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GeneGuard: A Modular Plasmid System Designed for Biosafety

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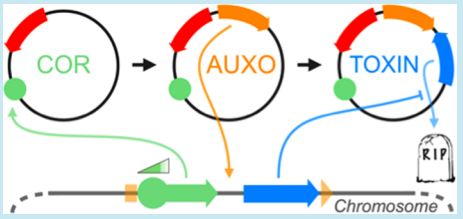
Supporting Information

ABSTRACT: Synthetic biology applications in biosensing, bioremediation, and biomining envision the use of engineered microbes beyond a contained laboratory. Deployment of such microbes in the environment raises concerns of unchecked cellular proliferation or unwanted spread of synthetic genes. While antibiotic-resistant plasmids are the most utilized vectors for introducing synthetic genes into bacteria, they are also inherently insecure, acting naturally to propagate DNA from one cell to another. To introduce security into bacterial synthetic biology, we here took on the task of completely reformatting plasmids to be dependent on their intended host strain and inherently disadvantageous for others. Using conditional origins of replication, rich-media compatible auxotrophies, and toxin−antitoxin pairs we constructed a mutually dependent host-plasmid platform, called GeneGuard. In this, replication initiators for the R6K or ColE2-P9 origins are provided *in trans* by a specified host, whose essential *thyA* or *dapA* gene is translocated from a genomic to a plasmid location. This reciprocal arrangement is stable for at least 100 generations without antibiotic selection and is compatible for use in LB medium and soil. Toxin genes ζ or Kid are also employed in an auxiliary manner to make the vector disadvantageous for strains not expressing their antitoxins. These devices, in isolation and in concert, severely reduce unintentional plasmid propagation in *E. coli* and *B. subtilis* and do not disrupt the intended *E. coli* host's growth dynamics. Our GeneGuard system comprises several versions of modular cargo-ready vectors, along with their requisite genomic integration cassettes, and is demonstrated here as an efficient vector for heavy-metal biosensors.



KEYWORDS: *biosafety, plasmids, synthetic biology, biosensors, environment, horizontal gene transfer*

Current microbial synthetic biology systems are predominantly built for use in contained bioreactors, for example, for the production of valuable compounds.[1] Other applications, by their very nature, are proposed for use outside the confines of a laboratory (e.g., biosensors,[2] bioremediation,[3] and biomining).[4] This leads to understandable concern over the release of genetically modified microbes (GMMs), including their unchecked proliferation and the possibility of "genetic pollution", (i.e., the undesired establishment of synthetic genetic material in other organisms).

In the past three decades, researchers have developed a catalogue of approaches for engineering microbes with the intention to address such GMM biosafety issues (e.g., "kill-switches", auxotrophies). Two recent reviews described the strengths and weaknesses of these methods with respect to synthetic biology[5,6] and highlighted how complete genomic recoding[7] and "xenobiology"[8] may offer solutions for the future. However, given that such technologies are in their infancy, a here-and-now solution is essential for biosensors and related GMM projects that are currently underway. In this work, we seek to fulfill this need by combining existing biosafety devices into what we believe is a robust strategy to counter unwanted horizontal gene transfer (HGT) of synthetic DNA.

Working with *E. coli*, we describe a plasmid-based system linked to its intended host strain via three separate mechanisms to ensure system redundancy. Although plasmids are extensively used in both synthetic biology and industrial biotechnology, as mobile genetic elements they are inherently an environmental biosafety concern (e.g., antibiotic resistance spread). Despite this, they offer two crucial advantages over the alternative strategy of placing synthetic genes into the host microbe genome: (i) they are easier to construct, test and tune compared to genomically integrated DNA; and (ii) via imperfect retention and/or a lack of selection,[9] they have a limited half-life when deployed into an environment. Furthermore, synthetic DNA placed within plasmids is not flanked by native genomic sequences, as it is with genomically integrated constructs. Such native flanking sequence gives the potential for synthetic DNA to homologously recombine into unmodified strains that possess significant genome homology to the intended host.

Our plasmid system, called GeneGuard, focuses on three device classes that lead to host-plasmid mutual dependency: (i) a conditional origin of replication (COR), in which the requisite plasmid replication initiator protein is provided *in trans*; (ii) complementation of an introduced host auxotrophy, with compatibility for use in common rich-media; and (iii) plasmid-encoding of a broad-spectrum toxin to select against plasmid spread by making the plasmid DNA itself disadvantageous to maintain by a wild-type bacterium. Importantly,

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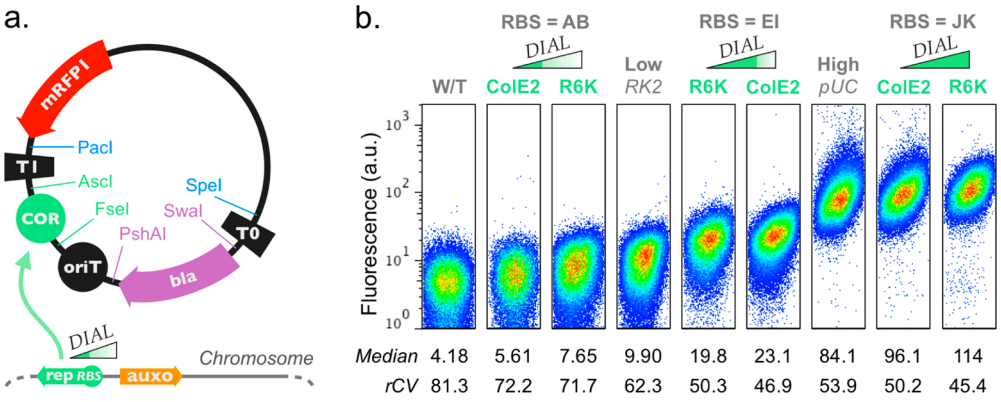

