

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 patented yeast gene technologies spreading outside the production facility. In this review, the different biocontainment technologies currently available for GMYs were evaluated. Interestingly, uniplex-type biocontainment approaches (UTBAs), which rely on nutrient auxotrophies induced by gene mutation or deletion or the expression of the simple kill switches apparatus, are still the major biocontainment approaches in use with GMY. While bacteria such as *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.



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## 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, such as flavor enhancers, oligosaccharides, vitamins, and amino acids [1]. GEMs are also employed for food enzyme production, including enzymes such as lactase, amylase, proteases, and phospholipases [1,2]. Another biotechnological field that benefits from the application of GEMs is bioremediation, which allows for the removal of pollutants (e.g., heavy metals) from water and soil [3]; 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 their ability to grow on inexpensive culture media and them having well-established fermentative technology [10]. One of the most popular yeasts employed as a GEM is *Saccharomyces cerevisiae*. *S. cerevisiae* has many different

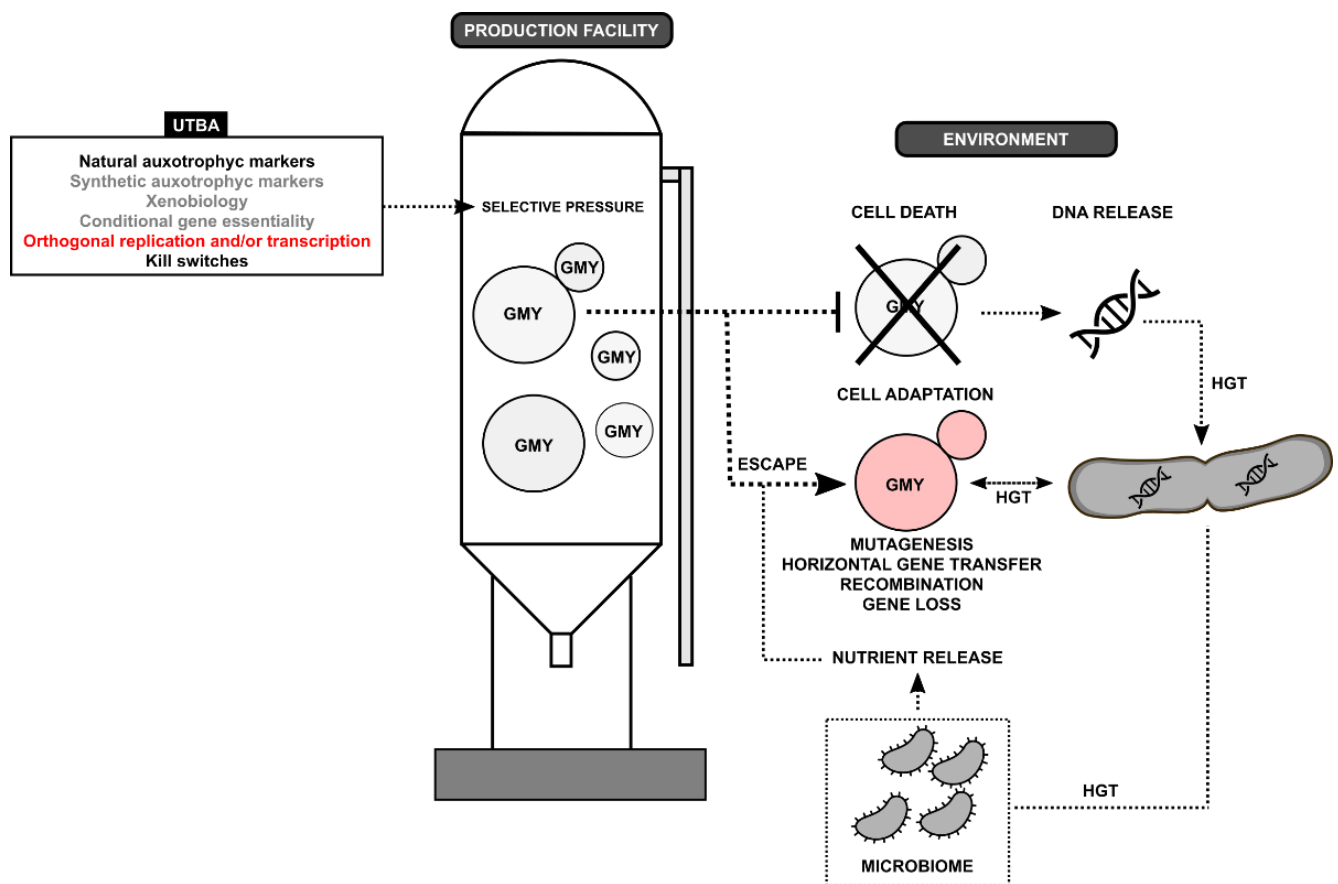
biotechnology applications, from the production of traditional fermented foods [11,12] to cell factories for the synthesis of chemicals and pharmaceuticals, such as bioethanol, propanol, butanol, artemisinic acid, and insulin precursor [10,13,14]. In addition to its biotechnological importance, *S. cerevisiae* 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 such as *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]. Methylotrophic yeasts (e.g., *Pichia pastoris* and *Ogataea polymorpha*) are very efficient in producing heterologous protein on an industrial scale, with many strains and molecular tools available for genetic/synthetic biology engineering [19,20]. Other examples of non-*Saccharomyces* species 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 the factory of any desired DNA sequence to “tailor-make” 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 (defined here as “production facilities”) [27,29] and restrict 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 spread of GMY/GEM into the environment (Figure 1). The National Institutes of Health (NIH) guidelines for research involving recombinant or synthetic nucleic acid molecules recommend an escape frequency of 1 cell per  $10^8$  cells as the 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) does 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 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 cannot be excluded, especially if those molecules confer a phenotype advantage to the native microbiome.

Different molecular approaches can be employed for GMY biocontainments, 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 failproof, since molecular and evolutive mecha-

nisms lead to the inactivation of biocontainment devices [36] (Figure 1). This is especially true when a biocontainment approach makes use of a single component/device (defined in this work as a “uniplex-type biocontainment approach” or UTBA; Table 1 and Figure 1), such as 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, the 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, such as mutagenesis, horizontal gene transfer (HGT), DNA recombination, and gene loss, result 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, 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 the production facility; gray, experimental technology not tested outside the production facility; red, technology not applied for GMYs and other microorganisms.

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

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

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

## 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 release 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 a 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 such as 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 confers 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 increases trehalose synthesis and leads 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 the mutagenesis rate in yeast cells [45], a condition that is observed for other auxotrophic markers such as *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*<sup>−</sup> cells) due to the accumulation of mutations in the 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 the *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, such as *Kluyveromyces lactis* [49]. However, it has been observed in some non-*Saccharomyces* species that auxotrophic gene deletion leads to a bradytroph/leak auxotroph phenotype, such as those observed for *PHA2* in *Pichia pastoris*, which is linked to L-phenylalanine biosynthesis [50]. In this case, the auxotrophic *phe*<sup>−</sup> 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 bradytroph 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 for overcoming the natural auxotrophies limitations is the use of synthetic amino acids and nitrogen bases that are not available outside the production facility and cannot be biologically supplied. This approach, termed “xenobiology” or “synthetic auxotrophies” (Figure 1), makes use of top-down synthetic biology techniques for engineering GMYs and creating new biocontainment strategies [35]. Another approach to circumventing 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 a 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 the 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 an orthogonal pyrrolysyl-tRNA synthetase/tRNA<sup>Pyl</sup><sub>CUA</sub> (PylRS/tRNA<sup>Pyl</sup><sub>CUA</sub>) 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 in 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<sup>Pyl</sup><sub>CUA</sub> [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 has been carried out [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]. Yeasts 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 engineered orthogonal codons, anticodons, and tRNA synthetases, including an *E. coli* tyrosine-tRNA synthetase (TyrRS) and an amber suppressor tRNA<sup>Tyr</sup><sub>CUA</sub>, to generate a library of TyrRS mutants that pair only with ncAAs [62]. Once this TyrRS library was transformed into an *S. cerevisiae* strain, five different ncAAs were incorporated into the 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 conducted by using the *E. coli* TyrRS/tRNA<sup>Tyr</sup><sub>CUA</sub> or leucyl-(Leu)RS/tRNA<sup>Leu5</sup><sub>CUA</sub> pairs for ncAA incorporation into proteins [60,64–72]. However, the use of GCE/OTS in yeast has some major challenges, including the low expression of tRNA<sup>Pyl</sup><sub>CUA</sub> 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], composes 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, such as (i) prospecting new archeal PylRS with higher ncAA incorporation efficiency in *S. cerevisiae* [76], (ii) selecting yeast strains with specific mutations (e.g., *yil014c-aΔ* and *alo1Δ*) for increasing ncAAs incorporation into tRNA<sup>Pyl</sup><sub>CUA</sub> [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 the recognition of quadruplet codons by an engineered orthogonal ribosome that allows for the expansion of the genetic code to 256 codons (this quadruplet codon-base OTS has been implemented more or less successfully 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) cannot be found 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 that are able to take up these precursors [84,85]. Thus, UBPs and XNAs can be like a “genetic firewall” [84], where HGT events could be avoided regarding the restrained aspects of XNA technology.

