Review

Synthetic Biology-Inspired Biocontainment Strategies of Therapeutic Genetically Engineered Bacteria

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ABSTRACT: With the rapid expansion of synthetic gene technologies and engineered bacteria for disease diagnosis or therapy, biosafety concerns have intensified. Substantial efforts have therefore been directed toward developing biocontainment systems that prevent the unintended release of engineered microorganisms and the horizontal transfer of synthetic genetic elements into natural ecosystems. Recent advances in synthetic biology have yielded a diverse suite of biocontainment strategies, including engineered biosafety genetic circuits, genetic isolation approaches, targeted degradation of genetic material, and physical encapsulation of microbial chassis. Furthermore, the incorporation of unnatural nucleic acids and noncanonical amino acid-based orthogonal replication, transcription, and translation systems has markedly improved the robustness and orthogonality of these containment platforms. In this review, we summarize the latest developments in biocontainment strategies for genetically engineered bacteria and discuss how these innovations may address current and emerging biosafety challenges.

Keywords: Biocontainment; Biosafety; Horizontal gene transfer; Auxotrophy; Unnatural nucleic acid; Noncanonical amino acid

1. Introduction

With the rapid advances of synthetic biology, the repertoire of artificially designed genetic components and genetically engineered bacteria has expanded substantially. These engineered bacteria have shown considerable promise in the diagnosis and treatment of various diseases, including gastrointestinal disorders and cancers [1–7]. Nevertheless, the unintentional release of such engineered constructs or microorganisms into the environment poses potential biosafety concerns [8–12]. A notable example is the use of antibiotic resistance genes as selectable markers in synthetic biology. Antibiotic resistance genes can be horizontally transferred to pathogenic microbes, facilitating the emergence of drug-resistant strains and complicating antimicrobial therapy [13]. Consequently, robust biosafety containment strategies are essential, particularly for engineered bacteria intended for clinical diagnostics and therapeutic applications [14–16].

Biosafety containment in synthetic biology encompasses two principal dimensions [17]. The first is preventing the environmental dissemination of engineered bacteria, typically through mechanisms that inhibit or eradicate cells escaping from designated settings. Current guidelines from the U.S. National Institutes of Health (NIH) recommend that the escape frequency of engineered microorganisms remain below 10^{-8} [18]. However, the rapid expansion in the number and diversity of engineered strains has intensified the need for even more stringent biocontainment thresholds. The second dimension involves minimizing the release of engineered genetic elements and subsequent uptake by environmental microorganisms [19,20]. Therefore, integrating biocontainment mechanisms directly into the genetic design of engineered bacteria based on synthetic biology principles has emerged as a foundational strategy to mitigate biosafety risks. This review outlines current biosafety containment strategies in synthetic biology, especially focusing on biosafety controls of genetically engineered bacteria developed for therapeutic applications. Then we discuss recent advances in orthogonal biocontainment systems, reducing the compromising risk via cross-talking with environmental microbes.

2. Preventing Genetically Engineered Bacteria from Escaping

Although most engineered bacteria have been designed based on lab model microbes, for example, *Escherichia coli*, and some strains are susceptible to growth conditions, the escape of the therapeutic bacteria from unintended sites could lead to adverse effects. To restrict the unintended spread of engineered bacteria, a central strategy involves controlling cellular survival by regulating the expression of essential genes or conditionally activating toxin genes. Some of these strategies are easily designed and have been well studied in the bacteria therapy.

2.1. Auxotrophy

Auxotrophy-based containment relies on the targeted disruption of essential genes, rendering engineered bacteria incapable of synthesizing critical metabolites such as nucleotides or amino acids. As a result, these strains can proliferate only when the missing metabolites are externally provided. In addition to deleting biosynthetic enzymes, inactivation of the corresponding transporter proteins can likewise induce auxotrophy, thereby forcing bacteria to depend on exogenous metabolic precursors for survival [21]. Because of its conceptual simplicity and ease of implementation, auxotrophy has been widely used as a biosafety containment strategy [22–27]. However, in complex environments containing diverse nutrients, engineered bacteria may circumvent auxotrophic constraints. Within the human body, a metabolically rich environment shaped by host tissues and the gut microbiota, auxotrophic strains may acquire essential metabolites directly from surrounding cells or restore metabolic capacity through horizontal gene transfer (HGT) from coexisting microorganisms. Importantly, many auxotrophic phenotypes are reliably expressed only under nutrient-limited conditions [21], as illustrated by NADPH-auxotrophic *E. coli* in mineral salts medium [28]. Consequently, careful selection and context-specific design of auxotrophic strategies are essential to ensure robust biocontainment of engineered bacteria.