Figure 1. Flow cytometry of DIAL strains hosting COR reporter plasmids. (a) Schematic of COR plasmid dependence on host DIAL strain, where plasmid copy number is tuned by the ribosome binding site (RBS) strength of the replication initiator protein transcript. (b) DIAL strains constitutively expressing both π and RepA at low (AB), medium (EI) and high (JK) levels were transformed with mRFP1 reporter plasmids containing the R6K (pSEVA117Rb) or ColE2 (pSEVA177Rb) COR, and fluorescence assessed at mid log growth phase by flow cytometry. Low-copy RK2 (pSEVA127Rb) and high-copy pUC (pSEVA167Rb) origins were also profiled as controls. Median fluorescence values and robust coefficient of variation (rCV) are indicated beneath each plot (n = 4 biological repeats; representatives shown). W/T, wild-type *E. coli* MC1061 used for DIAL strain construction; au, arbitrary units; *X*-axis, side scatter; RBS EI, RBS E for π, RBS I for RepA (see Supporting Information Table 1 for more detail).

GeneGuard does not need to utilize antibiotic resistance cassettes.

We assemble combinations of these devices using the Standard European Vector Architecture (SEVA[10]), and tune their expression using BIOFAB bicistronic domains.[11] The result is a collection of new "secure" plasmids that are able to replace those used as the vector DNA for most *E. coli* synthetic biology projects. We examine here their efficiency for use in the lab and provide a preliminary assessment of their compatibility for use in soil. Selected devices are also examined for their effects on host growth rate, and their ability to inhibit horizontal gene transfer is assessed via electroporation into *E. coli* and natural transformation into supercompetent *Bacillus subtilis*.[12] Finally, as a proof-of-principle, we show that heavy-metal biosensors built and hosted on commonly used plasmid vectors perform as well, if not better, in the GeneGuard system.

RESULTS AND DISCUSSION

To build the GeneGuard system we took three biosecurity device classes (CORs, auxotrophy complementation and toxin−antitoxin pairs), tested their functionality in *E. coli*, and combined them into a set of customizable plasmids built upon the modular SEVA plasmid architecture.[10]

Conditional Origins of Replication (CORs). Using pSEVA111[10] as a starting point, constitutive mRFP1 reporter vectors containing either the R6K[13] or ColE2 (-P9)[14] COR were built (pSEVA117Rb and pSEVA177Rb) and transformed into three different tunable plasmid copy number "DIAL" strain variants.[15] As a proxy for confirming copy number control, fluorescent output was assessed by flow cytometry (Figure 1; see Supporting Information Table 1 for comprehensive plasmid and strain details). Low-level expression of the replication initiator proteins for R6K and ColE2 (π and RepA, respectively) in DIAL strain AB resulted in near background levels of observable fluorescence. Medium-level expression (DIAL strain EI) gave an output higher than that observed from a low-copy number RK2 origin control (pSEVA127Rb; ∼4−15 per cell[16]), while R6K/ColE2 plasmids hosted under a high level of π/RepA expression (DIAL strain JK; previously unpublished) resulted in equivalent or greater fluorescence than that observed from a high-copy number pUC origin control (pSEVA167Rb; ∼100 per cell[17,18]). These results are consistent with the original DIAL strain data, which correlated well to quantitative PCR estimation of plasmid copy number.[15] Furthermore, the inability of these CORs to propagate in the absence of π or RepA was confirmed through repeatedly unsuccessful attempts to transform wild-type *E. coli* (i.e., no colonies were obtained unless DIAL strains were used). This confirms that COR plasmid replication is dependent on a specific host, thereby inhibiting establishment in other microbes.

Rich-media Compatible Auxotrophs. The use of antibiotic resistance genes is inappropriate for environmental applications not only due to concerns over HGT but also because of the impracticality of continually applying a selective agent to a GMM-deployed area. An alternative strategy for host-plasmid dependency is auxotrophic complementation. Common auxotrophies (e.g., amino acid biosynthesis knockouts) usually require defined minimal-media to ensure key metabolite absence, and typically result in a decreased growth rate. Of greater utility are auxotrophs compatible with rich-media (e.g., LB), such as knockouts in *E. coli* for thymidine (Δ*thyA*)[19] or diaminopimelic acid (DAP; Δ*dapA*).[20] Rich-media supplementation with small quantities of these metabolites supports the growth of knockouts, as does complementation via introduction of a plasmid carrying the relevant knocked-out gene.

Using the DIAL strains as a starting chassis, Δ*thyA* or Δ*dapA* auxotrophs were created using the λ Red recombinase method,[21,22] and integrants were verified by colony PCR. These auxotrophs were unable to grow in LB medium unless supplemented with the appropriate key metabolite. To complement these knockouts, the *thyA* and *dapA* open reading frames were amplified from *E. coli* MC1061 and separately inserted into the R6K and ColE2 COR reporter plasmids (after SEVA[10] and BioBrick[23] incompatible restriction sites had been removed, giving pSEVA117RbT or D, pSEVA177RbT or D). BIOFAB constitutive promoters and bicistronic designs (BCDs), configured for reliable levels of gene expression,[11] were used to heuristically tune *thyA* and *dapA* expression.