The developments of XNA technologies follow 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) the design of new DNA and/or RNA enzymes (XNAzymes) that are able to metabolize XNA polymers and/or substrates with an “alien” chemistry, such as 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, no interaction with the canonical components of the DNA/RNA metabolism [95]. Unfortunately, many different XNA technologies were not implemented in vivo, which renders their usage for GMY/GEM biocontainment still far away from a technical and practical viewpoint [96]. Finally, both GCE/OTS and XNA technologies were not tested in an open and uncontrolled environment (Figure 1), making their 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 are dependent on the presence of small molecule ligands, such as 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 conducted, including phosphite-dependent *Synechococcus elongatus* [98] and *Pseudomonas putida* [99]. In *S. cerevisiae*, a series of mutations in the *CDC10* gene have been identified, which codify an essential septin protein that can be rescued in the presence of small molecules, such as 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, similar 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”, has been applied for studies of the 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 environmental changes by activating/deactivating a type II toxin–antitoxin system CcdB/CcdA [113]. Besides this, specific and unspecific nucleases, auxotrophies, as well as type I toxin–antitoxin pairs have been employed to engineer kill switch circuits for biocontainment purposes, many of them in an MTBA format (Table 1) [34,56,114–117] in order to reduce the GEM escape frequency.

Considering nucleases for kill switch design, its usefulness for bacteria biocontainment has been shown. For example, the expression of the *EcoRI* endonuclease–*EcoRI* methylase pair combined with conditional gene essentiality in *E. coli* considerably lowers the escape frequency [34]. In this sense, combining *EcoRI* and *mf-Lon* protease in a strictly regulated gene circuit allowed for the development of the “Deadman” kill switch, which considerably lowered the escape frequency associated with a high genetic stability [114]. Other non-specific nucleases, such as *nucA* from *Serratia marcescens* [118] or nuclease A from *Staphylococcus aureus*, have been employed in different bacterial biocontainment projects [117].

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 [119]. 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 [119]. Interestingly, type II restriction enzymes have been expressed in yeast to study DNA damage and repair mechanisms [120–122]. Data from the expression of type II restriction enzymes in yeast cells point to a low survival and DNA damage tolerance, especially when blunt ends are induced by type II restriction enzymes (e.g., *PvuII*) [122]; 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) have been used in *E. coli* to design kill switches. These kill switch gene circuits make use of Cas3 [123] or Cas9 [124] and were successfully used to biocontain *E. coli* cells, both displaying an escape frequency  $\leq 10^{-8}$  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 for selectively eliminating the target strain from the microbiome [124,125]. 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 their pleiotropy, which targets different molecular mechanisms, such as DNA and mRNA synthesis, the cell cycle, nucleotide synthesis, and protein translation [126,127].

The prokaryotic TA system is classified into seven types numbered from I to VII [128,129], which act in different cell mechanisms. For example, the type I toxin–antitoxin system prevents toxin RNA translation through the binding of sRNA [128], while the type II toxin–antitoxin system is based on the interaction of an endoribonuclease and an inhibitor, forming a stable complex [130]. Type III toxin–antitoxin systems consist of the inhibition of the toxin protein by an antitoxin RNA [131]. Type IV toxin–antitoxin systems are composed of two proteins that do not form a complex; instead, the antitoxin acts as an antagonist of the toxin at its cellular targets [132]. The type V toxin–antitoxin system differs from the others by having its antitoxin cleave the toxin mRNA [133]. Type VI is composed of an antitoxin-based proteolytic adapter that degrades the toxin-based protein [134], while type VII is composed of an antitoxin that enzymatically neutralizes the toxin by post-translational modification [129].

Type I and II are historically employed for biocontainment gene circuit design, especially for *E. coli* [135]. For example, the type I *hok-sok* pair has been applied for the design of the conditional suicide of plasmid-containing *E. coli* cells in phosphate- [136] or tryptophan-limited [135] environments. Additionally, type I TA systems have been



used in synthetic biology projects related to GEM biocontainment [117]. Similarly, type II TA systems have been applied into kill switch circuits based on the use of the *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 loss of plasmids under the production facility [137].

In addition to GEM, the use of type I and II TA systems was proven to be effective for GMY biocontainment, such as the expression of the *relE-relB* system in *S. cerevisiae* [138]. When not being neutralized by the RelB antitoxin, the RelE toxin inhibits protein synthesis by cleaving mRNA that are being translated on the ribosome [139]. When RelB is neutralizing RelE, it displaces the toxin’s C-terminal and forms the RelBE complex [140]. To express this TA system in *S. cerevisiae*, two recombinant plasmids were constructed, one containing the *relE* toxin gene under the control of the galactose-induced *GAL1* promoter (pKP727) and another containing *relE*, regulated by the *GAL1* promoter, and the *relB* antitoxin gene, under the control of the 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. These data indicate that the toxic effects of *relE* can be minimized by the *relB* antitoxin in yeast [138]. Similar to the *relE-relB* system, the ribonuclease-associated toxin Kid (killing determinant) and its antitoxin Kis (killing suppressor) [141] 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  $\text{Cu}^{2+}$ -induced *CUP1* promoter was cloned into the recombinant integrative plasmid pRS303. In the presence of both methionine and  $\text{Cu}^{2+}$ , the expression of the Kis antitoxin is inhibited, while the expression of the Kid toxin is induced, leading to cell death [142].

Another TA system already tested in yeast is based on the  $\epsilon$ - $\zeta$  (*epsilon-zeta*) genes from the gram-positive bacteria *Streptococcus pyogenes* [143]. The  $\epsilon$ - $\zeta$  genes are organized in an operon together with the  $\omega$  (*omega*) gene, which codify a repressor that modulates the transcription of the  $\zeta$  toxin, the  $\epsilon$  antitoxin, and its own [143]. This TA system was expressed in a yeast two-hybrid system without including the  $\omega$  sequence, through which the toxicity of the  $\zeta$  protein was shown as well as the efficiency of the  $\epsilon$  protein in antagonizing its toxin [144]. Despite the efficiency of the  $\epsilon$ - $\zeta$  pair in inducing 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 [145,146]. However, the application of the *chpK-chpI* TA pair as a biocontainment system was not reported until now. In addition to *S. cerevisiae*, the expression of the endoribonuclease-associated toxin *mazF* from the *E. coli mazEF* module [147] in *Pichia pastoris* results in cell death and can be useful as a counter-selectable marker for genetic modification [148].