Auxotrophic designs intended for diagnostic or therapeutic engineered bacteria should preferentially target metabolites that are essential for prokaryotic survival but absent or scarce in mammalian systems. There are two commonly employed auxotrophy strategies (Figure 1a). Diaminopimelic acid (DAP) is a critical precursor produced by the *dapA* gene (4-hydroxy-tetrahydrodipicolinate synthase) in bacterial cell wall synthesis. Engineered strains lacking *dapA* cannot maintain proper cell wall integrity and therefore fail to survive [29,30]. DAP auxotrophy is commonly used in bacteria therapy because mammalian cells lack cell walls and do not synthesize DAP. Deoxythymidine (THY) generated via the *thyA* gene (thymidylate synthase) is required for dTTP biosynthesis and DNA replication. *thyA*-deficient strains become dependent on exogenous THY, which is present only at extremely low levels in mammalian tissues [31]. A notable

example of this approach is SYNB1891, an engineered *E. coli*–based immunotherapeutic strain developed by Synlogic. Designed to activate antigen-presenting cells through secretion of STING agonists, SYNB1891 incorporates dual knockouts of *dapA* and *thyA*, thereby preventing its proliferation in mice [32]. The strain has progressed to Phase I clinical evaluation [33]. Although auxotrophy is validated in well-defined bacterial metabolic pathways, incomplete annotation of metabolic networks in different chassis organisms [34] raises the possibility of metabolic compensation via unknown or alternative pathways. As such, rigorous experimental validation is essential when developing novel auxotrophic containment systems.
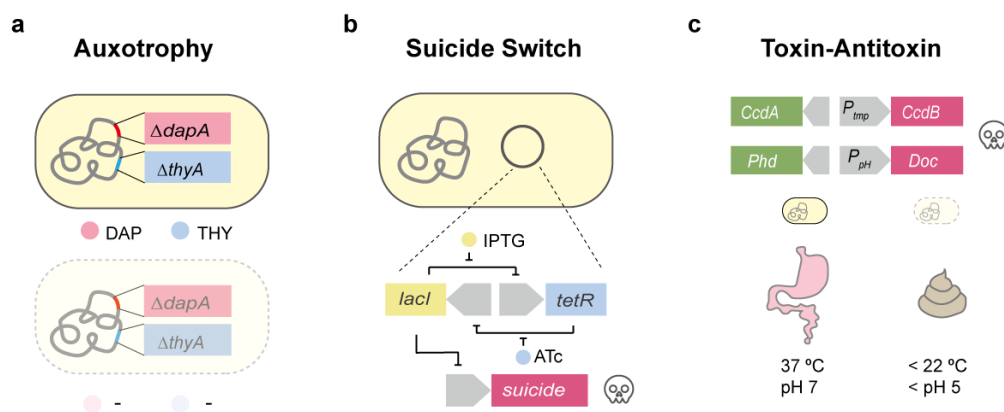

Figure 1. Biocontainment strategies prevent genetically engineered bacteria from escaping. (**a**) Autotrophy by gene knockout of *dapA* (4-hydroxy-tetrahydrodipicolinate synthase) or *thyA* (thymidylate synthase) [30,31]. DAP, diaminopimelic acid. THY, deoxythymidine. (**b**) Suicide switch based on transcriptional regulation [35]. LacI, lactose operon repressor. TetR, tetracycline repressor. IPTG, isopropyl β-D-1-thiogalactopyranoside. ATc, anhydrotetracycline. (**c**) The toxin-antitoxin system activates toxin expression to kill engineered bacteria in response to temperature or pH [36]. CcdB and Doc are toxins. CcdA and Phd are antitoxins. P_{tmp}, temperature-responsive promoter. P_{pH}, pH-responsive promoter.

2.2. Suicide Switches

Suicide gene–based regulatory switches enable the targeted elimination of engineered bacteria through inducible expression of toxic genes [37–39]. Compared with antibiotic-based control strategies, these systems offer notable advantages: they have no risk of disseminating antibiotic resistance genes and can achieve more rapid bacterial killing through protein-level regulatory mechanisms [40]. Such switches can be designed to activate suicide gene expression in response to defined chemical or environmental signals [41]. When stimuli such as temperature, pH, or specific metabolites are used as inducers, they enable the construction of environmentally responsive containment circuits. For example, the Deadman switch operates as a toggle system in which anhydrotetracycline (ATc) modulates the mutually repressive transcription factors TetR (tetracycline repressor) and LacI (lactose operon repressor), with LacI suppressing toxin expression (Figure 1b). Continuous ATc exposure is required to maintain bacterial viability, ATc removal initiates suicide gene activation. The system's efficacy can be further enhanced by supplementing IPTG (isopropyl β-D-1-thiogalactopyranoside), which directly induces toxin expression and improves killing efficiency [35]. More sophisticated designs integrate multi-layer regulatory logic, enabling simultaneous control by multiple inducers and further reducing the escape probability. The Passcode switch employs a LacI–GalR fusion protein together with additional regulators to create a circuit controlled by three inducers, permitting survival only under a specific input combination. Despite their potential, suicide gene switches frequently exhibit basal leakage. The expression of low-level toxins, even in the absence of inducers, can impair bacterial fitness. Over time, mutants that silence or disable the switch may accumulate and outcompete functional strains, ultimately compromising containment. Reducing mutation rates in engineered bacteria therefore represents a key strategy to improve the long-term stability and reliability of suicide switch systems.

