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Review

Biocontainment Techniques and Applications for Yeast Biotechnology

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Abstract: Biocontainment techniques for genetically modified yeasts (GMYs) are pivotal due to the importance of these organisms for biotechnological processes and also due to the design of new yeast strains by using synthetic biology tools and technologies. Due to the large genetic modifications that many yeast strains display, it is highly desirable to avoid the leakage of GMY cells into natural environments and, consequently, the spread of synthetic genes and circuits by horizontal or vertical gene transfer mechanisms within the microorganisms. Moreover, it is also desirable to avoid that patented yeast gene technologies spread outside the production facility. In this review, it was evaluated the different biocontainment technologies currently available for GMYs. Interestingly, uniplex-type biocontainment approaches (UTBAs), which relies on nutrient auxotrophies induced by gene mutation or deletion, or the expression of simple kill switches apparatus, are still the major biocontainment approaches still in use with GMY. While bacteria like *Escherichia coli* account for advanced biocontainment technologies based on synthetic biology and multiplex-type biocontainment approaches (MTBAs), GMYs are distant from this scenario due to many reasons. Thus, a comparison of different UTBAs and MTBAs applied for GMY and genetically engineered microorganisms (GEMs) was made, indicating the major advances of biocontainment techniques for GMYs.

Keywords: biocontainment; genetically engineered microorganisms; genetically modified yeasts; synthetic biology; gene circuits; auxotrophies; kill switches

1. Introduction

Genetically engineered or modified microorganisms (GEMs/GMMs) are the workhorse for basic and applied research as well as in industry. GEMs are employed in the food industry to improve protein synthesis or to generate small molecules that impact the nutritional value of a food, like flavor enhancers, oligosaccharides, vitamins, and amino acids [1]. GEMs are also employed for food enzyme production, including enzymes like lactase, amylase, proteases, and phospholipases [1,2]. Another biotechnological field that benefits from the application of GEMS is bioremediation, which allows the removal of pollutants (e.g., heavy metals) from water and soil [3], and finally different GEMs species are employed for the production of clinically important peptides/proteins (e.g., insulin) [4], vaccines [5], biofertilizers [6,7], biocontrol [8], and biofuels [9].

Considering eukaryotic GEMs, the yeasts constitute an important group of microorganisms with many industrial applications. Yeasts are used as a cell factory for the production of chemicals and biologicals due to its ability to grow on inexpensive culture media and have a well established

fermentative technology [10]. One of the most popular yeast employed as GEM is *Saccharomyces cerevisiae*. *S. cerevisiae* has many different biotechnology applications, from the production of traditional fermented foods [11,12] to cell factories for the synthesis of chemicals and pharmaceuticals, such as bioethanol, propanol, butanol, artemisinic acid, and insulin precursor [10,13,14]. In addition to its biotechnological importance, *S. cerevisiae* is considered an eukaryotic model organism for basic and applied research [15,16]. Other yeast species that have biotechnological importance include the halophilic/halotolerant yeast species like *Debaryomyces hansenii,* which can metabolize different types of carbon sources and synthesize high levels of lipids, xylitol, and flavonoids, making it an attractive model for metabolic engineering [17,18]. By its turn, methylotrophic yeasts (e.g., *Pichia pastoris* and *Ogataea polymorpha*) are very efficient in producing heterologous protein in industrial scale, with many strains and molecular tools available for genetic/synthetic biology engineering [19,20]. Other examples of non-*Saccharomyces* with industrial importance include *Arxula adeninivorans* and *Yarrowia lipolytica* for the production of therapeutic heterologous proteins [21]. In addition, yeast hybrid strains/chassis from the *Saccharomyces sensu stricto* complex for industrial purposes are gaining industrial importance [22]. Finally, advances in synthetic biology are leading to the generation of "synthetic microorganisms" by using the so-called "bottom-up approach", where isolated and well characterized biochemical components are modularly assembled in order to design artificial cells with specific phenotypes [23]. Another synthetic biology approach is the "top-down method", where a microbial genome is reduced to its essential genes, allowing to factory any desired DNA sequence to "tailor-made" a specific phenotype [24]. The top-down approach is currently being applied by the international Synthetic Yeast Genome project (known as the "Yeast 2.0" or "Sc2.0 project"), whose major objectives are to redesign all chromosomes of *S. cerevisiae* and generate the first synthetic eukaryote [24,25].