Promoter P14 coupled to BCD7 was picked as a prospective medium-strength combination and was found to be suitable for low-copy DIAL strain auxotroph complementation. In medium or high-copy DIAL strain auxotrophic variants; however, transformants were not obtainable. A second iteration using the weaker promoter P12 (~65% strength of P14) was found to exhibit wild-type growth characteristics in all DIAL strains used, indicating that a reasonable range of thymidylate synthase (*thyA*) or 4-hydroxy-tetrahydrodipicolinate synthase (*dapA*) is sufficient for key metabolite production.

The stability of our *thyA*/*dapA* plasmids was assessed at a low-copy number (DIAL strain AB), with their complementation of the introduced auxotrophy acting as sole selection pressure (Figure 2b). In the absence of selection, the probability of each daughter cell receiving a plasmid from its mother is not sufficient to support indefinite propagation in a population over time. This was confirmed by the R6K and ColE2 COR reporter plasmids (pSEVA117Rb and pSEVA177Rb) showing near complete depletion after ~100 cell divisions. In contrast, when auxotrophy complementation was employed, all cells analyzed retained the reporter plasmid (pSEVA117RbT or D, pSEVA177RbT or D). The previously used low-copy number RK2 origin control (pSEVA127Rb) was retained by approximately half of the population under the same conditions, while the high-copy pUC origin reporter plasmid (pSEVA167Rb) was lost from the population after ~60 to 70 divisions, its presence likely placing an unfavorable burden on the host due to the energetic cost of its high replication rate.

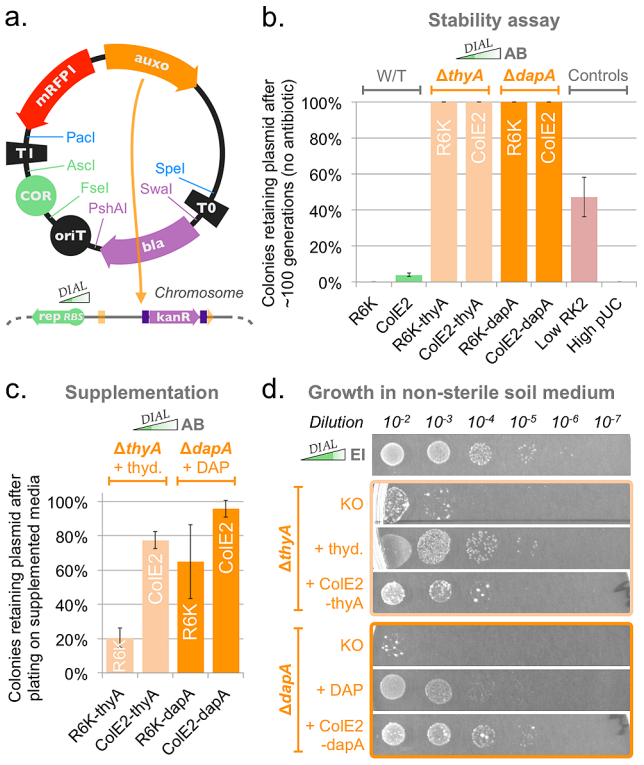

Figure 2. Plasmid stability in auxotrophic strains. (a) Schematic of DIAL strain auxotroph (Δ*thyA* or Δ*dapA*) dependence on complementing plasmid (*thyA*$^+$ or *dapA*$^+$). Orange arrow indicates complementation of knocked-out gene (remnants represented by orange bars on chromosome). (b) Stability assay measuring the proportion of colonies that retain their plasmid in low-copy DIAL strain AB after ~100 generations in liquid LB without antibiotic selection (n = 4 biological repeats; error bars = standard deviation). (c) Assay as above, but plated on agar containing key metabolite to assess plasmid stability when auxotrophic pressure is removed (n = 4 biological repeats; error bars = standard deviation). (d) Assessment nonsterile soil's ability to provide key metabolite to auxotrophs when added to liquid SOB and incubated overnight (dilution series subsequently plated on kanamycin-containing LB agar to suppress growth of other soil microbes). kanR, kanamycin resistance cassette; dark purple bars, FRT (flippase recognition target); thyd., thymidine; DAP, diaminopimelic acid; KO, knockout; R6K (pSEVA117Rb), ColE2 (pSEVA177Rb), R6K-thyA (pSEVA117RbT), ColE2-thyA (pSEVA177RbT), R6K-dapA (pSEVA117RbD), ColE2-dapA (pSEVA177RbD), low RK2 (pSEVA127Rb), high pUC (pSEVA167Rb) (see Supporting Information Table 1 for more detail).