### 3. Multiplex-Type Biocontainment Approaches

Multiplex-type biocontainment approaches (MTBA) (Table 1) have several advantages over UTBA. First, the combined use of different molecular mechanisms can reduce the selective evolutionary pressure observed for the majority of UTBA that results in mutagenic drift, HGT events, and small molecule supplementation by an ecologic niche that inactivates the biocontainment-associated gene or gene circuits [38]. Interestingly, an early work on *E. coli* showed that duplicating the biocontainment-associated gene could lower the mutational events that lead to biocontainment inactivation [149]. 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 [149]. In this sense, the use of synthetic biology approaches, such as redesigning 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 for reducing 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 on the Boolean gates “AND” and “NOR” have altered responses in laboratory-scale fermentations, negatively impacting both biomass production and gene circuit expression [150]. However, some successful applications of multiplex technologies in biocontainment were already described, which include 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 of engineered riboregulators that control the expression of essential genes and engineered nucleases that cleave the *E. coli* genome in the absence of exogenously supplied synthetic small molecules [34]. 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. The 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 such as 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 contribute to the lack of new GMY-linked biocontainment technologies are the complexity of the eukaryotic genome, the intracellular compartmentalization and biochemical specialization of organelles, as well as natural mechanisms that increase the genetic diversity of the yeast population, such as meiosis or even non-homologous recombination mechanisms, which lead 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 such as xenobiology and genetic code refactoring will become available for the development of different UTBAs and MTBAs for GMY.

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#### References

1. Hanlon, P.; Sewalt, V. GEMs: Genetically Engineered Microorganisms and the Regulatory Oversight of Their Uses in Modern Food Production. *Crit. Rev. Food Sci. Nutr.* **2021**, *61*, 959–970. [[CrossRef](#)] [[PubMed](#)]
2. Olempska-Beer, Z.S.; Merker, R.I.; Ditto, M.D.; DiNovi, M.J. Food-Processing Enzymes from Recombinant Microorganisms—A Review. *Regul. Toxicol. Pharmacol.* **2006**, *45*, 144–158. [[CrossRef](#)] [[PubMed](#)]
3. Chen, R.; Tu, H.; Chen, T. Potential Application of Living Microorganisms in the Detoxification of Heavy Metals. *Foods* **2022**, *11*, 1905. [[CrossRef](#)] [[PubMed](#)]
4. Singh, V.K.; Rai, S.; Singh, D.; Upadhyay, R.S. Application of Soil Microorganisms for Agricultural and Environmental Sustainability: A Review. In *Plant, Soil and Microbes in Tropical Ecosystems*; Dubey, S.K., Verma, S.K., Eds.; Rhizosphere Biology; Springer: Singapore, 2021; pp. 151–175; ISBN 9789811633645.
5. Shin, M.-K.; Yoo, H.S. Animal Vaccines Based on Orally Presented Yeast Recombinants. *Vaccine* **2013**, *31*, 4287–4292. [[CrossRef](#)] [[PubMed](#)]

6. Adetunji, C.O.; Anani, O.A.; Olaniyan, O.T.; Bodunrinde, R.E.; Osemwegie, O.O.; Ubi, B.E. Chapter 14—Sustainability of Biofertilizers and Other Allied Products from Genetically Modified Microorganisms. In *Biomass, Biofuels, Biochemicals*; Varjani, S., Pandey, A., Bhaskar, T., Mohan, S.V., Tsang, D.C.W., Eds.; Elsevier: Amsterdam, The Netherlands, 2022; pp. 363–393; ISBN 978-0-323-89855-3.
7. Ali, R.; Zulaykha, K.D.; Sajjad, N. Genetically Modified Microbes as Biofertilizers. In *Bioremediation and Biotechnology, Vol 4: Techniques for Noxious Substances Remediation*; Bhat, R.A., Hakeem, K.R., Eds.; Springer Nature: Cham, Switzerland, 2020; pp. 275–293; ISBN 978-3-030-48690-7.
8. Kebede, G.; Abera, S.; Haregu, S.; Yeshitila, A.; Palanivel, H. Genetically Modified Bacteria for Alleviating Agrochemical Impact on the Environment. In *Agrochemicals in Soil and Environment: Impacts and Remediation*; Naeem, M., Bremont, J.F.J., Ansari, A.A., Gill, S.S., Eds.; Springer Nature: Singapore, 2022; pp. 565–583; ISBN 9789811693106.
9. Ruchala, J.; Kurylenko, O.O.; Dmytruk, K.V.; Sibirny, A.A. Construction of Advanced Producers of First- and Second-Generation Ethanol in *Saccharomyces cerevisiae* and Selected Species of Non-Conventional Yeasts (*Scheffersomyces stipitis*, *Ogataea polymorpha*). *J. Ind. Microbiol. Biotechnol.* **2020**, *47*, 109–132. [[CrossRef](#)]
10. Wang, G.; Huang, M.; Nielsen, J. Exploring the Potential of *Saccharomyces cerevisiae* for Biopharmaceutical Protein Production. *Curr. Opin. Biotechnol.* **2017**, *48*, 77–84. [[CrossRef](#)] [[PubMed](#)]