Despite the importance of genetically modified yeasts (GMYs) there are concerns that the widespread use of GMYs could lead to a potential exchange of modified DNA molecules with other microorganisms in an ecosystem [26,27]. These concerns can be potentialized by considering that yeasts can exchange parts of their genomes with other microorganisms by interkingdom horizontal gene transfer [28], resulting in the widespread use of a transgene and/or a synthetic DNA molecule (Figure 1). Thus, highly effective biocontainment/safeguard strategies are needed to keep GMYs restricted to a laboratory and/or industrial environments (here defined as "production facilities") [27,29] and restricting the "escape frequency" of GMYs. The escape frequency is the consequence of a combination of different molecular mechanisms (e.g., mutagenesis, gene loss, recombination) as well as environmental/evolutionary processes that result in the widespread of GMY/GEM into the environment (Figure 1). The National Institutes of Health (NIH) guidelines for research involving recombinant or synthetic nucleic acid molecules recommends an escape frequency of one cell per 10^8 cells as standard [30].

Another major issue regarding the massive use of GMY/GEM is that the simple physico-chemical inactivation of cell biomass (e.g., by heat and/or pH treatment) do not avoid the DNA stability/persistence in the environment after its release from the cell (Figure 1). Data gathered from the PCR analysis of soil fertilized with the waste product of industrial GMY/GEM fermentations showed the presence of genes related to antibiotic resistance commonly found in synthetic plasmids [31]. Additionally, engineered DNA molecules can be detected in soil after several days or months after its introduction due to the intrinsic high stability of DNA molecules [32,33]. Interestingly, none of these studies observed the horizontal transfer of released engineered DNA molecules to the native soil microbiomes; however, the chance of horizontal gene transfer from released synthetic DNA molecules in the environment can not be excluded especially if those molecules confer phenotype advantage to the native microbiome.

Different molecular approaches can be employed for GMY biocontainment, including auxotrophic mutations, synthetic molecular mechanisms (xenobiology), and kill switches based on the expression of toxin-antitoxin [27,34] or exo-/endonucleases [29,35] (Figure 1). However, all of these approaches are not fail proof, since molecular and evolutive mechanisms lead to inactivation of biocontainment devices [36] (Figure 1). This is especially true when a biocontainment approach

makes use of a single component/device (in this work defined as "uniplex-type biocontainment approach" or UTBA; Table 1 and Figure 1), like natural auxotrophic markers. On the other hand, the combination of different biocontainment approaches ("multiplex-type biocontainment approach" or MTBA; Table 1) reduces its potential escape frequency at the expense of low cell fitness (Table 1) [27,34]. Paradoxically, the reduction in cell fitness increases the frequency of inactivated biocontainment devices [37]. Thus, an effective biocontainment strategy should consider different escape mechanisms, including mutagenic drift, environmental supplementation of nutrients and/or essential molecules, and horizontal gene transfer (HGT) [38].