2.3. Toxin-Antitoxin Systems

Toxin–antitoxin (TA) systems provide an effective strategy to mitigate the fitness costs often associated with suicide gene–based biocontainment circuits [42,43]. TA modules consist of a toxin, typically a small fast-acting protein, and a cognate antitoxin that may be either a protein or a noncoding RNA neutralizing toxin activity [44]. By maintaining low-level antitoxin expression to counteract basal toxin leakage in the absence of induction, TA systems can alleviate the detrimental effects of background toxicity, thereby preserving the functionality and stability of suicide gene switches. Among the best-characterized TA systems is the CcdB–CcdA pair. The CcdB toxin targets DNA gyrase, inducing double-stranded DNA breaks and cell death, whereas the CcdA antitoxin neutralizes CcdB through direct binding. In one application, a temperature-sensitive promoter governs expression of the CcdB–CcdA module in engineered strains designed for intestinal disease treatment. At the physiological temperature of 37 °C, CcdB expression is repressed to permit bacterial growth [45]. Upon excretion, exposure to ambient temperatures below 22 °C induces CcdB activation and triggers rapid bacterial killing (Figure 1c). The biocontainment robustness can be further enhanced by integrating multiple orthogonal TA modules. For example, combining the CcdB–CcdA system with the pH-responsive Doc–Phd pair has been shown to reduce escape frequencies to as low as 10^{-11} [36]. Doc toxin inhibits translation by phosphorylating the elongation factor EF-Tu, thereby preventing its interaction with tRNA, while the Phd antitoxin counteracts this effect. Reliable operation of TA-based biocontainment requires tightly regulated expression to maintain a precise balance between toxin and antitoxin expression levels. Perturbations in this balance may compromise biocontainment performance. Accordingly, the application of quantitative synthetic biology principles to TA circuit design offers a promising route to improving regulatory precision and long-term stability.

3. Limiting Horizontal Gene Transfer from Genetically Engineered Bacteria

Engineered bacteria can exchange genetic material with environmental microorganisms through HGT [46]. Such transfer events may disseminate engineered genetic elements, including antibiotic resistance genes, into natural microbial communities, potentially altering their composition and functional characteristics. Preventing and monitoring the dissemination of genetic components from engineered bacteria to environmental microorganisms remains a significant challenge. To reduce the risk of horizontal gene transfer, several strategies have been developed that employ genetic separation, target gene degradation, or physical encapsulation to limit the exposure of engineered genetic material to surrounding microbial communities.

3.1. Genetic Separation

Genetic separation is routinely applied in viral vector packaging, wherein the viral genome is partitioned to divide the structural and replication genes into distinct vectors, allowing viral assembly only when all components are present [47]. In contrast, segmenting the bacterial genome remains technically challenging since bacterial genomes are several orders of magnitude larger than viral genomes. Current strategies largely focus on partitioning specific genetic elements or selected genes within the genome to establish mutual dependency among the engineered components.

Geneguard is a plasmid-engineered bacterial mutual dependency system (Figure 2a) designed to prevent horizontal transfer of plasmids to environmental microorganisms [48]. In this system, the *thyA* gene is deleted from the engineered bacterial genome, while the plasmid carries a functional *thyA* to complement this deletion. Concurrently, the plasmid replication initiation gene *repA*, essential for the ColE2 plasmid origin of replication, is removed from the plasmid and integrated into the host chromosome. As a result, plasmid replication is confined to the engineered host, since any plasmid transferred to environmental

microbes cannot replicate in the absence of *repA*. Although Geneguard provides effective biocontainment in *E. coli*, its applicability to other bacterial species requires further validation.

