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Biotechnology for secure biocontainment designs in an emerging bioeconomy

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Genetically modified organisms (GMOs) have emerged as an integral component of a sustainable bioeconomy, with an array of applications in agriculture, bioenergy, and biomedicine. However, the rapid development of GMOs and associated synthetic biology approaches raises a number of biosecurity concerns related to environmental escape of GMOs, detection thereof, and impact upon native ecosystems. A myriad of genetic safeguards have been deployed in diverse microbial hosts, ranging from classical auxotrophies to global genome recoding. However, to realize the full potential of microbes as biocatalytic platforms in the bioeconomy, a deeper understanding of the fundamental principles governing microbial responsiveness to biocontainment constraints, and interactivity of GMOs with the environment, is required. Herein, we review recent analytical biotechnological advances and strategies to assess biocontainment and microbial bioproductivity, as well as opportunities for predictive systems biodesigns towards securing a viable bioeconomy.

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Introduction

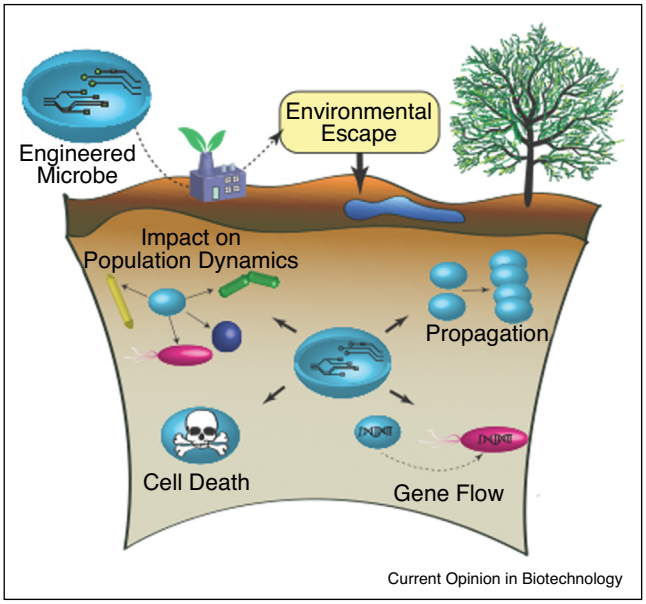
Concerns related to escape of transgenic organisms and potential exchange of transgenic DNA with native organisms in the ecosystem necessitates highly effective biocontainment strategies. To date, a number of synthetic biology-mediated biocontainment strategies have been developed that primarily rely on: (i) metabolic auxotrophy, (ii) inducible control of systems deleterious to cell health, and (iii) rewriting the genetic code using xenobiological and/or synthetic coding components, with varying degrees of efficacy [1•]. Many have successfully achieved or exceeded the U.S. National Institutes of Health guideline stating that a genetically modified organism (GMO) escape rate below 1 in 10^8 cells is considered to be acceptably safe [2,3]. However, a number of outstanding questions related to GMO stability, resilience, escape frequency, and performance and containment at scale and under variable environmental conditions have not been fully addressed (Figure 1). Additionally, the principles governing biocontainment and the impact of genetic safeguards upon microbial fitness and bioproductivity in industrial hosts remain to be explicated. Combinatorial approaches to incorporate multiple genetic safeguarding designs have yielded unpredictable results [4,5], underscoring the incomplete knowledgebase associated therewith. Further, these strategies have largely been evaluated in model microbial systems (e.g. *Escherichia coli*); translatability to non-model, industrial production hosts remain uncertain. Herein, we review current strategies for analyzing biocontainment in microbial systems and discuss future prospects for achieving predictive control of secure, high-productivity biodesigns.

Genetic safeguards for biocontainment

The first reported microbial biocontainment strategies primarily relied on engineered auxotrophies; the genetic knockout of a gene related to the synthesis of an essential metabolite [6–8]. Though generally effective for selection and biocontainment in the laboratory, achieving escape frequencies below 10^{-13} [8], such approaches tend to fail outside of strict laboratory conditions, as these essential metabolites can be scavenged from the natural environment, for example, soil, water, and native microbes [7]. Further compromising auxotrophic containment is the potential for intra-species or inter-species horizontal gene transfer (HGT), enabling reacquisition of essential genes. The disparity between escape frequencies in a lab versus a natural environment underscores the need to simulate natural conditions during the assessment of biocontainment strategies.