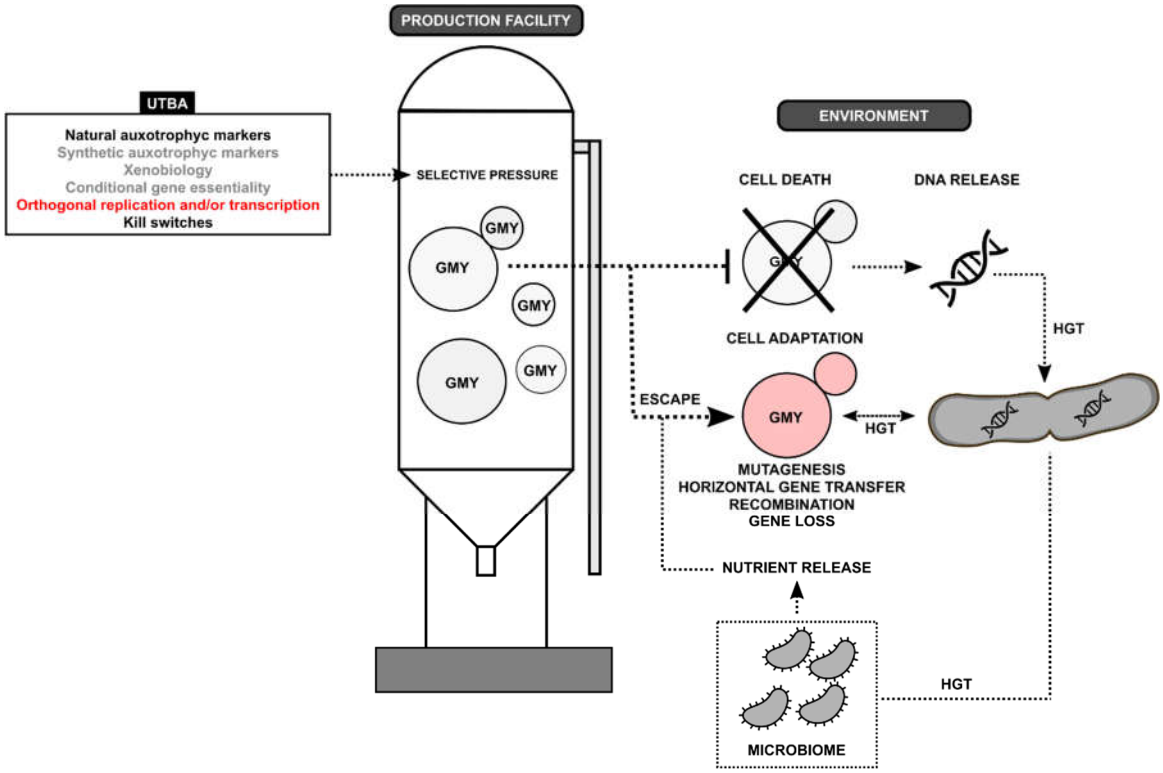

Figure 1. Diagram of major uniplex-type biocontainment approaches (UTBAs) applied for genetically modified yeast (GMY). Under different selective pressures induced by UTBAs, GMY cells are kept active/live in a production facility, where the absence of these selective pressures leads to cell death in an environment. However, a small fraction of GMY cells have the potential to escape the UTBA mechanisms, where different molecular mechanisms, like mutagenesis, horizontal gene transfer (HGT), DNA recombination, and gene loss, results in GMY adaptability to the environment. In some cases, the environment-associated microbiome could promote GMY adaptability by releasing nutrients that bypass UTBA (e.g., natural auxotrophies). On the other hand, GMY cell death can release synthetic/transgenic DNA molecules that remain stable in the environment and can be incorporated by HGT into different environment-associated microorganisms and thus changing the microbiome. The colors of UTBAs (inset) indicate the experimental status of the technology for GMYs: black, technology extensively tested inside and outside production facility; gray, experimental technology not tested outside production facility; red, technology not applied for GMYs and other microorganisms.

The purpose of this review manuscript is to focus on different UTBA and MTBA strategies for GMY biocontainment, and what approaches have been or not applied for industrially important GMYs.

Table 1. Advantages and disadvantages of different biocontainment approaches for genetically modified yeasts.

Biocontainment	Advantages	Disadvantages
Uniplex-type approach (UTBA)	1. Can be easy to implement for different yeast species. 2. Some approaches were extensively tested outside production facilities. 3. Orthogonal components diminish the impact of biocontainment apparatus on cellular physiology. 4. Can employ synthetic small molecules or DNA parts to prevent biocontainment escape. 5. Some UTBAs can be combined into a MTBA.	1. Can be inactivated by molecular and evolutive mechanisms. 2. Presence of metabolites from other organisms can surpass biocontainment. 3. Potential horizontal gene transfer events can surpass biocontainment mechanisms.
Multiplex-type approach (MTBA)	1. Designed with redundant or safeguard mechanisms for mutation tolerance. 2. Orthogonal circuits that respond to specific environmental inputs to induce cell death outside the production facility. 3. Can combine molecular mechanisms targeting different cellular components. 4. Can apply synthetic molecules or biological components.	1. Complex design and implementation. 2. Lower cell fitness and promotes the selection of inactive biocontainment devices. 3. Not extensively tested outside production facilities.

2. Uniplex-type biocontainment approaches

2.1. Natural auxotrophic markers

Natural auxotrophy (Figure 1) is a simple and widespread UTBA technique commonly applied to prevent the releasing and proliferation of GMYs outside the production facility. In general terms, natural auxotrophy can be defined as a nutritional deficiency induced by mutated genes resulting in an GMY strain that depends on the addition of the nutrient on its growth media [39]. The yeast *S. cerevisiae* has been used as a model for auxotrophic markers due to the facility of inducing deletion and/or point mutations in different genes associated with nutrient metabolism [39]. In this sense, genes linked to the metabolism of amino acids like L-histidine, L-leucine, L-tryptophan, and L-methionine [39], and nitrogen bases (e.g., adenine and uracil) [40] have been used as auxotrophic markers for decades.

Considering nitrogen base auxotrophies, the allele *ade2-1,* which contains a nonsense mutation (Glu64STOP) [41], is widely found in different laboratorial *S. cerevisiae* strains and confer adenine dependence. A major phenotypic characteristic of *ade2-1* is the development of a red ochre color due to the intracellular accumulation of oxidized adenine-associated metabolic intermediates [40,42], a phenotype that can be useful for red-white colony screening [43], redox biology [44] and drug discovery [40].