11. Kavšček, M.; Stražar, M.; Curk, T.; Natter, K.; Petrovič, U. Yeast as a Cell Factory: Current State and Perspectives. *Microb. Cell Factories.* **2015**, *14*, 94. [[CrossRef](#)]
12. Budroni, M.; Zara, G.; Ciani, M.; Comitini, F. *Saccharomyces* and Non-*Saccharomyces* Starter Yeasts. In *Brewing Technology*; IntechOpen: London, UK, 2017; ISBN 978-953-51-3342-1. [[CrossRef](#)]
13. Parapouli, M.; Vasileiadis, A.; Afendra, A.-S.; Hatziloukas, E. *Saccharomyces cerevisiae* and Its Industrial Applications. *AIMS Microbiol.* **2020**, *6*, 1–31. [[CrossRef](#)]
14. Nielsen, J. Production of Biopharmaceutical Proteins by Yeast. *Bioengineered* **2013**, *4*, 207–211. [[CrossRef](#)]
15. Petranovic, D.; Nielsen, J. Can Yeast Systems Biology Contribute to the Understanding of Human Disease? *Trends Biotechnol.* **2008**, *26*, 584–590. [[CrossRef](#)]
16. Mustacchi, R.; Hohmann, S.; Nielsen, J. Yeast Systems Biology to Unravel the Network of Life. *Yeast* **2006**, *23*, 227–238. [[CrossRef](#)] [[PubMed](#)]
17. Andreu, C.; Zarnowski, R.; Del Olmo, M. Recent Developments in the Biology and Biotechnological Applications of Halotolerant Yeasts. *World J. Microbiol. Biotechnol.* **2022**, *38*, 27. [[CrossRef](#)]
18. Strucko, T.; Andersen, N.L.; Mahler, M.R.; Martínez, J.L.; Mortensen, U.H. A CRISPR/Cas9 Method Facilitates Efficient Oligo-Mediated Gene Editing in *Debaryomyces hansenii*. *Synth. Biol.* **2021**, *6*, ysab031. [[CrossRef](#)] [[PubMed](#)]
19. Potvin, G.; Ahmad, A.; Zhang, Z. Bioprocess Engineering Aspects of Heterologous Protein Production in *Pichia pastoris*: A Review. *Biochem. Eng. J.* **2012**, *64*, 91–105. [[CrossRef](#)]
20. Yan, C.; Yu, W.; Zhai, X.; Yao, L.; Guo, X.; Gao, J.; Zhou, Y.J. Characterizing and Engineering Promoters for Metabolic Engineering of *Ogataea polymorpha*. *Synth. Syst. Biotechnol.* **2022**, *7*, 498–505. [[CrossRef](#)] [[PubMed](#)]
21. Gellissen, G.; Kunze, G.; Gaillardin, C.; Cregg, J.; Berardi, E.; Veenhuis, M.; Vanderklei, I. New Yeast Expression Platforms Based on Methylotrophic *Hansenula polymorpha* and *Pichia pastoris* and on Dimorphic *Arxula adeninivorans* and *Yarrowia lipolytica*—A Comparison. *FEMS Yeast Res.* **2005**, *5*, 1079–1096. [[CrossRef](#)]
22. Borneman, A.R.; Pretorius, I.S. Genomic Insights into the *Saccharomyces sensu stricto* Complex. *Genetics* **2015**, *199*, 281–291. [[CrossRef](#)]
23. Hirschi, S.; Ward, T.R.; Meier, W.P.; Müller, D.J.; Fotiadis, D. Synthetic Biology: Bottom-Up Assembly of Molecular Systems. *Chem. Rev.* **2022**, *122*, 16294–16328. [[CrossRef](#)]
24. Venter, J.C.; Glass, J.L.; Hutchison, C.A.; Vashee, S. Synthetic Chromosomes, Genomes, Viruses, and Cells. *Cell* **2022**, *185*, 2708–2724. [[CrossRef](#)]
25. Pretorius, I.S.; Boeke, J.D. Yeast 2.0—Connecting the Dots in the Construction of the World’s First Functional Synthetic Eukaryotic Genome. *FEMS Yeast Res.* **2018**, *18*, foy032. [[CrossRef](#)]
26. Arnolds, K.L.; Dahlin, L.R.; Ding, L.; Wu, C.; Yu, J.; Xiong, W.; Zuniga, C.; Suzuki, Y.; Zengler, K.; Linger, J.G.; et al. Biotechnology for Secure Biocontainment Designs in an Emerging Bioeconomy. *Curr. Opin. Biotechnol.* **2021**, *71*, 25–31. [[CrossRef](#)] [[PubMed](#)]
27. Cai, Y.; Agmon, N.; Choi, W.J.; Ubide, A.; Stracquadanio, G.; Caravelli, K.; Hao, H.; Bader, J.S.; Boeke, J.D. Intrinsic Biocontainment: Multiplex Genome Safeguards Combine Transcriptional and Recombinational Control of Essential Yeast Genes. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, 1803–1808. [[CrossRef](#)] [[PubMed](#)]
28. Gonçalves, P.; Gonçalves, C. Horizontal Gene Transfer in Yeasts. *Curr. Opin. Genet. Dev.* **2022**, *76*, 101950. [[CrossRef](#)]
29. Schmidt, M.; de Lorenzo, V. Synthetic Bugs on the Loose: Containment Options for Deeply Engineered (Micro)organisms. *Curr. Opin. Biotechnol.* **2016**, *38*, 90–96. [[CrossRef](#)]
30. NIH Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules (NIH Guidelines)—April 2019. 2019. Available online: [https://osp.od.nih.gov/wp-content/uploads/NIH\\_Guidelines.pdf](https://osp.od.nih.gov/wp-content/uploads/NIH_Guidelines.pdf) (accessed on 28 February 2023).
31. Andersen, J.T.; Schäfer, T.; Jørgensen, P.L.; Møller, S. Using Inactivated Microbial Biomass as Fertilizer: The Fate of Antibiotic Resistance Genes in the Environment. *Res. Microbiol.* **2001**, *152*, 823–833. [[CrossRef](#)]
32. Nielsen, K.M.; Johnsen, P.J.; Bensasson, D.; Daffonchio, D. Release and Persistence of Extracellular DNA in the Environment. *Environ. Biosaf. Res.* **2007**, *6*, 37–53. [[CrossRef](#)] [[PubMed](#)]
33. Recorbet, G.; Picard, C.; Normand, P.; Simonet, P. Kinetics of the Persistence of Chromosomal DNA from Genetically Engineered *Escherichia coli* Introduced into Soil. *Appl. Environ. Microbiol.* **1993**, *59*, 4289–4294. [[CrossRef](#)]

