabidopsis thaliana*	[43]
Dronpa	From FLARE (Fluorescent Apoptosis Regulator) protein	[44]
TULIPs (Twin Ubiquitin Ligase for Phytochrome SIgnaling)	Engineered LOV domain system	[45]
LOV-ipa	LOV domain-based system	[46]
LOV-SsrA	LOV domain-based system	[46]
PhyB/PIF6	*Arabidopsis thaliana*	[47]
PhyB/PIF3	*Arabidopsis thaliana*	[48]
FKF/GIGENTEA	*Arabidopsis thaliana*	[49]
oLID	*Avena sativa*	[50]
VVD	*Neurospora crassa*	[51,52]
EL222	*Neurospora crassa*	[53]
BphP1/PpsR2	*Rhodopseudomonas palustris*	[54]
BhP1/QPAS1	*Rhodopseudomonas palustris*	[55,56]
iLID	*Avena sativa*	[57,58]

For in vivo applications, light-induced dimerization offers spatiotemporal resolution unmatched by chemical-induced dimerization [42].

The CRY2-CIB system (cryptochrome circadian regulator 2-cryptochrome-interacting basic-helix-loop-helix 1), based on the interaction between Arabidopsis cryptochrome 2 and CIB1, has been widely used for optogenetic regulation of various cellular functions. In a study, the authors improved the versatility and tunability of the CRY2-CIB dimer system by generating second-generation CRY2 and CIB1 variants. They identified CRY2 truncations that showed improved dynamic range in response to light and minimal CIB1 truncations. They also identified CRY2 signaling mutants with altered photocycle, resulting in longer or shorter half-lives for CIB1 binding. Combining these mutations generated a second-generation photoactivatable Cre recombinase with enhanced dynamic range. This improved system allows precise spatial and temporal control of gene editing, making it suitable for creating a light-inducible biocontainment mechanism that can be inserted as a gene circuit [59].

Engineered photo switches, known as Magnets (Fig. 2b), provide a powerful optogenetic approach for controlling cellular proteins. Magnets comprise two photosensory units that become active only when both are simultaneously photoactivated, functioning similarly to an AND logic gate through electrostatic recognition. Their switch-off kinetics can vary from seconds to hours, depending on the system. In the absence of photoactivation, Magnets exhibit minimal dimerization activity. These photo switches are derived from VVD, a blue light-sensitive domain from the filamentous fungus *Neurospora crassa* [60,61].

Engineering single-chain photo switchable Cas9 (ps-Cas9) proteins (Fig. 2c) that can be controlled by light, allowing for precise temporal and spatial control of gene editing and transcriptional regulation, can be

used as a biocontainment measure. The ps-Cas9 proteins are designed using photodissociable dimeric fluorescent protein domains, which impede the DNA-binding aperture in the dark and open upon illumination, enabling DNA binding and effector activity. In a study, cells were exposed to 10 mW/cm^2 cyan light for 40 h and the results were comparable to those of a light-induced two-component Cas system. By incorporating the ps-Cas9 proteins into a gene circuit, the expression of these containment genes could be tightly regulated, allowing for activation only in specific conditions or upon exposure to light [62].

2.3. Temperature induction

Temperature can trigger the biocontainment of bacteria by using temperature-dependent transcriptional repressors to control bacterial gene expression. As shown in Table 2, these temperature-dependent transcriptional repressors can be integrated into thermal logic circuits, allowing for switch-like control of gene expression at specific temperature thresholds [63]. By incorporating these molecular bio switches into microbial therapy scenarios, such as activation using focused ultrasound or modulation of activity in response to a host fever, the bacteria can be controlled, and their viability can be regulated based on temperature. Additionally, temperature can induce self-destruction of the bacteria after fecal elimination, preventing their escape into the environment. This approach provides a way to couple endogenous or applied thermal signals to cellular function, providing the potential for precise control of bacterial behavior in various applications.

Lately, CRISPR-Cas9 has also been found to be applicable to temperature-based kill switches. Upon activation of non-permissive temperatures, CRISPR-Cas9 cleaves essential bacterial genes leading to cell death (Fig. 2c). Transcriptional repressors such as TetR and AcrIIA4 anti-CRISPR proteins can effectively inhibit Cas9 activation under standard conditions. A study successfully designed a kill switch incorporating these mechanisms for *Pseudomonas putida*, a bacterium of considerable biotechnological importance [67]. To ensure robust containment, it is crucial to implement secondary safeguards using diverse genetic circuits to prevent unintended lethal gene expression.