The depletion of adenine reserves in yeast cells increase trehalose synthesis and lead to cell cycle arrest, recapitulating the protective effects observed for desiccation stress tolerance. As a consequence, the adenine-deficient cells become viable for a longer period of time and evade biocontainment [42]. Complementally, it was observed that adenine auxotrophy increases mutagenesis rate in yeast cells [45], a condition that is observed for other auxotrophic markers like *leu2* and/or *lys2* alleles. Yeast cells carrying *leu2* and/or *lys2* alleles, when subjected to L-lysine or L-leucine starvation, display an increase in the number of respiratory deficient cells (*rho*$^-$ cells) due to the accumulation of mutations in mitochondrial genome, a condition termed "adaptive mutation" [46]. Additionally, adaptive mutations are linked to auxotrophy marker reversion to the wild-type state, as observed for *his4* marker in yeast under selective pressure [47].

In order to avoid the reversion of auxotrophic markers, a series of deletion gene markers have been generated for *S. cerevisiae* [48], as well as for other non-*Saccharomyces* species, like *Kluyveromyces lactis* [49]. However, it has been observed in some non-*Saccharomyces* species that auxotrophic gene deletion leads to a bradytroph/leak auxotroph phenotype, like those observed for *PHA2* in *Pichia pastoris,* which is linked to L-phenylalanine biosynthesis [50]. In this case, the auxotrophic *phe*$^-$ cells

are able to survive under L-phenylalanine starvation due to alternative L-phenylalanine biosynthesis mechanisms [50].

Considering the impact of auxotrophy in yeast metabolism, the effect of adaptive mutation in the selection of prototrophic cells, and bradytrophy due to the presence of poorly described biochemical pathways in non-*Saccharomyces* species, natural auxotrophy *per se* has strong limitations regarding its use as a biocontainment strategy. In addition, the presence of other microorganisms in the environment and/or production facility (Figure 1) can provide the auxotrophy-dependent nutrients for GMY mutants, bypassing the auxotrophy requirement [51].

A solution to overcome the natural auxotrophies limitations is the use of synthetic amino acids and nitrogen bases not available outside the production facility and can not be biologically supplied. This approach, termed "xenobiology" or "synthetic auxotrophies" (Figure 1), make use of top-down synthetic biology techniques to engineering GMYs and create new biocontainment strategies [35]. Another approach to circumvent the limitations of natural auxotrophies is the use of a so-called "conditional gene essentiality" (Figure 1), which employs promoter engineering to modify genes linked to nutrient metabolism and strictly regulate their expression by using synthetic molecules and/or orthogonal RNA polymerases [52].

2.2. Xenobiology and synthetic auxotrophies

Xenobiology focuses on the development of synthetic biological devices and systems that utilize non-canonical amino acids (ncAAs), nucleic acids with non-standard sugar backbone (xeno-nucleic acids or XNA) and non-natural nitrogen base pairs for different purposes, including the design of synthetic metabolic processes (neo-metabolism) [53], and biocontainment [52]. Many of xenobiology devices are designed by principle using orthogonality where synthetic components (e.g., proteins, RNAs, DNAs, and small molecules) are engineered for a purposed function and will not interfere with the natural biochemistry of a host cell [53,54]. The orthogonality also ensures that these components will not be used by natural biological systems, making it useful as a biocontainment strategy [55,56].

Biocontainment-based xenobiology is mostly centered on the use of different ncAAs for protein synthesis for both GEM and GMY. For example, the application of genetic code expansion (GCE) or orthogonal translation systems (OTSs) techniques [57,58] has been used with success in *Escherichia coli* biocontainment [56].

In bacteria, GCE/OTS relies on a orthogonal pyrrolysyl-tRNA synthetase/$tRNA^{Pyl}_{CUA}$ (PylRS/$tRNA^{Pyl}_{CUA}$) pair derived from *Methanosarcina barkeri, M. mazei* or *Methanocaldococcus jannaschii* to incorporate ncAAs in proteins [59,60], in the reassignment of the UAG amber codon, a rare stop codon in both *E. coli* and *S. cerevisiae* [61], and the deletion of the release factor 1 (RF1). Considering GCE for *E. coli* biocontainment, the genome of this bacterium was refactored by the introduction of a reassigned UAG codon into 22 essential genes together with a *Methanocaldococcus jannaschii* PylRS that is able to incorporate L-phenylalanine derivatives into $tRNA^{Pyl}_{CUA}$ [56], generating a synthetic auxotrophy. Data from this work indicated that the synthetic *E. coli* auxotroph cells were dependent on the addition of L-phenylalanine derivatives; moreover, these synthetic auxotrophs have undetectable escape frequencies in both solid and liquid culture media [56]. Additional work related to the creation of *E. coli* synthetic auxotroph strains have been made [38], pointing to the feasibility of this biocontainment technique for bacteria.