Engineered bacteria often harbor complex genetic constructs, which can impose substantial metabolic burdens and reduce genetic stability, potentially leading to the loss of functional or biocontainment elements. Distributing these functional and biocontainment components across multiple host strains to form bacterial consortia [49–51] can mitigate metabolic load on individual strains and enhance the stability of genetic elements [52,53]. To further improve biosafety, strains within the community can be engineered as auxotrophic mutants that rely on metabolite exchange. This interdependence ensures that, even if one strain escapes into the environment, it cannot replicate autonomously. Community design also enables strategic selection of chassis organisms according to functional requirements. For example, biocontainment elements can be incorporated into hosts with low mutation rates, whereas therapeutic genes can be expressed in auxotrophic hosts optimized for high production. This division of labor enhances both the functional performance and biosafety stability of therapeutic bacterial communities [54–57]. Bacterial population dynamics can be further regulated through quorum-sensing (QS) circuits, which enable coordinated behaviors at the community level [58]. For instance, the PluxI promoter can control antibiotic resistance gene expression in response to the autoinducer AHL. At high AHL concentrations, resistance is induced to maintain population density, whereas at low AHL levels, resistance is suppressed, leading to substantial bacterial death [59].
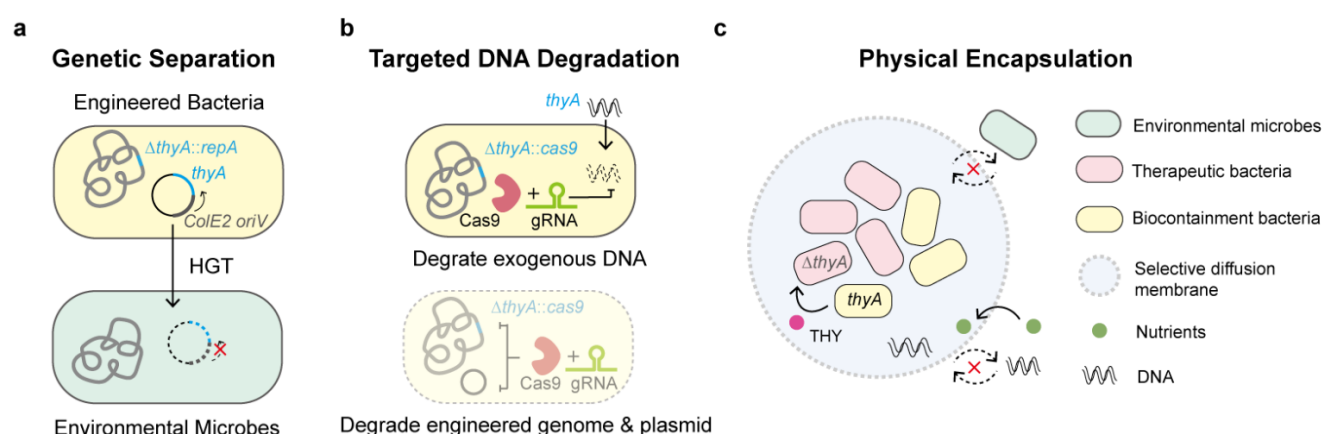

Figure 2. Biocontainment strategies mitigating horizontal gene transfer from genetically engineered bacteria. (**a**) Essential gene *thyA* or plasmid replication initiator *repA* is separately placed in a plasmid or the genome to prevent engineered plasmid horizontal gene transfer (HGT) from host bacteria to environmental microbes [48]. ColE2 oriV, ColE2 replication origin. (**b**) With the specificity of gene editing, CRISPR associated nuclease Cas9/gRNA (guide RNA) degrades exogenous DNA or engineered genome for biocontainment [60]. (**c**) Encapsulation of engineered bacteria provides physical barrier to insulate and protect therapeutic microbes [61]. Solid arrows indicate allowable transfer, while dashed arrows indicate unallowable transfer.

3.2. DNA Degradation

DNA degradation serves a dual function in biocontainment by inducing lethal double-strand breaks in the bacterial genome and selectively degrading plasmids to limit environmental dissemination of engineered genes. Clustered regularly interspaced short palindromic repeats (CRISPR)-based biocontainment systems have been widely adapted for this purpose. Biosafety platforms employing Cas (CRISPR-associated protein) nucleases such as Cas3 [62], Cas9 [63–66], and Cas12 [67,68] have achieved escape frequencies below 10^{-8}. For instance, a tetracycline and temperature regulated Cas9 switch can target the bacterial genome for cleavage, resulting in the complete clearance of engineered bacteria from the mouse gastrointestinal tract (Figure 2b). Auxotrophic strains are at risk of acquiring missing essential genes from environmental microbes, leading to compromising biocontainment. To preserve the reliability

of the auxotrophic safeguard, a *thyA*-targeting Cas9 system was introduced into a *thyA*-knockout auxotrophic strain, ensuring that any incoming exogenous thyA sequences are rapidly degraded [60]. Nevertheless, HGT may introduce alternative genes that bypass the auxotrophic dependency, and sequence variation in homologous genes (e.g., *thyA*) across environmental bacteria presents an additional challenge. Incorporating multiple guide RNAs (gRNAs) to target a broader range of potential exogenous sequences can mitigate this limitation and enhance the robustness of the containment system.

In addition to Cas proteins, other nucleases have been employed to achieve DNA degradation for biosafety purposes. Recently, Foo et al. engineered a temperature-sensitive intein-DNA endonuclease fusion protein to control the biocontainment of bacteria used in intestinal disease therapies [69]. In this system, the intein remains inactive at 37 °C, and its insertion disrupts proper folding of the endonuclease, allowing the engineered bacteria to survive and perform their therapeutic function within the mouse host. Upon excretion, exposure to lower ambient temperatures triggers intein splicing, restoring endonuclease activity, and promoting bacterial death. Notably, because this endonuclease lacks sequence specificity, it is unsuitable for applications requiring targeted gene degradation.