In another study, heat-sensitive engineered bacteria that can respond to thermal stimuli within 30 min were used. The bacteria were designed using a temperature-sensitive mutant of the cI protein derived from phage λ, cI857, which represses the promoter at low temperatures and unblocks at high temperatures to express the downstream target gene [64]. Such a system exploits the ability to maintain repression while growing at 37 °C and gives maximum yield in a relatively short time period which could be advantageous for large-scale projects.

2.4. pH induction

A previous study demonstrated that the addition of Tetrakis (quatrotriphenylphosphine palladium) solution to the α-hemolysin pore enabled pH-dependent control over the channel's opening and closing [68]. This innovation introduced a molecular switch capable of regulating molecular transport. The ability to control the α-hemolysin channel based on pH presents promising applications, such as "smart" drug delivery systems. By adjusting the pH, the channel can be precisely opened or closed, allowing controlled release of drugs or other molecules. At a specific pH, the channel remains closed, effectively halting the transport of substances and any associated biological activity. This mechanism could also be adapted into biocontainment systems to manage the release of potentially hazardous materials [69].

As previously explored in gene regulation and expression, pH-dependent promoters, as outlined in Table 3, can facilitate toxin production, enable the expression of dimerization-capable small molecules, and activate CRISPR-Cas9 complexes. In another investigation, the chemotaxis pathway of *E. coli* was analyzed using FRET (fluorescence resonance energy transfer) to study the robustness of ligand recognition by bacterial ligand-binding domains. This analysis revealed pH- and ligand concentration-dependent changes in these domains. A broad pH range of 5.5–10.5 was found to induce ligand binding, in contrast to cytosolic regions, which operate within a narrower pH range. The periplasmic ligand-binding domain exhibited consistent ligand recognition and signal transduction, highlighting its robust signaling capability. Tar chemoreceptors, present in *E. coli* and *Pseudomonas*, efficiently detect ligands across a wide pH spectrum, providing a reliable mechanism for signal detection, even at elevated pH levels. The adaptability of the periplasmic ligand-binding domain to fluctuating pH conditions makes it a valuable tool for environmental sensing and enhances bacterial adaptability [70].

3. Combinatorial system for enhanced biocontainment

Combinatorial systems for improved biocontainment utilize multiple kill switch mechanisms to strengthen containment and prevent the escape or survival of engineered genes. Effective biocontainment strategies must account for factors such as mutagenic drift, environmental conditions, and horizontal gene transfer. Various approaches have been developed to reduce the survival and proliferation of engineered microorganisms in challenging environments. By using a combinatorial approach, multiple safeguard designs are integrated, which may lead to unpredictable outcomes. This method also supports high-throughput screening of contaminants, enhancing the overall effectiveness of biocontainment efforts [79].

A previous study on pH emphasized that incorporating various ligand-binding domains, each responsive to specific ligands across a wide pH range, helps create a well-regulated system. In biocontainment, this combinatorial approach supports a controlled bacterial response, activating only under specific conditions, such as pH and ligand concentration. By requiring multiple signals to trigger gene expression or metabolic pathways, this system enhances the safety and containment of engineered bacteria, reducing the likelihood of escape and minimizing unintended interactions.

In another study, quorum sensing was used to develop a two-pillared mechanism for *Streptococcus pyogenes* and its biocontainment. Quorum sensing principles can derive relations between gene expression and

Table 2
Temperature sensitive promoters.

Name	Source	Temperature Range	Citation
pL/pR phage lambda promoters	Bacteriophage lambda	~42	[64]
DnaK promoter/ Phsp70	Heat shock genes, *Escherichia coli*	>37	[64]
GroE promoter/ Pchaperonin	Chaperonin genes, *Escherichia coli*	>37	[65]
trmE	*Pseudomonas syringae* (Lz4W)	>5	[65]
Hybrid galP1-cysG	*Escherichia coli*	<20	[66]

Table 3
pH sensitive promoters.