The generation of yeast ncAAS-dependent synthetic auxotrophs was achieved with very limited success [58,62]. Yeast are naturally able to incorporate ncAAs into proteins [61,63] and a GCE/OTS technique for *S. cerevisiae* was developed by Chin et al. [62]. In this work, the authors engineering orthogonal codons, anticodons and tRNA synthetase, including an *E. coli* tyrosine-tRNA synthetase (TyrRS) and a amber suppressor $tRNA^{Tyr}_{CUA}$, to generate a library of TyrRS mutants that pairs only with ncAAs [62]. Once this TyrRS library was transformed into a *S. cerevisiae* strain, five different ncAAs were incorporated into human superoxide dismutase 1 protein (hSOD1) [62].

Other relevant works related to the development of GCE/OTS for *S. cerevisiae* and *Pichia pastoris* have been made by using the *E. coli* TyrRS/$tRNA^{Tyr}_{CUA}$ or leucyl- (Leu)RS/$tRNA^{Leu5}_{CUA}$ pairs for ncAA

incorporation into proteins [60,64–72]. However, the use of GCE/OTS in yeast have some major challenges, including the low expression of $tRNA^{Pyl}_{CUA}$ in yeast due to the absence of intragenic promoter sequences A- and B-boxes [58,60]; moreover, the eukaryotic release factor 1 (eRF1), codified in yeast by the *SUP45* essential gene [73], compose the Sup45p-Sup35p complex that is necessary to end translation by binding into all three stop codons in yeast cells [74]. Comparatively, the *E. coli* RF1, which recognizes the UAG/UAA codons, can be deleted without major physiological impacts into the cell due to the functional superimposition with the release factor 2 (RF2) [75].

In order to circumvent the limitations inherently associated with the implementation of a GCE/OST-based biocontainment in GMY, different approaches were applied, like (i) prospecting new archeal PylRS with higher ncAA incorporation efficiency in *S. cerevisiae* [76], (ii) selecting yeast strains with specific mutations (e.g., *yil014c-a*Δ and *alo1*Δ) for increasing ncAAs incorporation into $tRNA^{Pyl}_{CUA}$ [77], (iii) improving *E. coli* TyrRS and LeuRS with enhanced ncAAs polyspecificity and efficiency by using random mutagenesis and directed selection [78], (iv) the use of an OTS based on recognition of quadruplet codons by an engineered orthogonal ribosome that allows to expand the genetic code to 256 codons; this quadruplet codon-base OTS have been implemented with more or less success in *E. coli* [58,79,80] and mammalian cells [81], and (v), the use of synthetic/unnatural nitrogen base pairs (UBPs) to expand the genetic code and increase the repertoire of ncAAs that can be used for protein synthesis in *E. coli* [82].

There are different approaches when considering the use of UPBs and XNAs for xenobiology and GMY/GEM biocontainment [83]. In fact, it is expected that XNA technology could be efficient for GMY/GEM biocontainment since the building blocks of XNAs (*e.g.*, nucleobases, sugar moieties and phosphate-modified groups) can not be find in natural environments and the GMY/GEM cells should be able to incorporate these building blocks into new XNA polymers by the usage of specialized polymerization enzymes and transmembrane proteins able to uptake this precursors [84,85]. Thus, UBPs and XNAs can be like a "genetic firewall" [84], where HGT events could be avoided to the restrained aspects of XNA technology.

The developments on XNA technologies follows two major mainstreams: (i) the use of unnatural nucleobases, sugar moieties and phosphate-modified groups (XNA substrates) to incorporate into XNAs or hybrid XNA/DNA/RNA polymers by canonical DNA and/or RNA polymerases [55,86] and (ii) design new DNA and/or RNA enzymes (XNAzymes) able to metabolize XNA polymers and/or substrates with an "alien" chemistry, like threose nucleic acid (TNA), cyclohexenyl nucleic acid (CeNA), arabino nucleic acid (ANA), 2'-fluoro-arabino nucleic acid (FANA), glycol nucleic acid (GNA), and locked nucleic acid (LNA) [87–94]. In all cases, the XNAs should display orthogonality in vivo with little or, preferentially, none interaction with the canonical components of DNA/RNA metabolism [95]. Unfortunately, many different XNA technologies were not implemented *in vivo*, which renders its usage for GMY/GEM biocontainment still far from technical and practical viewpoint [96]. Finally, both GCE/OTS and XNA technologies were not tested in an open and uncontrolled environment (Figure 1), making its behavior unpredictable for real biocontainment applications [26].