3.3. Phisical Encapsulation

Physical encapsulation is a key strategy for enhancing the biosafety of engineered bacteria. This approach employs polymeric materials, such as hydrogels, to insulate bacterial cells, establishing a stable physical barrier between the engineered organisms and the external environment (Figure 2c). The semi-permeable membranes formed by these polymers permit the selective diffusion of small molecules, such as nutrients, while restricting the passage of larger biomolecules (e.g., DNA) and bacteria. This confinement not only retains the engineered bacteria and their genetic elements within the material but also mitigates interference from environmental factors, including host immune responses, competing microorganisms, pH fluctuations, and ionic conditions [61,70,71]. Physical encapsulation holds significant promise for medical applications of diagnostic and therapeutic bacteria. Encapsulated bacteria can be localized at specific body sites to detect disease biomarkers or signaling molecules and perform site-specific diagnostic or therapeutic functions. For example, Gelatin methacrylate (GelMA)-based noncontact microbiota transplantation system (NMTS) microcapsules have been used to encapsulate engineered bacteria, protecting them from gastric acidity and bile salts in the gastrointestinal tract. This strategy prevents bacterial leakage and associated infections while preserving their anti-pathogenic activity [72]. Similarly, bacteria encapsulated in alginate and subsequently coated with biocompatible layers of hyaluronic acid (HA) and epigallocatechin gallate (EGCG) demonstrated enhanced intestinal colonization and sustained heme-responsive therapeutic protein secretion for monitoring and treating inflammatory bowel disease [73]. Encapsulation also facilitates integration of engineered bacteria with electronic components in personalized diagnostic devices. For instance, a microelectronic capsule containing engineered bacteria can be deployed in the gastrointestinal tract, where bacterial production of fluorescent proteins in response to inflammatory molecules is converted by integrated electronics into electrical signals, enabling real-time monitoring of intestinal inflammation [74]. Despite these advances, clinical translation of encapsulation strategies requires further validation of material stability and biocompatibility. Maintaining long-term bacterial viability and functionality, particularly for chronic disease applications, remains a major challenge. Furthermore, implantation of relatively large devices may require surgical procedures, imposing additional burdens on patients. The development of advanced biocompatible materials is expected to minimize immune rejection and inflammatory advert effects, facilitating broader clinical application.

4. Enhance the Orthogonality of Biocontainment Systems

A critical challenge in the development of biocontainment strategies is the potential failure of containment systems due to the horizontal acquisition of genetic fragments, spontaneous mutations, or the uptake of environmental metabolites. Enhancing the orthogonality of these systems is therefore essential to prevent functional interference between engineered bacteria and environmental microorganisms via metabolite exchange or genetic transfer.

4.1. Unnatural Nucleic Acids

The natural genetic code is composed of four canonical nucleotides, A, G, C, and T (or U in RNA), which encode and transmit genetic information via replication and transcription. Recently, non-canonical nucleotides have been developed to expand the genetic code and have been applied to biocontainment. For example, in *thyA*-knockout *E. coli*, which requires exogenous THY for dTTP synthesis and genome replication, gradual substitution of THY with 5-chlorouracil (Cl-U) in the medium leads to the evolution of a strain that depends exclusively on Cl-U for DNA replication. In this engineered auxotroph, approximately 90% of thymine residues in the genome were replaced by Cl-U. Because Cl-U is not naturally metabolized and must be supplied externally, this dependency provides a robust biocontainment strategy [75].