34. Gallagher, R.R.; Patel, J.R.; Interiano, A.L.; Rovner, A.J.; Isaacs, F.J. Multilayered Genetic Safeguards Limit Growth of Microorganisms to Defined Environments. *Nucleic Acids Res.* **2015**, *43*, 1945–1954. [[CrossRef](#)]
35. Lee, J.W.; Chan, C.T.Y.; Slomovic, S.; Collins, J.J. Next-Generation Biocontainment Systems for Engineered Organisms. *Nat. Chem. Biol.* **2018**, *14*, 530–537. [[CrossRef](#)]
36. Knudsen, S.M.; Karlström, O.H. Development of Efficient Suicide Mechanisms for Biological Containment of Bacteria. *Appl. Environ. Microbiol.* **1991**, *57*, 85–92. [[CrossRef](#)]
37. Kong, W.; Wanda, S.-Y.; Zhang, X.; Bollen, W.; Tinge, S.A.; Roland, K.L.; Curtiss, R. Regulated Programmed Lysis of Recombinant Salmonella in Host Tissues to Release Protective Antigens and Confer Biological Containment. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 9361–9366. [[CrossRef](#)] [[PubMed](#)]
38. Mandell, D.J.; Lajoie, M.J.; Mee, M.T.; Takeuchi, R.; Kuznetsov, G.; Norville, J.E.; Gregg, C.J.; Stoddard, B.L.; Church, G.M. Biocontainment of Genetically Modified Organisms by Synthetic Protein Design. *Nature* **2015**, *518*, 55–60. [[CrossRef](#)] [[PubMed](#)]
39. Pronk, J.T. Auxotrophic Yeast Strains in Fundamental and Applied Research. *Appl. Environ. Microbiol.* **2002**, *68*, 2095. [[CrossRef](#)] [[PubMed](#)]
40. Kokina, A.; Ozolina, Z.; Liepins, J. Purine Auxotrophy: Possible Applications beyond Genetic Marker. *Yeast* **2019**, *36*, 649–656. [[CrossRef](#)]
41. Ralser, M.; Kuhl, H.; Ralser, M.; Werber, M.; Lehrach, H.; Breitenbach, M.; Timmermann, B. The *Saccharomyces cerevisiae* W303-K6001 Cross-Platform Genome Sequence: Insights into Ancestry and Physiology of a Laboratory Mutt. *Open Biol.* **2012**, *2*, 120093. [[CrossRef](#)]
42. Kokina, A.; Kibilds, J.; Liepins, J. Adenine Auxotrophy—Be Aware: Some Effects of Adenine Auxotrophy in *Saccharomyces cerevisiae* Strain W303-1A. *FEMS Yeast Res.* **2014**, *14*, 697–707. [[CrossRef](#)]
43. Weng, Y.; Nickloff, J.A. Nonselective URA3 Colony-Color Assay in Yeast Ade1 or Ade2 Mutants. *BioTechniques* **1997**, *23*, 237–242. [[CrossRef](#)]
44. Bharathi, V.; Girdhar, A.; Prasad, A.; Verma, M.; Taneja, V.; Patel, B.K. Use of Ade1 and Ade2 Mutations for Development of a Versatile Red/White Colour Assay of Amyloid-Induced Oxidative Stress in *Saccharomyces cerevisiae*: Use of Ade1 and Ade2 Mutations for Development of Colour Assay. *Yeast* **2016**, *33*, 607–620. [[CrossRef](#)]
45. Achilli, A.; Matmati, N.; Casalone, E.; Morpurgo, G.; Lucaccioni, A.; Pavlov, Y.I.; Babudri, N. The Exceptionally High Rate of Spontaneous Mutations in the Polymerase Delta Proofreading Exonuclease-Deficient *Saccharomyces cerevisiae* Strain Starved for Adenine. *BMC Genet.* **2004**, *5*, 34. [[CrossRef](#)]
46. Heidenreich, E.; Wintersberger, U. Starvation for a Specific Amino Acid Induces High Frequencies of Rho<sup>-</sup> Mutants in *Saccharomyces cerevisiae*. *Curr. Genet.* **1997**, *31*, 408–413. [[CrossRef](#)]
47. Hall, B.G. Selection-Induced Mutations Occur in Yeast. *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 4300–4303. [[CrossRef](#)] [[PubMed](#)]
48. Brachmann, C.B.; Davies, A.; Cost, G.J.; Caputo, E.; Li, J.; Hieter, P.; Boeke, J.D. Designer Deletion Strains Derived from *Saccharomyces cerevisiae* S288C: A Useful Set of Strains and Plasmids for PCR-Mediated Gene Disruption and Other Applications. *Yeast* **1998**, *14*, 115–132. [[CrossRef](#)]
49. Heinisch, J.J.; Buchwald, U.; Gottschlich, A.; Heppeler, N.; Rodicio, R. A Tool Kit for Molecular Genetics of *Kluyveromyces lactis* Comprising a Congenic Strain Series and a Set of Versatile Vectors. *FEMS Yeast Res.* **2010**, *10*, 333–342. [[CrossRef](#)] [[PubMed](#)]
50. Ahmad, M.; Winkler, C.M.; Kolmbauer, M.; Pichler, H.; Schwab, H.; Emmerstorfer-Augustin, A. *Pichia pastoris* Protease-Deficient and Auxotrophic Strains Generated by a Novel, User-Friendly Vector Toolbox for Gene Deletion. *Yeast* **2019**, *36*, 557–570. [[CrossRef](#)]
51. Zhu, X.; Zhang, Z.; Jia, B.; Yuan, Y. Current Advances of Biocontainment Strategy in Synthetic Biology. *Chin. J. Chem. Eng.* **2023**, *56*, 141–151. [[CrossRef](#)]
52. Kim, D.; Lee, J.W. Genetic Biocontainment Systems for the Safe Use of Engineered Microorganisms. *Biotechnol. Bioprocess Eng.* **2020**, *25*, 974–984. [[CrossRef](#)]
53. Nieto-Domínguez, M.; Nikel, P.I. Intersecting Xenobiology and Neometabolism To Bring Novel Chemistries to Life. *ChemBioChem* **2020**, *21*, 2551–2571. [[CrossRef](#)]
54. Odar, C.; Winkler, M.; Wiltschi, B. Fluoro Amino Acids: A Rarity in Nature, yet a Prospect for Protein Engineering. *Biotechnol. J.* **2015**, *10*, 427–446. [[CrossRef](#)]
55. Handal-Marquez, P.; Anupama, A.; Pezo, V.; Marlière, P.; Herdewijn, P.; Pinheiro, V.B. Beneath the XNA World: Tools and Targets to Build Novel Biology. *Curr. Opin. Syst. Biol.* **2020**, *24*, 142–152. [[CrossRef](#)]
56. Rovner, A.J.; Haimovich, A.D.; Katz, S.R.; Li, Z.; Grome, M.W.; Gassaway, B.M.; Amiram, M.; Patel, J.R.; Gallagher, R.R.; Rinehart, J.; et al. Recoded Organisms Engineered to Depend on Synthetic Amino Acids. *Nature* **2015**, *518*, 89–93. [[CrossRef](#)]
57. de la Torre, D.; Chin, J.W. Reprogramming the Genetic Code. *Nat. Rev. Genet.* **2021**, *22*, 169–184. [[CrossRef](#)] [[PubMed](#)]
58. Sanders, J.; Hoffmann, S.A.; Green, A.P.; Cai, Y. New Opportunities for Genetic Code Expansion in Synthetic Yeast. *Curr. Opin. Biotechnol.* **2022**, *75*, 102691. [[CrossRef](#)] [[PubMed](#)]
59. Wan, W.; Tharp, J.M.; Liu, W.R. Pyrrolysyl-TRNA Synthetase: An Ordinary Enzyme but an Outstanding Genetic Code Expansion Tool. *Biochim. Biophys. Acta* **2014**, *1844*, 1059–1070. [[CrossRef](#)] [[PubMed](#)]
60. Hancock, S.M.; Uprety, R.; Deiters, A.; Chin, J.W. Expanding the Genetic Code of Yeast for Incorporation of Diverse Unnatural Amino Acids via a Pyrrolysyl-TRNA Synthetase/TRNA Pair. *J. Am. Chem. Soc.* **2010**, *132*, 14819–14824. [[CrossRef](#)] [[PubMed](#)]
61. Wiltschi, B. Incorporation of Non-Canonical Amino Acids into Proteins in Yeast. *Fungal Genet. Biol.* **2016**, *89*, 137–156. [[CrossRef](#)]
62. Chin, J.W.; Cropp, T.A.; Anderson, J.C.; Mukherji, M.; Zhang, Z.; Schultz, P.G. An Expanded Eukaryotic Genetic Code. *Science* **2003**, *301*, 964–967. [[CrossRef](#)]
63. Budisa, N.; Wenger, W.; Wiltschi, B. Residue-Specific Global Fluorination of *Candida antarctica* Lipase B in *Pichia pastoris*. *Mol. Biosyst.* **2010**, *6*, 1630–1639. [[CrossRef](#)]
64. Ai, H.-W.; Shen, W.; Brustad, E.; Schultz, P.G. Genetically Encoded Alkenes in Yeast. *Angew. Chem. Int. Ed.* **2010**, *49*, 935–937. [[CrossRef](#)]