Another approach related to the induction of synthetic auxotrophies in *E. coli* is based on the selection of essential proteins whose structure and activity is dependent on the presence of a small molecule ligands, like the so called "synthetic auxotrophs based on ligand-dependent essential genes" (SLiDE) technique [97]. In this work, the authors were able to select five essential genes in *E. coli* by applying protein engineering and saturation mutagenesis and generate proteins dependent on benzothiazole. The authors related a very low escape frequency ($< 3 \times 10^{-11}$) under laboratory assays [97]. Similar works on bacterial synthetic auxotrophs have been made, including phosphite-dependent *Synechococcus elongatus* [98] and *Pseudomonas putida* [99]. In *S. cerevisiae* it has been identified a series of mutations in *CDC10* gene, which codify an essential septin protein that can be rescued in the presence of small molecules, like guanidinium ion [100,101]. Although the authors of this work have not applied the *CDC10* conditional mutants for biocontainment, the data may indicate new techniques based on chemical rescue for GMY biocontainment.

2.3. Conditional gene essentiality

The conditional essentiality is, similarly to auxotrophy or xenobiology, a plethora of molecular techniques where essential genes that codify for proteins and enzymes needed for cell growth and maintenance are modified to have its expression regulated by external agents [52]. In this regard, the conditional gene essentiality has been applied for biocontainment in *S. cerevisiae, E. coli* and other bacteria with a low escape frequency (Figure 1) [27,52]. In *S. cerevisiae,* conditional gene essentiality was employed to regulate the expression of histone genes [27], fatty acid synthetase, mitochondrial and cytoplasmic histidine tRNA synthetase (HisRS), RNA polymerase II subunit B, and interorganellar chaperones and GTPases related to vesicular transport and fusion [102]. These genes have their original regulatory sequences replaced by a combination of promoters whose activities are modulated by galactose and estradiol [27]. Moreover, the orthogonality is ensured in this biocontainment system by providing a "fail-safe" recombination-induced lethality mechanism based on small molecule dependent Cre recombinase [27,103].

2.4. Orthogonal DNA replication and RNA transcription

Another potential strategy for GMY biocontainment is to implement orthogonal DNA and RNA polymerases [51]. In yeast, an orthogonal DNA replication system derived from the *Kluyveromyces lactis* cytoplasmic plasmid pGKL1/2 named "OrthoRep" have been applied for studies of in vivo continuous evolution of target genes due to the high error-prone activity of DNA polymerase 1 (DNAP1) and DNA polymerase 2 (DNAP2), both codified by pGLK1/2 [104–106]. Similar in vitro works were also described by using the orthogonal DNA polymerase derived from bacteriophage phi29 to generate a self-contained synthetic transcription and translation-coupled DNA replication system [107–109]. Additionally, orthogonal DNA replication systems, including XNA polymerases and/or engineered DNA polymerases [110], are being developed for xenobiology applications. However, the use of different orthogonal DNA replication mechanisms as a biocontainment system for GMY/GEM was not reported until now, pointing to an unexplored research field (Figure 1).

Like orthogonal DNA replication systems, orthogonal RNA transcription systems are also underexplored in the context of GMY/GEM biocontainment. Some examples include orthogonal genic circuits based on the use of bacteriophage T7 RNA polymerase in bacteria and yeast for different biotechnological purposes [111,112]. However, no biocontainment applications using T7 RNA polymerase-based techniques for GMY/GEM were described until now (Figure 1).

2.5. Nuclease-based kill switches

Kill switches are defined as gene circuits activated by specific environmental inputs, resulting in the expression of lethal genes that lead to cell death [113]. Different kill switch designs have been used for GEM biocontainment (Figure 1), including gene circuits that respond to environment changes by activating/deactivating a type II toxin-antitoxin system CcdB/CcdA [113]. Besides it, specific and unspecific nucleases, auxotrophies, as well as type I toxin-antitoxin pairs have been employed to engineer kill switches circuits for biocontainment purposes, many of them in a MTBA format (Table 1) [56,114–118] in order to reduce the GEM escape frequency.

Considering nucleases for kill switch design, it has been shown its usefulness for bacteria biocontainment. For example, the expression of *Eco*RI endonuclease-*Eco*RI methylase pair combined with conditional gene essentiality in *E. coli* considerably lowers the escape frequency [117]. In this sense, combining *Eco*RI and mf-Lon protease in a strictly regulated gene circuit allowed the development of the "Deadman" kill switch, which considerably lowered the escape frequency associated with a high genetic stability [114]. Other non-specific nucleases, like *nucA* from *Serratia marcescens* [119] or nuclease A from *Staphylococcus aureus* have been employed in different bacterial biocontainment projects [118].