Name	Source	pH range for induction of transcription	Citation
P170	*Lactococcus lactis*	6.0 to 6.5 in stationary phase	[71]
Pgas	*Aspergillus niger*	<5.0	[72]
F-ATPase Operon Promoter	*Streptoccus mutans*	~7.0	[73]
malTp1-malTp10	*Escherichia coli*	>6.0	[74]
gadA	*Escherichia coli*	~7.0	[75]
gadC	*Shigella flexniri*	<6.5	[76]
P-atp2	*Corynebacterium glutamicum*	8.0 to 10.0	[77]
K-12	*Escherichia coli*	5.0 to 6.0	[78]

their population density. In this case, they could portray the interplay between pH decrease at high population density and the facilitation of the high-affinity secretory peptide interactions. This, in turn, causes disease progression and cell death. The method exploits the intricacies of bacterial signaling in non-permissive environmental conditions [80].

Combinatorial systems offer several advantages for enhanced biocontainment [81]. First, by incorporating multiple mechanisms for containment, the chances of escape or survival of genetically modified organisms are significantly reduced. Second, combinatorial systems can provide a higher level of specificity and control over the activation or deactivation of containment mechanisms. Third, by using multiple mechanisms, there is a reduced risk of off-target effects or unintended consequences. Fourth, combinatorial systems can offer increased adaptability and flexibility in different environments or under varying conditions [82,83].

GEOs were developed for the betterment of human life. A combinatorial system can be designed using synthetic microbial consortia and the available literature on microbial signaling pathways and cell interactions. Each strain in a GEO is designed to co-exist by providing nutrients essential to the community. They exhibit auxotrophy and require multiple nutrients, which the consortium will supply. Escaping from their respective environment will inhibit the growth or death of the GEO. CRISPR and quorum sensing based systems, along with the help of the genetic circuits, help precisely control the gene expression, which can be applied to kill the GEO in case of an escape [84,85]. Furthermore, combinatorial systems can also incorporate regulatory circuits and feedback loops to enhance the control and reliability of the biocontainment strategy [86]. While introducing genetic circuits within the chassis, nonessential genes must be identified, as shown in Table 4, and in-silico disruption of each gene in the updated chassis model can be used to assess the percentage change in the growth rate of the chassis. In silico disruption in vivo, the best candidate deletions must be disrupted to confirm the predicted scores and assess the suitability for a gene insert [87].

Various environmental changes in tandem can serve as triggers for an inducible promoter to express toxins, CRISPR-Cas systems, or proteins that have dimerization ability to prevent the action of essential metabolic enzymes and thus pathways. Rational design and toxin/antitoxin titering approach has been previously used to produce and screen a library of potential constructs. The constructs that show the most evolutionary stability are selected [14]. Multi-layered kill switches have previously been demonstrated to exhibit stability for 110 generations [87]. Following the strain selection, plasmid construction and gene insertion kill switches must be evaluated for their biocontainment efficacy in controlled environments, followed by variable environments by simulating possible disruptors. Strains in which the required circuit has been inserted are assessed by sequencing to determine the location of the "kill switch circuit" in the genome. Then, the strains can be grown through passaging. The inactivation of the kill switch mechanism must be assessed by adding the required inducer and repressor particles and evaluating if the respective signals create the desired outcome, usually the reduction of cell population size. If the desired result is not being attained, then the genome can be analyzed for frameshift mutations to assess how the accumulation of mutations with an increase in passage number has inactivated the circuit. The kill switch circuit must be analyzed to ensure that it is not producing a significant selection pressure in the strain without the required signal.

Table 4
Commonly used organisms in Genetic Engineering of organisms and sites for insertion of combinatorial genetic biocontainment systems.