65. Wang, Q.; Wang, L. New Methods Enabling Efficient Incorporation of Unnatural Amino Acids in Yeast. *J. Am. Chem. Soc.* **2008**, *130*, 6066–6067. [[CrossRef](#)]
66. Chen, S.; Schultz, P.G.; Brock, A. An Improved System for the Generation and Analysis of Mutant Proteins Containing Unnatural Amino Acids in *Saccharomyces cerevisiae*. *J. Mol. Biol.* **2007**, *371*, 112–122. [[CrossRef](#)]
67. Brustad, E.; Bushey, M.L.; Brock, A.; Chittuluru, J.; Schultz, P.G. A Promiscuous Aminoacyl-TRNA Synthetase That Incorporates Cysteine, Methionine, and Alanine Homologs into Proteins. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 6004–6006. [[CrossRef](#)] [[PubMed](#)]
68. Tippmann, E.M.; Schultz, P.G. A Genetically Encoded Metallocene Containing Amino Acid. *Tetrahedron* **2007**, *63*, 6182–6184. [[CrossRef](#)]
69. Lemke, E.A.; Summerer, D.; Geierstanger, B.H.; Brittain, S.M.; Schultz, P.G. Control of Protein Phosphorylation with a Genetically Encoded Photocaged Amino Acid. *Nat. Chem. Biol.* **2007**, *3*, 769–772. [[CrossRef](#)] [[PubMed](#)]
70. Summerer, D.; Chen, S.; Wu, N.; Deiters, A.; Chin, J.W.; Schultz, P.G. A Genetically Encoded Fluorescent Amino Acid. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 9785–9789. [[CrossRef](#)]
71. Lee, H.S.; Guo, J.; Lemke, E.A.; Dimla, R.D.; Schultz, P.G. Genetic Incorporation of a Small, Environmentally Sensitive, Fluorescent Probe into Proteins in *Saccharomyces cerevisiae*. *J. Am. Chem. Soc.* **2009**, *131*, 12921–12923. [[CrossRef](#)]
72. Young, T.S.; Ahmad, I.; Brock, A.; Schultz, P.G. Expanding the Genetic Repertoire of the Methylotrophic Yeast *Pichia pastoris*. *Biochemistry* **2009**, *48*, 2643–2653. [[CrossRef](#)]
73. Himmelfarb, H.J.; Maicas, E.; Friesen, J.D. Isolation of the SUP45 Omnipotent Suppressor Gene of *Saccharomyces cerevisiae* and Characterization of Its Gene Product. *Mol. Cell. Biol.* **1985**, *5*, 816–822.
74. Stansfield, I.; Jones, K.M.; Kushnirov, V.V.; Dagkesamanskaya, A.R.; Poznyakovski, A.I.; Paushkin, S.V.; Nierras, C.R.; Cox, B.S.; Ter-Avanesyan, M.D.; Tuite, M.F. The Products of the SUP45 (ERF1) and SUP35 Genes Interact to Mediate Translation Termination in *Saccharomyces cerevisiae*. *EMBO J.* **1995**, *14*, 4365–4373. [[CrossRef](#)]
75. Lajoie, M.J.; Rovner, A.J.; Goodman, D.B.; Aerni, H.-R.; Haimovich, A.D.; Kuznetsov, G.; Mercer, J.A.; Wang, H.H.; Carr, P.A.; Mosberg, J.A.; et al. Genomically Recoded Organisms Expand Biological Functions. *Science* **2013**, *342*, 357–360. [[CrossRef](#)]
76. Stieglitz, J.T.; Lahiri, P.; Stout, M.I.; Van Deventer, J.A. Exploration of Methanomethylophilus Albus Pyrrolysyl-TRNA Synthetase Activity in Yeast. *ACS Synth. Biol.* **2022**, *11*, 1824–1834. [[CrossRef](#)]
77. Zackin, M.T.; Stieglitz, J.T.; Van Deventer, J.A. Genome-Wide Screen for Enhanced Noncanonical Amino Acid Incorporation in Yeast. *ACS Synth. Biol.* **2022**, *11*, 3669–3680. [[CrossRef](#)]
78. Stieglitz, J.T.; Van Deventer, J.A. High-Throughput Aminoacyl-TRNA Synthetase Engineering for Genetic Code Expansion in Yeast. *ACS Synth. Biol.* **2022**, *11*, 2284–2299. [[CrossRef](#)]
79. Dunkelmann, D.L.; Oehm, S.B.; Beattie, A.T.; Chin, J.W. A 68-Codon Genetic Code to Incorporate Four Distinct Non-Canonical Amino Acids Enabled by Automated Orthogonal mRNA Design. *Nat. Chem.* **2021**, *13*, 1110–1117. [[CrossRef](#)] [[PubMed](#)]
80. Hankore, E.D.; Zhang, L.; Chen, Y.; Liu, K.; Niu, W.; Guo, J. Genetic Incorporation of Noncanonical Amino Acids Using Two Mutually Orthogonal Quadruplet Codons. *ACS Synth. Biol.* **2019**, *8*, 1168–1174. [[CrossRef](#)] [[PubMed](#)]
81. Niu, W.; Schultz, P.G.; Guo, J. An Expanded Genetic Code in Mammalian Cells with a Functional Quadruplet Codon. *ACS Chem. Biol.* **2013**, *8*, 1640–1645. [[CrossRef](#)] [[PubMed](#)]
82. Fischer, E.C.; Hashimoto, K.; Zhang, Y.; Feldman, A.W.; Dien, V.T.; Karadeema, R.J.; Adhikary, R.; Ledbetter, M.P.; Krishnamurthy, R.; Romesberg, F.E. New Codons for Efficient Production of Unnatural Proteins in a Semi-Synthetic Organism. *Nat. Chem. Biol.* **2020**, *16*, 570–576. [[CrossRef](#)]
83. Nie, P.; Bai, Y.; Mei, H. Synthetic Life with Alternative Nucleic Acids as Genetic Materials. *Molecules* **2020**, *25*, 3483. [[CrossRef](#)]
84. Torres, L.; Krüger, A.; Csibra, E.; Gianni, E.; Pinheiro, V.B. Synthetic Biology Approaches to Biological Containment: Pre-Emptively Tackling Potential Risks. *Essays Biochem.* **2016**, *60*, 393–410. [[CrossRef](#)]
85. Herdewijn, P.; Marlière, P. Toward Safe Genetically Modified Organisms through the Chemical Diversification of Nucleic Acids. *Chem. Biodivers.* **2009**, *6*, 791–808. [[CrossRef](#)]
86. Dhami, K.; Malyshev, D.A.; Ordoukhanian, P.; Kubelka, T.; Hocek, M.; Romesberg, F.E. Systematic Exploration of a Class of Hydrophobic Unnatural Base Pairs Yields Multiple New Candidates for the Expansion of the Genetic Alphabet. *Nucleic Acids Res.* **2014**, *42*, 10235–10244. [[CrossRef](#)]
87. Pinheiro, V.B.; Holliger, P. The XNA World: Progress towards Replication and Evolution of Synthetic Genetic Polymers. *Curr. Opin. Chem. Biol.* **2012**, *16*, 245–252. [[CrossRef](#)] [[PubMed](#)]
88. Hendrix, C.; Rosemeyer, H.; Verheggen, I.; Van Aerschot, A.; Seela, F.; Herdewijn, P. 1', 5'-Anhydrohexitol Oligonucleotides: Synthesis, Base Pairing and Recognition by Regular Oligodeoxyribonucleotides and Oligoribonucleotides. *Chem.–A Eur. J.* **1997**, *3*, 110–120. [[CrossRef](#)]
89. Schöning, K.U.; Scholz, P.; Guntha, S.; Wu, X.; Krishnamurthy, R.; Eschenmoser, A. Chemical Etiology of Nucleic Acid Structure: The  $\alpha$ -Threofuranosyl-(3'→2') Oligonucleotide System. *Science* **2000**, *290*, 1347–1351. [[CrossRef](#)] [[PubMed](#)]
90. Wang, J.; Verbeure, B.; Luyten, I.; Lescrier, E.; Froeyen, M.; Hendrix, C.; Rosemeyer, H.; Seela, F.; Van Aerschot, A.; Herdewijn, P. Cyclohexene Nucleic Acids (CeNA): Serum Stable Oligonucleotides That Activate RNase H and Increase Duplex Stability with Complementary RNA. *J. Am. Chem. Soc.* **2000**, *122*, 8595–8602. [[CrossRef](#)]
91. Noronha, A.M.; Wilds, C.J.; Lok, C.N.; Viazovkina, K.; Arion, D.; Parniak, M.A.; Damha, M.J. Synthesis and Biophysical Properties of Arabinonucleic Acids (ANA): Circular Dichroic Spectra, Melting Temperatures, and Ribonuclease H Susceptibility of ANA·RNA Hybrid Duplexes. *Biochemistry* **2000**, *39*, 7050–7062. [[CrossRef](#)]