In *S. cerevisiae,* a kill switch based on the conditional expression of *S. marcescens nucA* under the control of a glucose-repressed *ADH2*/*GAPDH* hybrid promoter has been proposed [120]. This work showed that under non repressible conditions, the *nucA* was efficiently expressed leading to yeast cell death in both laboratorial and soil microcosm assays [120]. Interestingly, type II restriction enzymes have been expressed in yeast to study DNA damage and repair mechanisms [121–123]. Data

from the expression of type II restriction enzymes in yeast cells point to a low survival and DNA damage tolerance, specially when blunt ends are induced by type II restriction enzymes (e.g., *Pvu*II) [123]; however, the use of type II restriction enzymes as part of a kill switch mechanism in yeast cells for biocontainment purposes was not described until now.

In addition to conventional exo- and endonucleases, the CRISPR-associated nucleases (Cas) that have been used in *E. coli* to design kill switches. These kill switches gene circuits make use of Cas3 [124] or Cas9 [125] and were successfully used to biocontain *E. coli* cells, both displaying an escape frequency ≤ 10^{-8} cells. A major advantage of the Cas-based kill switches is the use of guide RNAS (gRNAs) to select specific DNA sequence targets or microorganism strains, allowing to selectively eliminate the target strain from microbiome [125,126]. Until now, no Cas-based kill switches were reported for GMY.

2.6. Kill switches based on type I and II toxin-antitoxin systems

Another kill switch extensively applied in GEM biocontainment is based on type I and II toxin-antitoxin (TA) systems. These kill switch-based TA systems efficiently lead to controlled cell death outside the production facility and can be easily engineered in GEM/GYM cells (Figure 1). What makes the TA systems so attractive for biocontainment applications is its pleiotropy, which targets different molecular mechanisms, like DNA and mRNA synthesis, cell cycle, nucleotide synthesis, and protein translation [127,128].

The prokaryotic TA system is classified into seven types numbered from I to VII [130], which act in different cell mechanisms. For example, type I toxin-antitoxin system prevents toxin RNA translation through the binding of sRNA [129], while type II toxin-antitoxin system is based on the interaction of an endoribonuclease and an inhibitor, forming a stable complex [131]. By its turn, type III toxin-antitoxin system consists of the inhibition of the toxin protein by an antitoxin RNA [132]. Type IV toxin-antitoxin system is composed of two proteins that do not form a complex; instead, the antitoxin acts as an antagonist of the toxin at its cellular targets [133]. The type V toxin-antitoxin system differs from the others by having its antitoxin cleave the toxin mRNA [134]. Type VI is composed of an antitoxin-based proteolytic adapter that degrades the toxin-based protein [135], while type VII is composed of an antitoxin that enzymatically neutralizes the toxin by post-translational modification [130].

Type I and II are historically employed for biocontainment gene circuit design, especially for *E. coli* [136]. For example, the type I *hok-sok* pair have been applied for the design of conditional suicide of plasmid-containing *E. coli* cells in phosphate- [137] or tryptophan-limited [136] environments. Additionally, type I TA systems have been used in synthetic biology projects related to GEM biocontainment [118]. Similarly, type II TA systems have been applied into kill switch circuits based on the use of *ccdB-ccdA* pair regulated by a bi-stable cI/Cro memory switch ('essentializer' circuit) and by a cold-inducible promoter ('cryodeath' circuit) [113]. Finally, type II TA systems have been applied for the design of 'plasmid addiction' systems to prevent the lost of plasmids under production facility [138]

In addition to GEM, the use of type I and I TA systems was proven to be effective for GMY biocontainment, like the expression of the *relE-relB* system in *S. cerevisiae* [139]. When not being neutralized by the RelB antitoxin, the RelE toxin inhibits protein synthesis by cleaving mRNA that are being translated on the ribosome [140]. When RelB is neutralizing RelE it displaces the toxin''s C-terminal and forms the RelBE complex [141]. To express this TA system in *S. cerevisiae* two recombinant plasmids were constructed, one containing the *relE* toxin gene under the control of galactose-induced *GAL1* promoter (pKP727) and another containing *relE* regulated by *GAL1* promoter and the *relB* antitoxin gene under the control of methionine-repressible *MET25* promoter (pKP1006). Yeast cells transformed with the plasmid pKP727 showed visible growth inhibitory effects induced by the *relE* toxin; on the other hand, yeast transformed with the plasmid pKP1006 showed a higher growth rate. This data indicates that the toxic effects of *relE* can be minimized by *relB* antitoxin in yeast [139]. Similar to the *relE-relB* system, the ribonuclease-associated toxin Kid (killing determinant) and its antitoxin Kis (killing suppressor) [142] have been used in *S. cerevisiae* as a

potential biocontainment system. For this purpose, an expression system containing the antitoxin Kis controlled by the *MET25* promoter and the Kid toxin controlled by the Cu^{2+}-induced *CUP1* promoter was cloned into the recombinant integrative plasmid pRS303. In the presence of both methionine and Cu^{2+}, the expression of Kis antitoxin is inhibited while the expression of Kid toxin is induced, leading to cell death [143].