While these two auxotrophy systems provide an antibiotic-free method of selecting for plasmids, their stability can easily be disturbed by the addition to the media of the relevant key metabolite (Figure 2c; *thyA*$^+$ plasmids are lost more quickly than *dapA*$^+$). This indicates the importance of choosing auxotrophies based on metabolites that are absent from the intended application environment. As an example, the performance of the Δ*thyA* and Δ*dapA* *E. coli* DIAL strains in garden soil was investigated. Soil was taken and used as a supplement for overnight cultures of auxotrophic medium-copy DIAL strains (EI) in SOB (Figure 2d). Both Δ*thyA* and Δ*dapA* strains grew poorly in nonsterile soil, with Δ*dapA* resulting in the fewest *E. coli* colonies. (Observation of larger, noncoliform colonies indicates the presence of additional kanamycin-resistant microbes in the soil used.) When supplemented with either the appropriate key metabolite or complementation plasmid (e.g., pSEVA177RbT or D), knockout strain growth density returned to wild-type levels. When sterile soil was used (Supporting Information Figure 1) in order to remove any potential competition effect that native microbes may have on key metabolite utilization, the Δ*dapA* strain still failed to thrive, implying that there is insufficient environmental DAP to support knockout growth.[24,25] The growth of Δ*thyA*, however, was restored, indicating the presence of adequate thymidine nucleoside in the sterilized soil sample. Although this indicates potential for the eventual disruption of plasmid propagation, such imperfect retention may actually be preferred from a biosafety standpoint. Gradual plasmid loss, when combined with a host fitness deficit, should lead to GMMs that survive for months in the environment, rather than years. This is preferable for reducing the long-term chance of genetic pollution, and could theoretically be tuned for specific applications. For example, an auxotrophy based on an abundant environmental metabolite could be used with contained biosensors (e.g., those housed in a pregnancy-test-like device[2]), thereby ensuring a rapid loss of plasmid retention pressure in the event of GMM leakage.

Toxin−Antitoxin Systems As a Negative Selection Pressure. A GMM that undergoes environmental deployment may contain synthetic genes that are advantageous for other organisms to acquire. To further reduce the likelihood of plasmid acquisition beyond a specified host, DNA-encoded broad-spectrum toxins were investigated as a means of exerting negative selection pressure on wild-type microbes. With plasmid-encoded toxins, host immunity is provided *in trans* via genomic integration of the cognate antitoxin. Full dependency on the expression or function of a toxin−antitoxin pair is a major flaw in previous "kill-switch" designs, as an inability to protect against inactivating mutations ultimately

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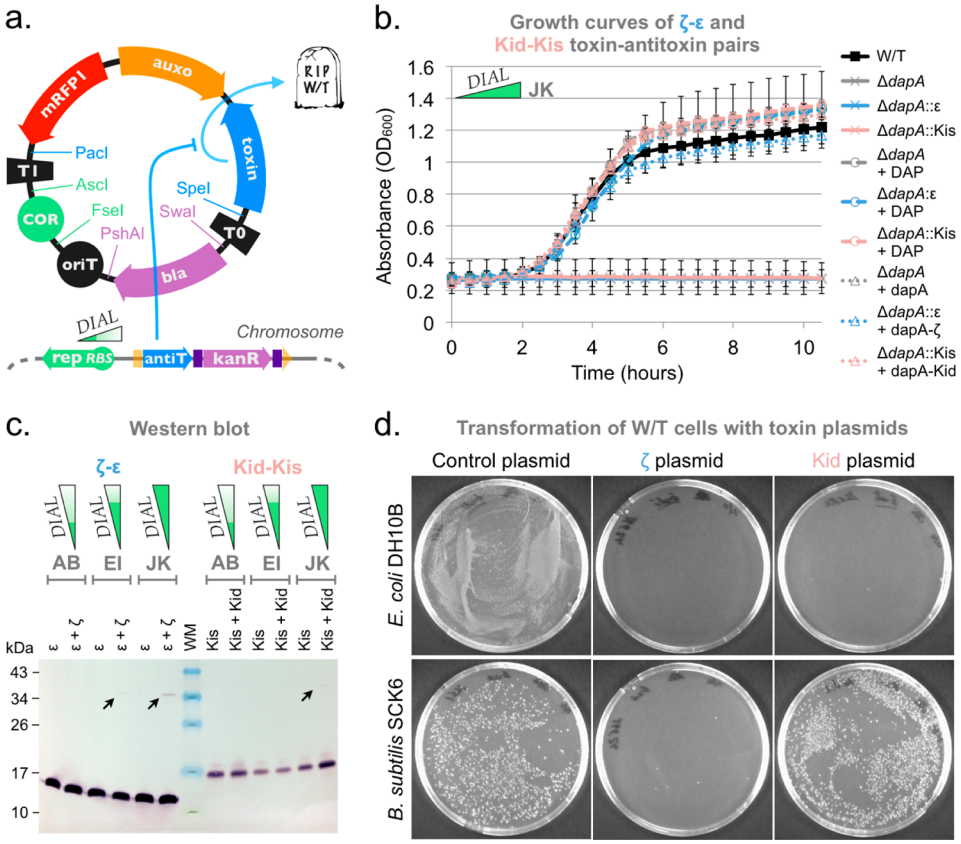