Natural nucleotides form base pairs through complementary hydrogen bonding, whereas unnatural nucleotides can utilize alternative pairing mechanisms. For example, the dNaM-dTPT3 pair interacts via hydrophobic forces. When plasmids containing dNaM–dTPT3 are introduced into *E. coli*, these unnatural nucleotides can be faithfully replicated in daughter plasmids [76] (Figure 3a). Because dNaM and dTPT3 cannot cross the bacterial membrane passively, the host *E. coli* must be engineered to genomically integrate the PtNTT2 transporter gene for active importing of these nucleotides. As a result, plasmids harboring dNaM-dTPT3 cannot replicate in wild-type bacteria lacking this specific transporter, providing an effective biocontainment mechanism. Plasmids containing dNaM-dTPT3 can also be transcribed into RNA, and ribosomes are capable of interpreting codons containing these unnatural nucleotides. For instance, incorporation of a mutant *serT* gene produces a tRNA^{Ser} with a TPT3-containing anticodon (GYU, where Y = TPT3), which decodes the unnatural codon AXC (X = NaM) as serine [77]. However, both the number and positional context of unnatural nucleotides within codons influence their orthogonality. Not all sequences support correct codon-anticodon pairing. Several orthogonal codon-anticodon pairs, including AXC–GYU, GXC–GYC, and AGX–XCU, have been identified for use in protein translation. Additional unnatural base pairs, such as 5SICS–NaM, TPT3–CNMO, and TAT1–5FM, have also been developed to expand the genetic repertoire.
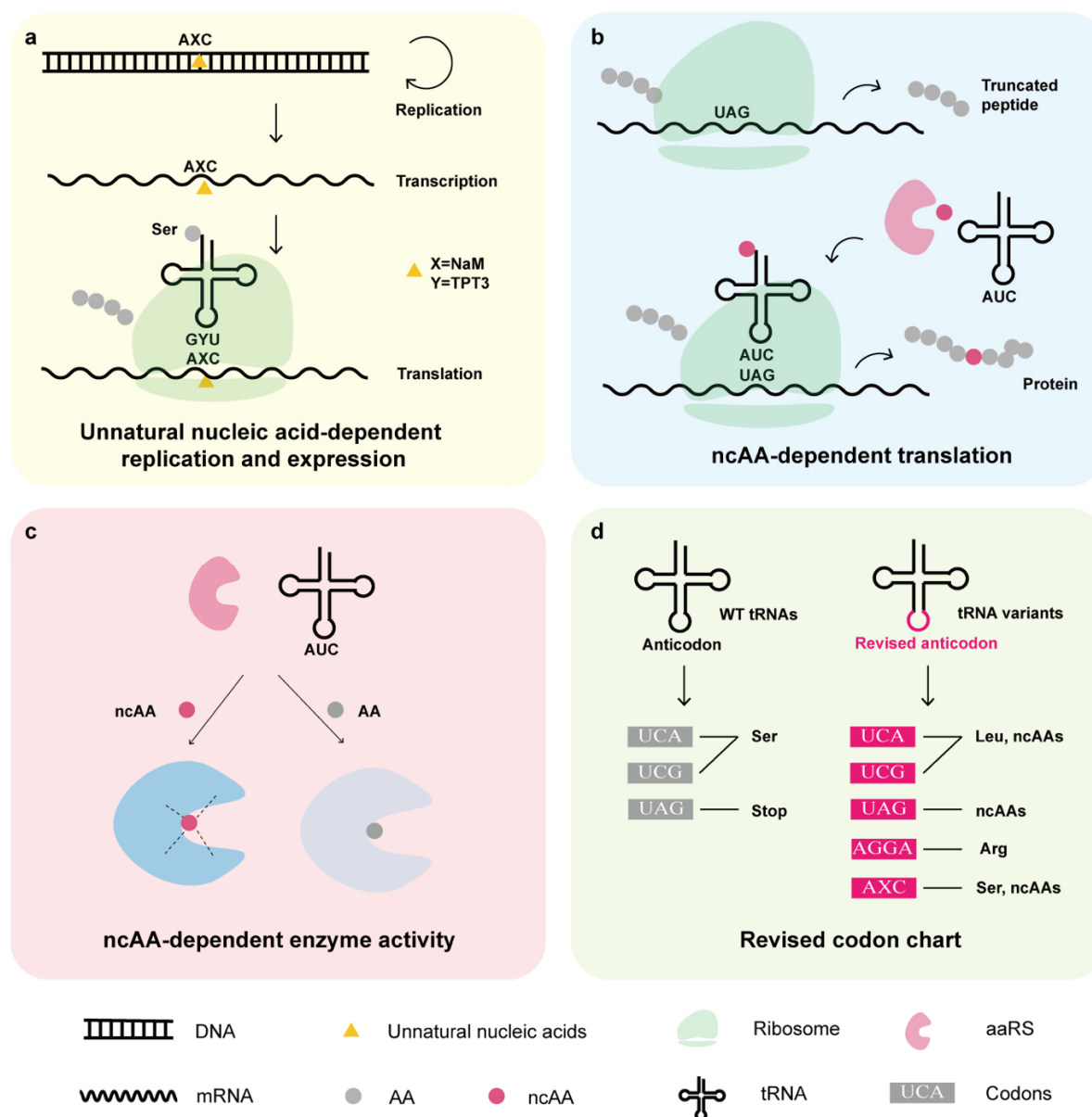
Figure 3. Synthetic replication, transcription, and translation systems enhance the orthogonality of the biocontainment strategy. (**a**) Unnatural nucleic acids are employed by DNA replication and RNA transcription [77]. (**b**) Noncanonical amino acids (ncAA) regulate essential gene translation by reading through the premature stop codons [78]. (**c**) The unique chemical structures of ncAAs confer an additional layer of biocontainment by modulating enzyme activity. (**d**) Genome-wide engineering reconstructs the codon chart and reduces cross-talking with environmental microbes [79]. AA, canonical amino acids. ncAA, noncanonical amino acids. aaRS, aminoacyl-tRNA synthetase.