92. Kalota, A.; Karabon, L.; Swider, C.R.; Viazovkina, E.; Elzagheid, M.; Damha, M.J.; Gewirtz, A.M. 2'-Deoxy-2'-Fluoro- $\beta$ -D-Arabinonucleic Acid (2'F-ANA) Modified Oligonucleotides (ON) Effect Highly Efficient, and Persistent, Gene Silencing. *Nucleic Acids Res.* **2006**, *34*, 451–461. [[CrossRef](#)]
93. Zhang, L.; Peritz, A.; Meggers, E. A Simple Glycol Nucleic Acid. *J. Am. Chem. Soc.* **2005**, *127*, 4174–4175. [[CrossRef](#)]
94. Singh, S.K.; Koshkin, A.A.; Wengel, J.; Nielsen, P. LNA (Locked Nucleic Acids): Synthesis and High-Affinity Nucleic Acid Recognition. *Chem. Commun.* **1998**, *4*, 455–456. [[CrossRef](#)]
95. Liu, C.; Cozens, C.; Jaziri, F.; Rozenski, J.; Maréchal, A.; Dumbre, S.; Pezo, V.; Marlière, P.; Pinheiro, V.B.; Groaz, E.; et al. Phosphonomethyl Oligonucleotides as Backbone-Modified Artificial Genetic Polymers. *J. Am. Chem. Soc.* **2018**, *140*, 6690–6699. [[CrossRef](#)]
96. Diwo, C.; Budisa, N. Alternative Biochemistries for Alien Life: Basic Concepts and Requirements for the Design of a Robust Biocontainment System in Genetic Isolation. *Genes* **2019**, *10*, 17. [[CrossRef](#)]
97. Lopez, G.; Anderson, J.C. Synthetic Auxotrophs with Ligand-Dependent Essential Genes for a BL21(DE3) Biosafety Strain. *ACS Synth. Biol.* **2015**, *4*, 1279–1286. [[CrossRef](#)] [[PubMed](#)]
98. Motomura, K.; Sano, K.; Watanabe, S.; Kanbara, A.; Gamal Nasser, A.-H.; Ikeda, T.; Ishida, T.; Funabashi, H.; Kuroda, A.; Hirota, R. Synthetic Phosphorus Metabolic Pathway for Biosafety and Contamination Management of Cyanobacterial Cultivation. *ACS Synth. Biol.* **2018**, *7*, 2189–2198. [[CrossRef](#)] [[PubMed](#)]
99. Asin-Garcia, E.; Batianis, C.; Li, Y.; Fawcett, J.D.; de Jong, I.; dos Santos, V.A.P.M. Phosphite Synthetic Auxotrophy as an Effective Biocontainment Strategy for the Industrial Chassis *Pseudomonas putida*. *Microb. Cell Factories* **2022**, *21*, 156. [[CrossRef](#)] [[PubMed](#)]
100. Johnson, C.R.; Steingesser, M.G.; Weems, A.D.; Khan, A.; Gladfelter, A.; Bertin, A.; McMurray, M.A. Guanidine Hydrochloride Reactivates an Ancient Septin Hetero-Oligomer Assembly Pathway in Budding Yeast. *eLife* **2020**, *9*, e54355. [[CrossRef](#)]
101. Hassell, D.S.; Steingesser, M.G.; Denney, A.S.; Johnson, C.R.; McMurray, M.A. Chemical Rescue of Mutant Proteins in Living *Saccharomyces cerevisiae* Cells by Naturally Occurring Small Molecules. *G3 GenesGenomesGenetics* **2021**, *11*, jkab252. [[CrossRef](#)]
102. Agmon, N.; Tang, Z.; Yang, K.; Sutter, B.; Ikushima, S.; Cai, Y.; Caravelli, K.; Martin, J.A.; Sun, X.; Choi, W.J.; et al. Low Escape-Rate Genome Safeguards with Minimal Molecular Perturbation of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* **2017**, *114*, E1470–E1479. [[CrossRef](#)]
103. Lindstrom, D.L.; Gottschling, D.E. The Mother Enrichment Program: A Genetic System for Facile Replicative Life Span Analysis in *Saccharomyces cerevisiae*. *Genetics* **2009**, *183*, 413–422. [[CrossRef](#)]
104. Arzumanyan, G.A.; Gabriel, K.N.; Ravikumar, A.; Javanpour, A.A.; Liu, C.C. Mutually Orthogonal DNA Replication Systems In Vivo. *ACS Synth. Biol.* **2018**, *7*, 1722–1729. [[CrossRef](#)]
105. Ravikumar, A.; Arzumanyan, G.A.; Obadi, M.K.A.; Javanpour, A.A.; Liu, C.C. Scalable, Continuous Evolution of Genes at Mutation Rates above Genomic Error Thresholds. *Cell* **2018**, *175*, 1946–1957.e13. [[CrossRef](#)]
106. Strak, M.J.R.; Boyd, A.; Mileham, A.J.; Ramonos, M.A. The Plasmid-Encoded Killer System of *Kluyveromyces lactis*: A Review. *Yeast* **1990**, *6*, 1–29. [[CrossRef](#)]
107. van Nies, P.; Westerlaken, I.; Blanken, D.; Salas, M.; Mencía, M.; Danelon, C. Self-Replication of DNA by Its Encoded Proteins in Liposome-Based Synthetic Cells. *Nat. Commun.* **2018**, *9*, 1583. [[CrossRef](#)] [[PubMed](#)]
108. Sakatani, Y.; Ichihashi, N.; Kazuta, Y.; Yomo, T. A Transcription and Translation-Coupled DNA Replication System Using Rolling-Circle Replication. *Sci. Rep.* **2015**, *5*, 10404. [[CrossRef](#)] [[PubMed](#)]
109. Libicher, K.; Hornberger, R.; Heymann, M.; Mutschler, H. In Vitro Self-Replication and Multicistronic Expression of Large Synthetic Genomes. *Nat. Commun.* **2020**, *11*, 904. [[CrossRef](#)]
110. Medina, E.; Yik, E.J.; Herdewijn, P.; Chaput, J.C. Functional Comparison of Laboratory-Evolved XNA Polymerases for Synthetic Biology. *ACS Synth. Biol.* **2021**, *10*, 1429–1437. [[CrossRef](#)] [[PubMed](#)]
111. Kar, S.; Ellington, A.D. Construction of Synthetic T7 RNA Polymerase Expression Systems. *Methods* **2018**, *143*, 110–120. [[CrossRef](#)] [[PubMed](#)]
112. Morse, N.J.; Wagner, J.M.; Reed, K.B.; Gopal, M.R.; Laufer, L.H.; Alper, H.S. T7 Polymerase Expression of Guide RNAs In Vivo Allows Exportable CRISPR-Cas9 Editing in Multiple Yeast Hosts. *ACS Synth. Biol.* **2018**, *7*, 1075–1084. [[CrossRef](#)]
113. Stirling, F.; Bitzan, L.; O'Keefe, S.; Redfield, E.; Oliver, J.W.K.; Way, J.; Silver, P.A. Rational Design of Evolutionarily Stable Microbial Kill Switches. *Mol. Cell* **2017**, *68*, 686–697.e3. [[CrossRef](#)] [[PubMed](#)]
114. Chan, C.T.Y.; Lee, J.W.; Cameron, D.E.; Bashor, C.J.; Collins, J.J. “Deadman” and “Passcode” Microbial Kill Switches for Bacterial Containment. *Nat. Chem. Biol.* **2016**, *12*, 82–86. [[CrossRef](#)]
115. Halvorsen, T.M.; Ricci, D.P.; Park, D.M.; Jiao, Y.; Yung, M.C. Comparison of Kill Switch Toxins in Plant-Beneficial *Pseudomonas fluorescens* Reveals Drivers of Lethality, Stability, and Escape. *ACS Synth. Biol.* **2022**, *11*, 3785–3796. [[CrossRef](#)]
116. Wright, O.; Delmans, M.; Stan, G.-B.; Ellis, T. GeneGuard: A Modular Plasmid System Designed for Biosafety. *ACS Synth. Biol.* **2015**, *4*, 307–316. [[CrossRef](#)]
117. Whitford, C.M.; Dymek, S.; Kerkhoff, D.; März, C.; Schmidt, O.; Edich, M.; Droste, J.; Pucker, B.; Rückert, C.; Kalinowski, J. Auxotrophy to Xeno-DNA: An Exploration of Combinatorial Mechanisms for a High-Fidelity Biosafety System for Synthetic Biology Applications. *J. Biol. Eng.* **2018**, *12*, 13. [[CrossRef](#)] [[PubMed](#)]
118. Ahrenholtz, I.; Lorenz, M.G.; Wackernagel, W. A Conditional Suicide System in *Escherichia coli* Based on the Intracellular Degradation of DNA. *Appl. Environ. Microbiol.* **1994**, *60*, 3746–3751. [[CrossRef](#)] [[PubMed](#)]
119. Balan, A.; Schenberg, A.C.G. A Conditional Suicide System for *Saccharomyces cerevisiae* Relying on the Intracellular Production of the *Serratia marcescens* Nuclease. *Yeast* **2005**, *22*, 203–212. [[CrossRef](#)]