Figure 3. Use of $\zeta-\varepsilon$ and Kid-Kis toxin−antitoxin pairs. (a) Schematic of how a toxin-encoding plasmid may prove deleterious if taken up by wild-type cells, while the specified host cell possesses genome-encoded immunity. (b) Growth curves of high-copy DIAL strain JK with various combinations of Δ*dapA* auxotrophy, chromosomally integrated ε or Kis antitoxin, and plasmid-encoded ζ or Kid toxin (ColE2 COR used; $n = 3$ biological repeats; error bars = standard deviation). For Δ*thyA* auxotrophy, and other plasmid copy numbers, see Supporting Information Figure 2. (c) Western blot of various DIAL strains constitutively expressing integrated ε (11.5 kDa) or Kis (10.2 kDa) antitoxins alone, as well as with plasmid-encoded ζ (33.2 kDa) or Kid (12.7 kDa) toxins. All toxins/antitoxins are His-tagged at the C-terminus; putative toxin bands are arrowed. (d) Transformation assessment of ability of wild-type cells to maintain toxin plasmid in the absence of integrated antitoxin. W/T, wild-type; antiT, antitoxin; WM, weight marker; dapA (pSEVA177RbD); dapA-ζ (pSEVA177RbDZh); dapA-Kid (pSEVA177RbDKh); control plasmid (pSEVA3b61); ζ plasmid (pSEVA3b6Zh); Kid plasmid (pSEVA3b6Kh).

leads to safety system failure.[5,26] In GeneGuard, a toxin−antitoxin pair is instead employed in an auxiliary manner, that is, continued function is not critical to the overall integrity of the system.

As toxin activity is required to be broad-spectrum to work against a variety of environmental microbes, a broad-host-range constitutive promoter was used to maximize the likelihood of expression, and the toxins ζ (from *Streptococcus pyogenes* plasmid pSM19035)[27,28] and Kid (from *E. coli* plasmid R1)[29] were investigated. These proteins are reported to be growth-inhibitory (i.e., bacteriostatic) when expressed in Gram-positive, Gram-negative, and even some eukaryotic hosts. It is important to note that the term "toxin" relates to their activity as cytosolic enzymes: ζ interferes with the beginning of peptidoglycan synthesis,[30] while Kid is a sequence-specific endoribonuclease.[31] The poisoning of nearby organisms is therefore highly unlikely, as any released enzyme would require cellular internalization before toxicity could occur; its degradation is more probable.

His-tagged antitoxin genes for ε[27] or Kis[29] were paired with promoter P1 and ThrA-BCD2[11] and genome-integrated as per previously (i.e., auxotrophs were also simultaneously created; Figure 3a). While integration fixes antitoxin gene copy number, plasmid-encoded toxin levels will vary depending on the host DIAL strain used. A balance is therefore required so that toxin gene dosage at a high plasmid copy number does not overwhelm the available antitoxin, while maintaining an adequate expression of toxin such that a low-copy dosage remains toxic to wild-type cells lacking the antidote. This was achieved through tuning toxin expression levels with a designed, *E. coli* and *B. subtilis* compatible, spoVG RBS[32] paired with a similarly compatible constitutive Pveg2 promoter. His-tagged ζ and Kid genes were inserted into the *thyA*/*dapA* complementation plasmids (containing the ColE2 COR) so that both the auxotrophy complementation and toxin open reading frames converged upon a bidirectional terminator flanked by synthetic insulating spacers.[33]

The use of spoVG2 gave tolerable levels of ζ in all antitoxin-expressing DIAL strains. Kid expression, however, required down-tuning for its plasmid to be acceptable to the high-copy DIAL strain JK (the spoVG5 promoter, at ∼27% of the strength[32] of spoVG2, was settled upon). Both the ζ and Kid plasmids (pSEVA177RbTZh or TKh, DZh, or DKh), when hosted by their respective antitoxin/auxotrophic DIAL strains (AB, EI, or JK), did not perturb the growth rate from that of the wild-type (Figure 3b; Supporting Information Figure 2). Expression of antitoxin was confirmed via Western blot (Figure 3c) with toxin expression only just visible for both types in the

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high-copy DIAL strain JK. The strong antitoxin bands observed indicate that greater expression of both toxins should be possible: ζ is neutralized by ε at a 1:1 ratio,[30] while two molecules of Kid are neutralized by each Kis.[34] Greater expression was not found to be possible for Kid, however, as demonstrated by the need to tune its translation down with spoVG5.

To confirm toxin activity in cells lacking antitoxin, the above plasmids were transformed into the low, medium and high-copy number auxotrophic DIAL strains that lacked the relevant antitoxin gene, and as intended, no colonies were immediately obtainable. After prolonged incubation for ∼72 h, however, thousands of small colonies appeared on low-copy DIAL strain AB plates hosting the Kid toxin. This possibly indicates insufficient Kid expression for complete bacteriostasis. In addition, when the selection pressure of auxotrophy complementation was removed through the use of prototrophic DIAL strains, several healthy colonies were obtained from each toxin plasmid transformation. This was due to deleterious *recA*-mediated homologous recombination between two similarly orientated pVeg promoters within our test plasmids, leading to toxin gene removal (pVeg also drove mRFP1 expression). This event was not seen in our auxotrophic DIAL strains, as such recombination also leads to the deletion of the essential complementation gene (see Figure 3a).

To assess toxin activity in a Gram-positive bacterium, toxin genes were transferred to a SEVA shuttle backbone containing a dual replication origin (pUC and pTHT15) and a chloramphenicol resistance cassette compatible for use in both *E. coli* and *B. subtilis*.[35] These constructs (pSEVA3b6Zh or Kh) contained no mRFP1 reporter, eliminating the previous source of recombination-mediated toxin deletion. To simulate a transformation that is more environmentally realistic than electroporation, a competence-inducible *B. subtilis* strain (SCK6[12]) was used. While electroporation of *E. coli* DH10B gave no colonies for either the ζ or Kid toxin plasmids (Figure 3d), several ζ colonies and hundreds of Kid colonies resulted when transforming *B. subtilis* SCK6. The lack of Kid toxicity in *B. subtilis* SCK6 is likely due to the pre-existing genomic presence of a Kis antitoxin homologue, such as YdcD.[36] Taken together, the data presented reinforces the futility of relying on toxin integrity over time, and supports our approach of only utilizing kill-switch devices in an auxiliary role. Table 1 summarizes the ability of the described devices to influence plasmid propagation.