Another approach to biocontainment relies on the conditional expression of proteins toxic to cells. For example, membrane destabilizing proteins, can be controlled via a small molecule that represses expression of the toxic gene such that if the organism escapes into nature the absence

Figure 1



Potential environmental fates of GMOs in native ecosystems.

of the repressor will lead to toxic gene expression and subsequent cell death [9,10]. To address potential for HGT, researchers have utilized activation of nucleases as a kill switch, which kills cells through destruction of their DNA; once degraded, native and transgenic DNA is no longer available for transfer to wild type organisms [9,11,12]. Additional strategies using toxin-antitoxin pairing systems have also proved effective [12]. More recent work on kill switches has involved multi-layer, programmable control, such as creation of a logic gate, wherein cell survival requires the input of two small molecules, and the absence of a third [13••]. A notable limitation of basic kill switches is the relative ease for cells to mutate their DNA and thus escape biocontainment, which could be mitigated by strategies that reduce host mutation rates or through the use of redundant devices [14].

Many recent approaches to biocontainment rely on linking essential gene functionality to availability of non-native small molecules, nucleotides, or amino acids. Synthetic auxotrophy has recently been demonstrated through the use of non-canonical amino acids (ncAA). In this approach, a rare codon is first reassigned to a synonymous codon to create an unused codon in the genome; this codon can then be used for the incorporation of a non-standard amino acid into essential genes [15•]. This makes cell survival dependent on an exogenous supply of the ncAA, and if applied to transgenes, would greatly decrease the implications of HGT [7,16•,17,18••]. Such genetically recoded *E. coli* strains have also proven to effectively confer biocontainment advantages, including resistance to viruses and impairment of conjugative plasmids by multiple orders of magnitude [19]. However, to date, the fitness of genetically recoded organisms in natural ecosystems has not been evaluated, nor has it been assessed in industrially relevant organisms. While not explicitly tested for biocontainment, the recent development of xeno-nucleic acid (XNA) polymers that have either a unique sugar back bone or base pairing with unnatural nucleotides [20–22] could prove exceptionally useful in preventing HGT and containment of genetically modified organisms to a defined environment.

The above technologies represent promising biocontainment strategies. However, at present, many of these strategies are difficult to implement at large scale and in non-model microbes, requiring extensive genomic redesign or recoding. Further, costs associated with supplementation of exogenous metabolites or xenomolecules at industrial deployment scales may prove economically unviable. Lastly, the impact of xenobiological molecules upon native ecosystems remains to be comprehensively evaluated. Accordingly, it is imperative to consider biocontainment design and assessment within the context of an industrial process, not just a laboratory model system.

Assessment of biocontainment designs

While great strides have been made in the development of novel biocontainment strategies, the analytical approaches deployed to assess their performance have remained largely unchanged. To date, growth in liquid media or on agar plates has been used to assess the escape risk in the majority of biocontainment studies [5,9–11,23]. Although useful to establish a baseline of efficacy, there are notable drawbacks and limitations with media and plate-based assays. These assays are designed to quantitatively evaluate single strategies at a time, but are not adept at examining many combinations concurrently. Additionally, plate assays can fail to capture transient resistance and persisters [4,10]. More importantly, these experiments fail to capture the complexity of an environmental release, nor can they account for the potential impacts industrial-scale growth may have on the efficacy of biocontainment modules. High-throughput screening of biocontainment modules could be accelerated by the use of combinatorial biocontainment libraries and pooled screening strategies to assess both bioproductivity and stability of biocontainment. Such approaches could be complemented by recent advances in DNA barcoding technologies, which present an effective means to track biocontainment designs in combinatorial space [24].

There have been some notable efforts to address the complexity a microbe may face upon environmental escape, where both abiotic and biotic factors may influence the stability of the strain. Blood agars, soil extracts, bacterial lysate, or wild-type bacteria have been used to introduce some complexity into culture conditions [4,7,8,16•,25]. The importance of these efforts is

highlighted by the fact that escape frequency increased in a number of these experiments, with metabolic auxotrophs being particularly unreliable [7,16•,25]. Another crucial question is how the changing conditions and increased stress of large-scale industrial production might impact both bio-productivity and biocontainment. Some have begun to explore the effect of increased scales as well as long-term cultivation, however with volumes capping out at 1−10 L and time-courses maxing out at 21 days, these efforts still fall well short of recapitulating industrial deployment conditions [4,16•]. An assessment of the integrity of the AND and NOR genetic circuits in *E. coli* using 10 L fermenters identified a reduction of overall growth and output as well as the failure of one of the input gates in the AND circuit, neither of which were observed in smaller flask cultures, underscoring the impact changing conditions may have on both bioproductivity and circuit control [26•]. These results highlight the fact that benchtop assays need to be followed by more robust analyses that stimulate industrial growth and environmental release. Importantly, these analyses need to be standardized and adopted by industrial and scientific communities for comparative analyses.