120. Lewis, L.K.; Kirchner, J.M.; Resnick, M.A. Requirement for End-Joining and Checkpoint Functions, but Not RAD52-Mediated Recombination, after EcoRI Endonuclease Cleavage of *Saccharomyces cerevisiae* DNA. *Mol. Cell. Biol.* **1998**, *18*, 1891–1902. [[CrossRef](#)]
121. Barnes, G.; Rine, J. Regulated Expression of Endonuclease EcoRI in *Saccharomyces cerevisiae*: Nuclear Entry and Biological Consequences. *Proc. Natl. Acad. Sci. USA* **1985**, *82*, 1354–1358. [[CrossRef](#)] [[PubMed](#)]
122. Westmoreland, J.W.; Summers, J.A.; Holland, C.L.; Resnick, M.A.; Lewis, L.K. Blunt-Ended DNA Double-Strand Breaks Induced by Endonucleases PvuII and EcoRV Are Poor Substrates for Repair in *Saccharomyces cerevisiae*. *DNA Repair* **2010**, *9*, 617–626. [[CrossRef](#)] [[PubMed](#)]
123. Caliendo, B.J.; Voigt, C.A. Targeted DNA Degradation Using a CRISPR Device Stably Carried in the Host Genome. *Nat. Commun.* **2015**, *6*, 6989. [[CrossRef](#)]
124. Rottinghaus, A.G.; Ferreiro, A.; Fishbein, S.R.S.; Dantas, G.; Moon, T.S. Genetically Stable CRISPR-Based Kill Switches for Engineered Microbes. *Nat. Commun.* **2022**, *13*, 672. [[CrossRef](#)]
125. Rottinghaus, A.G.; Vo, S.; Moon, T.S. Computational Design of CRISPR Guide RNAs to Enable Strain-Specific Control of Microbial Consortia. *Proc. Natl. Acad. Sci. USA* **2023**, *120*, e2213154120. [[CrossRef](#)]
126. Pantoja Angles, A.; Valle-Pérez, A.U.; Hauser, C.; Mahfouz, M.M. Microbial Biocontainment Systems for Clinical, Agricultural, and Industrial Applications. *Front. Bioeng. Biotechnol.* **2022**, *10*, 830200. [[CrossRef](#)]
127. Yeo, C.A.; Abu Bakar, F.; Chan, W.T.; Espinosa, M.; Hari Krishna, J.A. Heterologous Expression of Toxins from Bacterial Toxin-Antitoxin Systems in Eukaryotic Cells: Strategies and Applications. *Toxins* **2016**, *8*, 49. [[CrossRef](#)] [[PubMed](#)]
128. Yamaguchi, Y.; Park, J.-H.; Inouye, M. Toxin-Antitoxin Systems in Bacteria and Archaea. *Annu. Rev. Genet.* **2011**, *45*, 61–79. [[CrossRef](#)] [[PubMed](#)]
129. Wang, X.; Yao, J.; Sun, Y.-C.; Wood, T.K. Type VII Toxin/Antitoxin Classification System for Antitoxins That Enzymatically Neutralize Toxins. *Trends Microbiol.* **2021**, *29*, 388–393. [[CrossRef](#)] [[PubMed](#)]
130. Kamruzzaman, M.; Wu, A.Y.; Iredell, J.R. Biological Functions of Type II Toxin-Antitoxin Systems in Bacteria. *Microorganisms* **2021**, *9*, 1276. [[CrossRef](#)]
131. Fineran, P.C.; Blower, T.R.; Foulds, I.J.; Humphreys, D.P.; Lilley, K.S.; Salmond, G.P.C. The Phage Abortive Infection System, ToxIN, Functions as a Protein-RNA Toxin-Antitoxin Pair. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 894–899. [[CrossRef](#)]
132. Masuda, H.; Tan, Q.; Awano, N.; Wu, K.-P.; Inouye, M. YeeU Enhances the Bundling of Cytoskeletal Polymers of MreB and FtsZ, Antagonizing the CbtA (YeeV) Toxicity in *Escherichia coli*. *Mol. Microbiol.* **2012**, *84*, 979–989. [[CrossRef](#)]
133. Wang, X.; Lord, D.M.; Cheng, H.-Y.; Osbourne, D.O.; Hong, S.H.; Sanchez-Torres, V.; Quiroga, C.; Zheng, K.; Herrmann, T.; Peti, W.; et al. A New Type V Toxin-Antitoxin System Where MRNA for Toxin GhoT Is Cleaved by Antitoxin GhoS. *Nat. Chem. Biol.* **2012**, *8*, 855–861. [[CrossRef](#)]
134. Aakre, C.D.; Phung, T.N.; Huang, D.; Laub, M.T. A Bacterial Toxin Inhibits DNA Replication Elongation through a Direct Interaction with the  $\beta$  Sliding Clamp. *Mol. Cell* **2013**, *52*, 617–628. [[CrossRef](#)]
135. Bej, A.K.; Perlin, M.H.; Atlas, R.M. Model Suicide Vector for Containment of Genetically Engineered Microorganisms. *Appl. Environ. Microbiol.* **1988**, *54*, 2472–2477. [[CrossRef](#)]
136. Schweder, T.; Schmidt, I.; Herrmann, H.; Neubauer, P.; Hecker, M.; Hofmann, K. An Expression Vector System Providing Plasmid Stability and Conditional Suicide of Plasmid-Containing Cells. *Appl. Microbiol. Biotechnol.* **1992**, *38*, 91–93. [[CrossRef](#)]
137. Kroll, J.; Klintner, S.; Schneider, C.; Voss, I.; Steinbüchel, A. Plasmid Addiction Systems: Perspectives and Applications in Biotechnology. *Microb. Biotechnol.* **2010**, *3*, 634–657. [[CrossRef](#)]
138. Kristoffersen, P.; Jensen, G.B.; Gerdes, K.; Piškur, J. Bacterial Toxin-Antitoxin Gene System as Containment Control in Yeast Cells. *Appl. Environ. Microbiol.* **2000**, *66*, 5524–5526. [[CrossRef](#)] [[PubMed](#)]
139. Pedersen, K.; Zavialov, A.V.; Pavlov, M.Y.; Elf, J.; Gerdes, K.; Ehrenberg, M. The Bacterial Toxin RelE Displays Codon-Specific Cleavage of MRNAs in the Ribosomal A Site. *Cell* **2003**, *112*, 131–140. [[CrossRef](#)]
140. Li, G.-Y.; Zhang, Y.; Inouye, M.; Ikura, M. Inhibitory Mechanism of *Escherichia coli* RelE-RelB Toxin-Antitoxin Module Involves a Helix Displacement Near an MRNA Interferase Active Site. *J. Biol. Chem.* **2009**, *284*, 14628–14636. [[CrossRef](#)] [[PubMed](#)]
141. Monti, M.C.; Hernández-Arriaga, A.M.; Kamphuis, M.B.; López-Villarejo, J.; Heck, A.J.R.; Boelens, R.; Díaz-Orejas, R.; van den Heuvel, R.H.H. Interactions of Kid-Kis Toxin-Antitoxin Complexes with the ParD Operator-Promoter Region of Plasmid R1 Are Piloted by the Kis Antitoxin and Tuned by the Stoichiometry of Kid-Kis Oligomers. *Nucleic Acids Res.* **2007**, *35*, 1737–1749. [[CrossRef](#)]
142. de la Cueva-Méndez, G.; Mills, A.D.; Clay-Farrace, L.; Díaz-Orejas, R.; Laskey, R.A. Regulatable Killing of Eukaryotic Cells by the Prokaryotic Proteins Kid and Kis. *EMBO J.* **2003**, *22*, 246–251. [[CrossRef](#)]
143. Zielenkiewicz, U.; Ceglowski, P. The Toxin-Antitoxin System of the Streptococcal Plasmid PSM19035. *J. Bacteriol.* **2005**, *187*, 6094–6105. [[CrossRef](#)] [[PubMed](#)]
144. Zielenkiewicz, U.; Kowalewska, M.; Kaczor, C.; Ceglowski, P. In Vivo Interactions between Toxin-Antitoxin Proteins Epsilon and Zeta of Streptococcal Plasmid PSM19035 in *Saccharomyces cerevisiae*. *J. Bacteriol.* **2009**, *191*, 3677–3684. [[CrossRef](#)] [[PubMed](#)]
145. Picardeau, M.; Le Dantec, C.; Richard, G.-F.; Saint Girons, I. The Spirochetal ChpK-Chromosomal Toxin-Antitoxin Locus Induces Growth Inhibition of Yeast and Mycobacteria. *FEMS Microbiol. Lett.* **2003**, *229*, 277–281. [[CrossRef](#)]
146. Picardeau, M.; Ren, S.; Saint Girons, I. Killing Effect and Antitoxic Activity of The *Leptospira Interrogans* Toxin-Antitoxin System In *Escherichia coli*. *J. Bacteriol.* **2001**, *183*, 6494–6497. [[CrossRef](#)]

147. Engelberg-Kulka, H.; Hazan, R.; Amitai, S. MazEF: A Chromosomal Toxin-Antitoxin Module That Triggers Programmed Cell Death in Bacteria. *J. Cell Sci.* **2005**, *118*, 4327–4332. [[CrossRef](#)]
148. Yang, J.; Jiang, W.; Yang, S. MazF as a Counter-Selectable Marker for Unmarked Genetic Modification of *Pichia pastoris*. *FEMS Yeast Res.* **2009**, *9*, 600–609. [[CrossRef](#)]
149. Knudsen, S.; Saadbye, P.; Hansen, L.H.; Collier, A.; Jacobsen, B.L.; Schlundt, J.; Karlström, O.H. Development and Testing of Improved Suicide Functions for Biological Containment of Bacteria. *Appl. Environ. Microbiol.* **1995**, *61*, 985–991. [[CrossRef](#)]
150. Moser, F.; Broers, N.J.; Hartmans, S.; Tamsir, A.; Kerkman, R.; Roubos, J.A.; Bovenberg, R.; Voigt, C.A. Genetic Circuit Performance under Conditions Relevant for Industrial Bioreactors. *ACS Synth. Biol.* **2012**, *1*, 555–564. [[CrossRef](#)]

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