GeneGuard Genomic and Vector Cassettes. Following assessment of the individual devices, various COR, auxotrophy, and toxin−antitoxin combinations were arranged into a complete GeneGuard system (Figure 4). A genomic integration cassette was designed to serve as a PCR template for the λ Red-mediated genomic insertion method, and consists of genes for the COR replication initiator, an antitoxin and a kanamycin selection cassette, all flanked by the necessary 5′ and 3′ homology arms for *thyA* or *dapA* knockout creation. A low-copy RK2 origin is used in this plasmid to minimize gene dosage problems (excess antitoxin production was itself found to exhibit toxicity), and a constitutive mRFP1 marker allows for easy identification of false-positives during the integration procedure (i.e., template plasmid carryover). Once a knockout is verified, P1 transduction can be performed if desired, and the FRT-flanked kanamycin selection cassette may be excised using the pCP20-encoded FLP recombinase.[21] Note that the kanamycin resistance gene is not strictly necessary when

Table 1. Summary of COR, Auxotrophy Complementation and Toxin Device Effects on Host Cell Propagation[a]

	pSEVA3b61 Control	pSEVA117Rb R6K COR	pSEVA177Rb ColE2 COR	pSEVA3b6Zh ζ toxin	pSEVA3b6Kh Kid toxin	pSEVA177RbT ColE2 + thyA	pSEVA177RbD ColE2 + dapA	pSEVA177RbDZh ColE2 + dapA + ζ	pSEVA177RbDKh ColE2 + dapA + Kid
E. coli MC1061	>1000 (n = 3)	0 (n = 2)	0 (n = 2)	<10 (n = 2)	<10 (n = 2)	–	–	–	–
E. coli DIAL[b]	>1000 (n = 2)	>1000 (n = 3)	>1000 (n = 3)	<10 (n = 4)	<10 (n = 2)	>1000 (n = 2)	>1000 (n = 2)	<10 (n = 3)[c]	<100 (n = 3)[c]
E. coli DIAL auxo[b]	–	–	–	–	–	>1000 (n = 5)	>1000 (n = 5)	0 (n = 6)	0 (n = 6)
E. coli DIAL auxo/antitoxin[b]	–	–	–	>1000 (n = 2)	>1000 (n = 2)	>1000 (n = 2)	>1000 (n = 1)	>1000 (n = 6)	>1000 (n = 6)
B. subtilis SCK6	>100 (n = 3)	0 (n = 2)[d]	<10 (n = 2)[d]	<10 (n = 3)	>100 (n = 3)	–	–	–	–

[a]Approximate number of colonies resulting from transformation of plasmid containing the various devices into each cell line. [b]Low, medium or high plasmid copy number DIAL strains gave similar results for each plasmid (strains with cognate auxotrophies/antitoxins used where appropriate). [c]Homologous recombination led to deletion of toxin gene. [d]pSEVA3b17Rb or pSEVA3b77Rb used respectively for testing R6K or ColE2 in *B. subtilis* due to antibiotic resistance cassette compatibility. Dashes indicate experiment not performed as considered unnecessary. For full plasmid and strain details, see Supporting Information Table 1.

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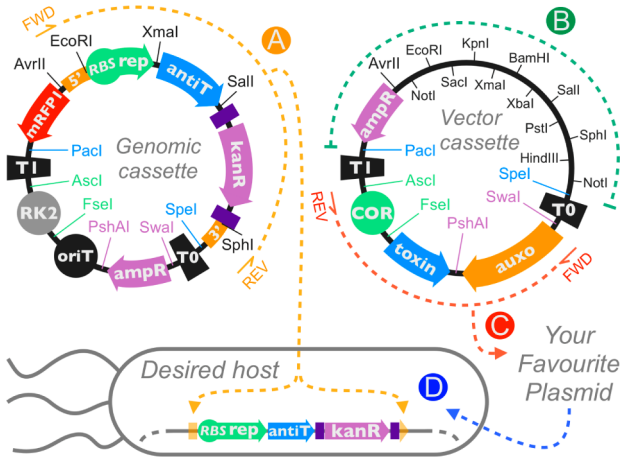

Figure 4. Schematic of the GeneGuard system. The genomic cassette (a) consists of replication initiator, antitoxin and FRT-bound kanamycin resistance genes, flanked by ~280 to 500 bp of 5′/3′ UTR sequence from the *thyA* or *dapA* genes (total cassette size of ~3.6–3.8 kbp). The vector cassette (b) hosts cargo DNA via a pUC18-derived multicloning site that contains a removable antibiotic resistance gene between the PacI and AvrII sites. To retrofit existing plasmids, the COR/toxin/auxotrophy cassette (c) may be PCR-amplified and swapped with the existing origin/antibiotic resistance region. After construction, GeneGuard-derived plasmids are dependent on host cells that contain the requisite genomic cassette (d).