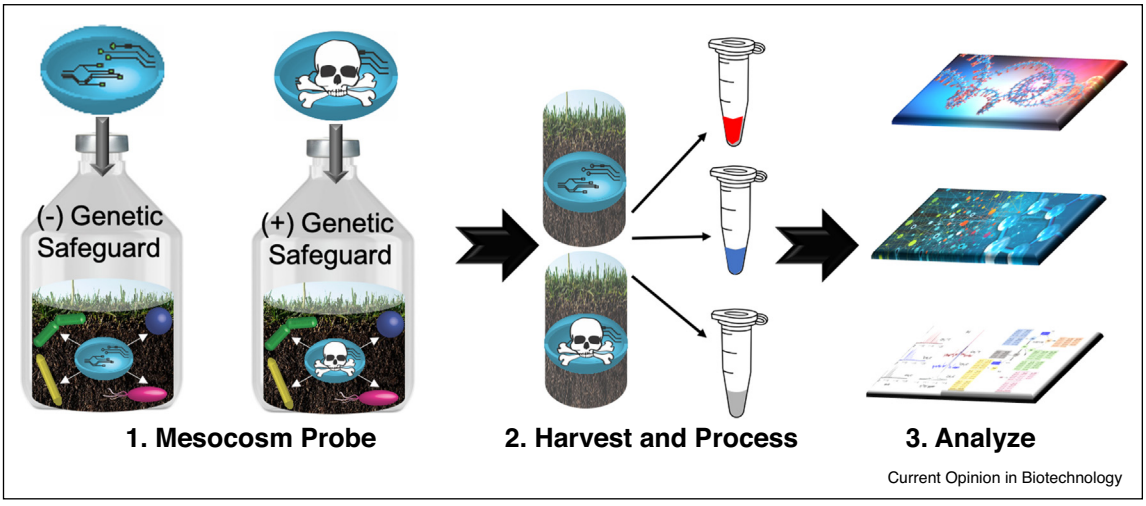
Mesocosm studies can act as stop-gap between lab-based evaluations and *in situ* deployment, and have long been utilized to assess environmental perturbations, such as the survival of pathogens, gene flow between transgenic and native organisms, phenotyping genetically modified microbes, and the impacts of pollutants [27–31]. By mimicking environmental conditions, mesocosms are well suited not only to investigate the stability of a biocontainment module in a realistic escape scenario but also to provide insights on the potential impacts of release aside from just propagation, such as HGT or metabolic shifts in a multitrophic environment [32–35].

Multi-omic analyses of mesocosms are needed for a comprehensive exploration of system level impacts of GMO release. Transcriptomic and metabolomic analyses would enable correlation of transcriptional regulation to biosynthetic outputs, including native metabolite pools and engineered bioproduct biosynthesis, as functions of biocontainment and environmental parameters (Figure 2). Phylogenomics of microbial communities in mesocosms would allow for the assessment of how GMO propagation, HGT, or bioproduct release might impact population dynamics and ecosystem stability in a complex community. The data from these efforts would not only allow for a comprehensive risk analysis of a potential GMO release, but also shed light on what underlying ecosystem characteristics contribute to the stability or the escape potential of a particular microbe under the constraints of genetic safeguards. Importantly, these data can be incorporated into models allowing researchers to predict metabolic interactions and transcriptional and translational responsiveness driven by particular genetic safeguards within well-defined ecosystems [36••].

Data integration and predictive design

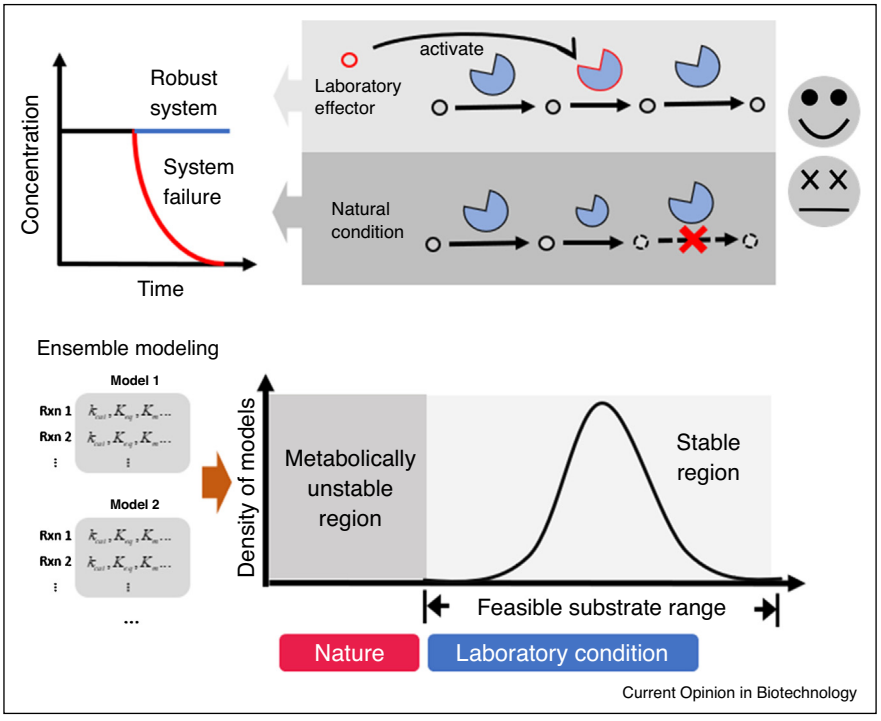
Recent advances in computational modeling present a means to contextualize multi-omic data to link genotypes with phenotypes, and ultimately provide the basis for predictive design and control capacity. For example, genome-scale metabolic models (GEMs) have emerged