4.2. Noncanonical Amino Acids

Proteins composed of 20 canonical amino acids are direct executors of biological functions. To expand amino acid diversity, multiple methods have been developed to incorporate structurally unique noncanonical amino acids (ncAAs) into proteins [78]. Among these, genetic code expansion enables the site-specific insertion of ncAAs into proteins in living cells using orthogonal tRNA/aminoacyl-tRNA synthetase (aaRS) pairs [80]. The orthogonal aaRS catalyzes the formation of ncAA-tRNA, which then pairs via its anticodon to a designated codon, typically a premature stop codon such as TAG in the mRNA, resulting in the incorporation of the ncAA at a specific site in the protein [81] (Figure 3b). This technology has been adapted to create synthetically engineered auxotrophic strains [82]. In bacteria equipped with an orthogonal tRNA/aaRS pair, introducing a premature stop codon into an essential gene prevents the

synthesis of the full-length essential protein, leading to bacterial death [83,84]. Viability is restored only when the corresponding ncAA is supplied externally. Unlike conventional auxotrophy, which relies on gene knockout, these synthetic auxotrophies exhibit significantly lower escape frequencies, as ncAAs are generally unavailable in natural environments. Further reduction in escape rates can be achieved by incorporating premature stop codons into multiple essential genes simultaneously. This approach has been applied to various engineered bacteria, including the therapeutic strain *E. coli* Nissle 1917 [85,86]. In one study, systematic screening of 155 premature stop codons introduced into 22 essential genes of *E. coli* identified that simultaneously targeting *murG*, *dnaA*, and *serS* reduces the bacterial escape rate to below 10^{-11} [87].

Orthogonal tRNA/aaRS pairs do not exhibit absolute specificity for noncanonical amino acid incorporation. In the absence of the designated ncAA, the orthogonal synthetase may mischarge a structurally similar canonical amino acid at the target codon. Even low-level misincorporation can generate trace amounts of essential proteins, potentially compromising biocontainment. To overcome this limitation, protein structure prediction and directed evolution have been employed to engineer enzymes whose activity depends exclusively on the unique chemical structure of the ncAA, thereby preventing escape via incorporation of canonical amino acids (Figure 3c) [88,89]. For example, L-4,4′-biphenylalanine (bipA) has a substantially larger aromatic side chain than any natural amino acid. Incorporation of bipA into an enzyme active site, combined with complementary mutations in surrounding residues, renders enzymatic activity strictly dependent on bipA. Substitution with any natural amino acid abolishes the enzyme function [90]. Introducing bipA dependency into multiple essential proteins in *E. coli* has reduced the escape frequency to 10^{-12}. In another approach, chorismate mutase (CM), a homodimeric enzyme in aromatic amino acid biosynthesis stabilized by a Tyr72-mediated salt bridge, was redesigned by replacing Tyr72 with a TAG codon. Using the orthogonal tRNA/aaRS system to incorporate benzoyl-L-phenylalanine (pBzF) at this site enables dimerization via π–π stacking of pBzF side chains, making bacterial viability strictly dependent on exogenous pBzF for active CM formation [91]. Similarly, the essential gene *manA*, encoding mannose-6-phosphate isomerase, requires His264 for zinc binding and catalytic activity. By remodeling the active site through mutagenesis and incorporating 3-methylhistidine (MeH) at the His264TAG position via the orthogonal system, ManA activity becomes dependent on MeH rather than histidine for zinc coordination and enzymatic function [92].

Engineered tRNA modifications in combination with synthetic genomics enable the reassignment of codon-amino acid relationships [79] (Figure 3d). For example, the fully recoded *E. coli* strain Syn61 was constructed with a genome containing only 61 codons. The serine codons TCA and TCG, along with the stop codon TAG, were liberated from their canonical assignments and reassigned to various ncAA [93]. Consequently, plasmids harboring these reassigned codons cannot produce functional proteins in environmental microorganisms that lack the corresponding orthogonal tRNA/aaRS pairs, limiting the horizontal transfer of bioactive genes. In another study, the anticodons of tRNALeu were mutated to generate tRNALeuUGA and tRNALeuCGA, which recognize the TCA and TCG codons, respectively. Introduction of these engineered tRNAs into Syn61 effectively reassigned these serine codons to leucine [94]. Further expansion of the genetic code has been achieved through tRNAs with quadruplet anticodons, enabling decoding of four-base codons. For instance, the native tRNA$^{Arg}_{CCU}$ was redesigned as tRNA$^{Arg}_{UCCU}$. Essential genes, such as the replication initiation factor *TrfA*, were modified to include an AGGA quadruplet codon, making bacterial viability strictly dependent on tRNAArgUCCU, thereby providing a dual safeguard against both bacterial escape and functional gene transfer [95]. The integration of unnatural nucleotide-based replication systems with ncAA incorporation further enables translation at codons containing synthetic bases. In one example, the codon AXC (X = NaM) is introduced into a target gene, and an engineered *E. coli* strain equipped with an orthogonal tRNA/aaRS pair decodes it by a tRNA with the anticodon GYT (Y = TPT3), then inserts a ncAA at the specified site [77]. Collectively, these

codon reassignment strategies not only reduce bacterial escape rates but also restrict the functional transfer of genetic material to natural microorganisms.

4.3. Synthetic Genome

Genetic mutations in engineered bacteria can compromise the functionality of synthetic biology components within biocontainment systems. Engineering of DNA replication, recombination, and repair can reduce mutation rates, thereby maintaining the integrity of the biocontainment system. For example, continuous culture of *E. coli* harboring a suicide gene switch for four days led to insertion mutations within the suicide gene. Deletion of the genomic insertion sequence elements SI1 and SI5 effectively suppressed these mutations, decreasing the escape frequency of the engineered bacteria by over three orders of magnitude [35].