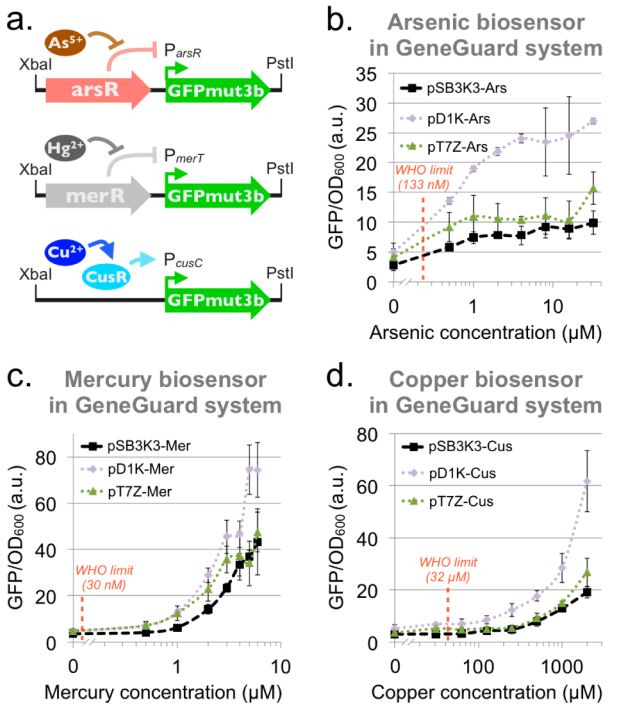

Figure 5. GeneGuard system applied to heavy-metal biosensors. (a) Schematic of biosensors inserted into GeneGuard plasmids. Arsenic relieves ArsR repression of ParsR; mercury relieves MerR repression of PmerT; and copper enables CusR activation of PcusC. Each of these promoters is linked to the reporter GFPmut3b. (b) Dose response curves in *E. coli* DH10B for the arsenic biosensor in its original plasmid (pSB3K3 contains a medium-copy p15A origin, requires kanamycin selection), and its performance when ported to GeneGuard variants pD1K (*dapA*, R6K COR, Kid toxin; no antibiotic selection used) and pT7Z (*thyA*, ColE2 COR, ζ toxin; no antibiotic selection used) with the requisite genomic cassettes inserted to support a medium-copy plasmid number (n = 4 biological repeats; error bars = standard deviation). (c) Dose response curves for the mercury biosensor, as per part b. (d) Dose response curves for the copper biosensor, as per part b. W/T, wild-type; au, arbitrary units; WHO, World Health Organization.[43] For low and high-copy GeneGuard plasmid results, see Supporting Information Figure 3.

integrating at *thyA*, as trimethoprim may instead be used as a counter-selection agent.[19]

The accompanying vector cassette consists of a COR region and toxin and auxotrophy complementation genes. With a total size of ~2.7 kbp, this compact cassette may be directly amplified by PCR and used to simultaneously replace the replication origin and resistance gene of a pre-existing plasmid (i.e., retrofitting) or the vector cassette itself can be used as a readymade plasmid backbone for the insertion of cargo DNA. To ease initial plasmid manipulation in standard cloning strains, a removable antibiotic resistance cassette is also included in the multicloning site of the vector cassette backbone.

Using the GeneGuard System for Heavy-Metal Biosensors. The GeneGuard system must satisfy two criteria for use in environmental synthetic biology applications: (i) it must increase biosafety but (ii) not disrupt application functionality. To demonstrate that our plasmids fit the second criterion, we took previously described arsenic, mercury, and copper biosensors[37] and compared their performance when hosted on their original backbone to that of two different medium-copy GeneGuard plasmids (Figure 5). When the pD1K backbone (*dapA*$^+$, R6K COR, Kid toxin) is used, fluorescence output at all heavy-metal concentrations is beneficially elevated in comparison to the original medium-copy number pSB3K3 BioBrick plasmid. When pT7Z (*thyA*$^+$, ColE2 COR, ζ toxin) is used, the biosensors have a dose response profile more equivalent to that of the original pSB3K3 backbone. This proves that the GeneGuard system does not hamper cargo function, and hence that it could be used for real-world deployment.

The useful but unexpected increase in dynamic signal range for pD1K-hosted biosensors (see Supporting Information Figure 3) is likely due to greater than expected π replication initiator production, resulting in a higher than desired plasmid copy number. This illustrates the importance of the genomic cassette's architecture: whereas in the original DIAL strains the replication initiator genes were placed in disparate genomic regions, here they were inserted immediately downstream of the native *thyA* or *dapA* promoter region and may therefore suffer from transcriptional read-through. Inversion of the replication initiator gene in a future iteration of the GeneGuard genomic cassette would address this.

Our GeneGuard system, while simple in concept, is sturdier in design when compared to other complicated biosecurity solutions.[38,39] Through the use of three distinct mechanisms, it has redundancy in its design and is highly unlikely to provide any benefit to wild-type cells, therefore limiting the potential for genetic pollution. A COR sequence on its own is of little use, unless receiving microbes already host similar initiator-encoding plasmids (and even then plasmid incompatibility will likely result). The acquisition of a constitutive auxotrophy complementation gene also provides little utility; it may lead to greater flux through a pathway, but as thymidine and DAP biosynthesis genes are already pervasive, this is unlikely to confer a significant benefit. The toxins have been selected specifically to produce a negative selection pressure, and even if their activity is neutralized via mutation or through the presence of a pre-existing antitoxin, no evolutionary advantage

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will ensue. While this version of GeneGuard is designed to work in *E. coli*, application to other environmentally relevant bacteria is possible. For example, thymidine and DAP auxotrophies have been experimented with in *Pseudomonas fluorescens*[24,25] and *P. putida*[40] for bioremediation roles, while *B. subtilis* required the deletion of two discontiguous thymidylate synthetase genes to create a thymidine auxotroph.[41] It is important to note, however, that each species requires context-specific optimization of GeneGuard device expression and function, a nontrivial task if plasmid construction and propagation during this optimization relies on using *E. coli* cloning strains.