Organism	Possible genes for deletion	Biological relevance	Citation
Corynebacterium glutamicum	cg1361, porB, mepA, ung, mutM	Industrial production of amino acids.	[88]
Cyanobacteria anabeana	ava2679, alr2887, alr3608, all4388, all2508	Used in nitrogen fixation.	[89]
Rhodococcus erythropolis	aqdA1B1C1, aqdA2B2C2, rodA, parA	Used in bioremediation to remove toxic organic solvents.	[90]
Escherichia coli	yacF, yacG, yacH, ruvA, yabB, adhE, sfcA	Base organism for testing synthetic biology circuits.	[91,92]
Lactococcus lacti	LysP, AcaP, FywP	Production of curd.	[93]
Bifidobacterium animalis	Balat1410, AR668, gene 0348, gene 0208	Probiotic strain.	[94]
Pseudomonas putida	PP4378, PP2357-PP2363	Applications of *P. putida* range from bioeconomy chemicals to biosynthetic drugs.	[95]

4. Conclusions and perspectives

In conclusion, the development and implementation of synthetic biology-based kill switch mechanisms offer a promising approach to enhance biocontainment and prevent the escape of GEOs [58]. Current limitations of the kill switch and combinatorial systems include the potential for mutations to lead to surviving subpopulations, which undermines containment efforts [5]. George et al., identified several limitations to the biocontainment strategies, such as the inability to predict the release of the organism into the environment, the lack of ability to mimic different environmental conditions in a lab model, and the high cost associated with the implementation of the biocontainment systems in industries which increases their operating cost and reduces their financial sustainability [5]. Further, George et al., encouraged redefining the biocontainment strategy, thereby incorporating an acceptable amount of genetic material distribution within the specific parameters to balance ecological concerns, societal risks, and other environmental issues. Arnolds et al., suggested incorporating computational tools such as the metabolic environmental models and GEMs to build a secure system suitable for specific conditions [8]. The authors also recommended conducting mesocosm research as it will resemble the natural ecosystem more precisely and help assess the gene flow, consequences of the GMOs, and their stability.

Potential risks and concerns associated with synthetic biology-based kill switch mechanisms include the possibility of unintended consequences. Unintended consequences may arise if the kill switch mechanism malfunctions or becomes activated in unintended circumstances, potentially leading to negative impacts on the environment or human health. To mitigate these risks, thorough testing and evaluation of kill switch mechanisms is crucial [96]. To bridge the gap between laboratory research and applications in the real world, interdisciplinary collaborations, standardized protocols, and robust testing frameworks are required to overcome the challenges faced due to horizontal gene transfer, the metabolic demand of the host organism, and the lack of standardized regulation. A bidirectional system was proposed to increase efficiency and overall stability by adopting a toxin-antitoxin system and inducible CRISPR switches to prevent the escape of genetic material [36]. Lee et al., proposed a combinatorial system comprising microbial strains, which are interdependent and cell-free systems, to overcome the challenges associated with the escape of genetic material and other constraints [83]. They also highlight the necessity of developing standardized testing protocols, functional redundancies with multiple fail-safes, and realistic evaluations to create a sustainable biocontainment solution.

Combination of kill switches and genetic regulatory mechanisms that are dependent on different induction methods such as chemical, environmental signals and genetic circuits could be used as a genetic insert together to create molecules that are expressed only in the presence of all the required conditions and thus ensure a safer containment method. Dimerization and engineered promoters in combination with antitoxin/toxin systems or Deadman-Passcode switches show the highest promise

and the easiest detectability *in vitro* studies for the same. Combinatorial kill switches are particularly promising in complex environments where the dependency on one signal is not possible due to the disturbances in the systems and could prove unsafe due to the nature of the system; such complex environments for GEO are particularly observed in gut microbiota studies and wastewater treatment plants studies [96].

Future research directions for improving biocontainment strategies involve exploring and developing new lethal genes, regulatory elements, and toxin-antitoxin modules. Furthermore, the use of orthogonal systems could provide a more robust and absolute containment solution for GEOs. This could be achieved by incorporating additional layers of containment mechanisms, such as compartmentalization and cell-free systems, and further refining the engineering of genetic circuits. In conclusion, the development of synthetic biology-based kill switch mechanisms for biocontainment of GEOs is an ongoing and evolving field.

CRediT authorship contribution statement

Shreya Varma: Writing – original draft, Visualization, Methodology, Data curation, Conceptualization. **Khushi Ash Gulati:** Writing – original draft, Data curation, Conceptualization. **Janani Sriramakrishnan:** Validation, Formal analysis, Conceptualization. **Riyaa Kedar Ganla:** Writing – original draft, Investigation, Formal analysis, Data curation. **Ritu Raval:** Writing – review & editing, Supervision, Resources, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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