Another TA system already tested in yeast is based on the ε-ζ (*epsilon-zeta)* genes from the gram-positive bacteria *Streptococcus pyogenes* [144]. The ε-ζ genes are organized in an operon together with the ω (*omega*) gene, which codify a repressor that modulates the transcription of the ζ toxin, ε antitoxin and its own [144]. This TA system was expressed in a yeast two-hybrid system without including the ω sequence, through which the toxicity of the ζ protein was shown as well as the efficiency of the ε protein in antagonizing its toxin [145]. Despite the efficiency of ε-ζ pair in to induce yeast cell death, its use as a biocontainment system for GMY was not attempted until now.

Finally, it was observed that the expression of the *chpK-chpI* TA pair from *Lepstospira interrogans* in *E. coli* and *S. cerevisiae* modulates their cell growth [146,147]. However, the application of *chpK-chpI* TA pair as a biocontainment system was not reported until now. In addition to *S. cerevisiae*, the expression of endoribonuclease-associated toxin *mazF* from the *E. coli mazEF* module [148] in *Pichia pastoris* result in cell death and can be useful as a counter-selectable marker for genetic modification [149].

3. Multiplex-type biocontainment approaches

Multiplex-type biocontainment approaches (MTBA) (Table 1) have several advantages over UTBAs. First, the combined use of different molecular mechanisms can reduce the selective evolutionary pressure observed for the majority of UTBAs that results in mutagenic drift, HGT events and small molecule supplementation by an ecologic niche that inactivate the biocontainment-associated gene or gene circuits [38]. Interestingly, an early work on *E. coli* showed that duplicating the biocontainment-associaciated gene could lower the mutational events that lead to biocontainment inactivation [150]. The same authors suggest that the use of multiple copies of biocontainment-associated genes, with different regulatory mechanisms and independent molecular targets could also diminish the populational evolutionary pressure to negatively select mutated, non-functional biocontainment circuits [150]. In this sense, the use of synthetic biology approaches, like redesign the organism genome by incorporating expanded genetic code alphabets, the application of orthogonal DNA/RNA devices and/or systems, or XNA technologies could be a solution to reduce the evolutionary pressure to inactivate the biocontainment in GMY/GEM populations [27,52–54,83,84]. Unfortunately, such approaches are far away for direct biocontainment applications in eukaryotic cells, especially yeasts. Even for prokaryotes, these technologies are mainly restricted to *E. coli* and many of them were never tested in non-laboratorial conditions. Even considering the production facility environment, it has been reported that synthetic transcriptional circuits based in the boolean gates 'AND' and 'NOR' have altered responses in laboratory-scale fermentations, negatively impacting both biomass production and gene circuit expression [151]. However, some successful applications of multiplex technologies in biocontainment were already described, which includes the 'Deadman' and 'Passcode' kill switches in *E. coli* [114] and the transcriptional and recombinational control of essential genes in *S. cerevisiae* [27]. Another approach is the use of overlapping 'safeguards' composed by engineered riboregulators that control the expression of essential genes and engineered nucleases that cleaves *E. coli* genome when in the absence of exogenously supplied synthetic small molecules [117]. These examples reinforce the idea that redundancy combined with the use of different molecular approaches are crucial factors for the design of a resilient biocontainment technology.

4. Conclusions and perspectives

More than ever, the development of new biocontainment technologies for GMY is imperative facing the availability of synthetic biology tools and/or fermentation technologies. Data gathered so far showed that the technologies available for GMY biocontainment are far away from what is

available for GEMs (e.g., *E. coli*). While technologies like xenobiology and MTBAs are available for *E. coli,* the same is not true for yeasts that mainly rely on natural auxotrophies or other non-redundant UTBAs for biocontainment. The major factors that contributes to the lack of new GMY-linked biocontainment technologies are the complexity of eukaryotic genome, the intracellular compartmentalization and biochemical specialization of organelles, as well as natural mechanisms that increase the genetic diversity of the yeast population, like meiosis or even non-homologous recombination mechanisms, which leads to the inactivation of biocontainment circuits. However, with the advances in top-down synthetic biology approaches for eukaryote genomes (e.g., 'Yeast 2.0'), it is expected that technologies like xenobiology and genetic code refactoring become available for the development of different UTBAs and MTBAs for GMY.

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