Increasing the copy number of target genes can mitigate the functional consequences of mutations in synthetic biological components. Sequencing of escaped engineered bacteria has revealed mutations in genetic switches as a primary cause of biocontainment failure. Introducing multiple copies of regulatory genes reduces the likelihood of complete inactivation, as the probability that all copies of a multi-copy gene are simultaneously mutated is substantially lower than for a single-copy gene [96]. Moreover, integration of target genes into the host genome provides greater genetic stability than plasmid-based expression. Engineered bacteria with synthetically streamlined genomes may further reduce mutational uncertainty. Genome minimization achieved by removing non-essential genes enhances control over metabolic pathways and creates capacity for additional synthetic components. For instance, while the native genome of *Mycoplasma mycoides* spans 1079 kb, the synthesized JVCI-syn3.0 strain has a reduced genome of 531 kb [97]. Similarly, pangenome analysis of *E. coli* identified 243 essential genes out of 867, highlighting substantial potential for genomic reduction [98].

5. Conclusions and Perspective

The expanding application of synthetic biology has intensified biosafety concerns. Although rapidly advancing biocontainment strategies can effectively limit the dissemination and replication of engineered bacteria and genetic elements in the environment (Table 1), several challenges remain. Auxotrophic designs targeting specific essential genes are widely employed in therapeutic bacteria. Strategies incorporating ncAA or unnatural nucleic acids further reduce the risk of cross-feeding by environmental metabolites. However, the orthogonality of such systems requires improvement. Metabolomic profiling can inform the distribution and availability of metabolites in diverse environments, guiding the selection of feasible auxotrophic designs. Additionally, expanding the repertoire of tRNA/aaRS pairs from diverse species, coupled with protein structure prediction, is expected to yield orthogonal pairs with enhanced specificity. Environmentally responsive suicide gene switches can restrict bacterial escape, and toxin-antitoxin systems mitigate the fitness costs associated with basal expression of toxic genes. Nevertheless, the introduction of complex regulatory circuits imposes metabolic burdens. Advances in quantitative synthetic biology and artificial intelligence now enable rational design of sophisticated gene circuits and metabolic pathways, optimizing the expression of both functional and biocontainment genes [99]. Genetic separation allows the construction of host–plasmid mutual dependency systems or the distribution of functions across microbial communities, reducing HGT between engineered and environmental bacteria. To date, most biocontainment systems have been validated only in laboratory model strains. Future efforts should expand to include diverse host species, such as naturally occurring strains, to facilitate complex community-level behaviors. Genetic mutations in biocontainment elements remain a major cause of escape. Engineering DNA replication and repair pathways can reduce mutation rates, while a deeper understanding of genome function can inform the design of minimized, synthetic genomes with predictable genetic backgrounds.

Cell-free systems offer a radical solution by eliminating the risk of bacterial escape entirely. Looking ahead, integrated multi-layered containment strategies represent a critical research frontier. For instance, combining orthogonal replication and translation machinery could establish fully synthetic systems based on engineered DNA, RNA, proteins, and metabolites, substantially reducing the leakage of viable bacteria or functional genetic elements. When coupled with physical containment approaches, such as polymer-based encapsulation [100–102], these strategies have the potential to enable safer and more effective diagnostic and therapeutic bacterial products. Finally, it is challenging to track the escaping events of genetically engineered bacteria or genetic components in a complex environment, particularly when the escaping rate of the biocontainment system is low. Next-generation sequencing is promising for detecting escaping events if the escaped DNA or RNA can be distinguished from unescaped microorganisms using specific barcoding technologies [103–105].

Table 1. Summary of different biocontainment strategies.

Biocontainment Strategies	Summarizing
Auxotrophy	Most commonly used. Simple and easy to implement. Cross-talking with environmental metabolites.
Suicide switch	Rapid bacterial clearance. Without the risk of antibiotic resistance gene. Toxin leakage impairs fitness.
Toxin-Antitoxin system	Alleviate background toxicity.
Genetic separation	Reduce bacterial escaping and horizontal gene transfer. Further engineering on host genomes.
DNA degradation	Target DNA cleavage for biosafety control.
Physical encapsulation	Physical isolation of engineered bacteria and genetic components. Need further investigation for biocompatibility.
Unnatural nucleic acids	Orthogonal replication-transcription system. The number and context influence the orthogonality.
Noncanonical amino acids	An orthogonal translational system to reduce the escaping rate.
Synthetic genome	Synthetic genome to minimize genetic components and cross-talking. Need large-scale genome engineering.

Statement of the Use of Generative AI and AI-Assisted Technologies in the Writing Process

During the preparation of this manuscript, the author(s) used ChatGPT (OpenAI GPT-4.1, 2025) in order to polish language. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the published article.

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