Figure 2



Schematic overview of mesocosm analyses.
Representative soil samples probed in the absence and presence of GMO strains can serve as valuable test beds to assess HGT, phylogenomic, and functional multi-omic analyses.

Figure 3



Predictive bio-secure design based on ensemble modeling and metabolic robustness analysis.

as a powerful tool to define the complete metabolic capacity of an organism, presenting a global representation of its metabolism and gene–protein(enzyme)–reaction–metabolite connections [37]. GEMs are solved using flux balance analysis, a linear programming optimization method, in which biomass generation is frequently used as an objective function as a proxy for growth. This optimization process predicts the usage of reactions through the metabolic networks to achieve a maximum or minimum flux of the objective function [38]. GEMs thus serve as a powerful tool to both identify biocontainment gene targets as well as metabolic targets for optimization of bioproduction [36••].

An extension of GEMs are integrated metabolic and gene expression models (ME-models), which compute the molecular constitution of cells as a function of genetic and environmental parameters [39,40]. The ME-model explicitly predicts transcription and protein abundances, thus experimentally obtained multi-omics data can be evaluated within a comprehensive modeling framework [41]. The ME-model is not restricted to metabolism and can accurately predict the synthesis of proteins, usage of metals, and translation effectors. Importantly, the energetic costs associated with all aspects of these processes are also represented, which can further inform optimization of bioproduction in the context of biocontainment constraints.

Recent advances have further expanded computational capacity to enable construction of genome-scale models for co-cultures and communities, facilitating the analysis of symbiotic associations and interactions in microbial systems [42–45,46••,47]. Such community modeling approaches have been deployed to predict networks of interactions including syntrophic metabolite exchanges and determinants in community assembly [45,46••]. Understanding these dynamic interactions is crucial for delineating carbon metabolism in changing environments. These analyses have also been leveraged to determine if genetic mutations, often lethal for organisms in axenic cultures, could be rescued in co-culture [36••,45]. Accounting for all possible ways GMOs might overcome otherwise lethal perturbations in a community context will be critical for fail-safe strain designs.

Bio-secure designs can additionally benefit from state-of-the-art metabolic robustness analysis [48,49]. This approach offers a unique capability to predict and evaluate the fitness of a dynamic biological system, especially when the system exhibits differential behavior in laboratory and natural conditions. In a modeled biocontainment circuit (Figure 3), the GMO microbe will

exhibit steady-state metabolism when a laboratory effector stabilizes the function of a key enzyme. In an uncontrolled environment, where the effector is absent, the targeted enzyme will be down regulated, thus depleting the pool of a vital metabolite(s) and resulting in instability of the GMO during escape. Metabolic robustness can thus be deployed in the context of biocontainment modules by ensemble modeling [50,51], where a set of models with different kinetic data are parameterized and perturbed by varying maximum rate (V_{max}), which is largely proportional to the control level of the enzyme. This approach counts the probability of system failure per perturbation. Such approaches can ultimately pinpoint new metabolic targets for optimal bio-secure design, as well as assess viability of a modified laboratory organism in response to environmental changes.

Concluding remarks

The promise of a sustainable and profitable bioeconomy depends on the successful development and deployment of biocontainment strategies that are as secure at industrial scale and under environmental conditions as they are on the benchtop, without sacrificing the productive capacity of GMO strains. Systems level analyses, as described above, will enable identification of key regulatory networks that are differentially controlled in response to genetic safeguards and subsequent environmental escape. Large-scale deployment and simulated environmental release via mesocosms will allow for rigorous testing of biocontainment modules as well as provide data regarding the robustness and fitness of engineered microbes. Robustness analyses and GEM will facilitate integration of multi-omic and phenotypic data, as well as provide a framework for the rational design of biocontainment modules. These efforts will elucidate the underlying mechanisms that govern the efficacy of biocontainment and metabolic fitness. The resultant knowledgebase will provide a blueprint for predictive design and establish the basis for transferability to future biocatalysts, paving the way to a sustainable bioeconomy.

Conflict of interest statement

Nothing declared.

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