GeneGuard, as presented here, represents the state-of-the-art for *E. coli* plasmid biosecurity. In our opinion, the best combination of parts profiled would be the ColE2 COR combined with Δ*dapA* complementation (soil lacks sufficient DAP) and the ζ toxin (effective against *E. coli* and *B. subtilis*). Our system could further be improved to limit successful HGT by refactoring vector parts to have minimal homology to all known microbial genomes (e.g., codon shuffling of the auxotrophy complementation genes) or plasmids, and through the future addition of alternative parts into the modular set. In addition, integration of the replication initiator and antitoxin genes at different genomic loci would further decrease the chance of both parts transposing to, or with, the synthetic plasmid into other cells. This, however, would require additional genomic manipulation. In conclusion, adoption of GeneGuard for environmental synthetic biology would be a beneficial move, as common biosafety concerns can be addressed without detriment to end-use applications. Whether this satisfies local and/or supranational regulations, however, is an ongoing debate. A recent workshop[42] held on this subject, covering past examples and current regulatory issues, discusses and summarizes the prospects and hurdles faced for future deployment requests of GMMs.

METHODS

Media and General Materials. Microbes were propagated in LB broth/agar, supplemented as appropriate with thymidine (20 mg/mL stock prepared in water) or diaminopimelic acid (DAP; 20 mg/mL stock prepared in water, with 10 M NaOH added dropwise until solute dissolved) to a final concentration of 50 μg/mL.[44−46] (Thymine, despite a previous report,[19] was unable to support Δ*thyA* cells.) Ampicillin was used at 100 μg/mL; kanamycin at 25 μg/mL; and chloramphenicol at 6 μg/mL. Sterile polystyrene 96-well plates from Corning B.V. Life Sciences were used for all assays, and sealed with Breathe-Easy sealing membrane (Sigma-Aldrich Company Ltd.) prior to any incubation step. *B. subtilis* SCK6[12] was purchased from the Bacillus Genetic Stock Center (http://www.bgsc.org/).

Device Construction. Oligos were ordered from Integrated DNA Technologies BVBA, and larger DNA pieces as GeneArt Strings from Life Technologies Ltd. Phusion High-Fidelity DNA polymerase (20 μL reactions; New England Biolabs (U.K.) Ltd.) was used according to the manufacturer's instructions for part construction, along with touchdown PCR[47] and overlap extension PCR[48] for device assembly/point mutations (for full sequence details and GenBank accession numbers, see Supporting Information Table 1). For colony PCR, REDTaq ReadyMix (10 μL reactions; Sigma-Aldrich Company Ltd.) was used according to the manufacturer's instructions on colony scrapings from sterile toothpicks. Sequence verification was performed by Source BioScience Plc.

Flow Cytometry. Using 96-well plates, 2 μL of overnight culture was used to seed 200 μL of fresh LB (with ampicillin) and incubated to mid log phase (30 °C, ∼710 rpm, for ∼3 h). After dilution in water, samples were analyzed using a FACScan (Becton Dickinson Co.) that had been upgraded by Cytek Development Inc. and coupled to an Automated Machine Sampler system (Cytek Development Inc.). Excitation of mRFP1 was at 561 nm, with emission monitored at 615/25 nm. Data was collected using CellQuest Pro (v5.1.1; Becton Dickinson Co.), and processed using FlowJo (v7.6.5; Tree Star Inc.).

Genomic Integration. Genomic integration was performed as previously described.[21] For each reaction, 166 μL overnight culture of desired host strain (pretransformed with pKD46; encodes arabinose-inducible λ Red recombinase system) was used to seed 8.3 mL LB ($^1/_{50}$ dilution; ampicillin) and grown at 30 °C, ∼225 rpm, for 1 h. Arabinose was then added to 0.05% (∼3.3 mM; 20% stock), and incubation continued until OD_{600} ≈ 0.4. After washing and concentrating cells to 50 μL in 20% glycerol, 200 to 400 ng of PCR product to be inserted (amplified from ∼100 pg template plasmid; purified, resuspended in water) was added for electroporation. Transformed cells were recovered for 2 h at 37 °C, ∼225 rpm, pelleted and plated on LB agar (with kanamycin, plus key metabolite to supplement the introduced auxotrophy), and incubated overnight at 37 °C. Typically ∼50 to 500 colonies were obtained, the large majority of which were successful integrants. It is worth noting that the integration of the PCR products (∼3.8 kbp) used in this work is pushing the known limits of the λ Red method.[49] In addition, thiamine pyrophosphate[50] knockouts (TPP; Δ*thiL*) were also attempted (TPP supplemented to 4.6 μg/mL[51,52]), but for unknown reasons, we were unable to isolate an auxotroph.

Plasmid Stability Assay. Using 96-well plates, 0.2 μL of overnight culture under antibiotic selection pressure (ampicillin) was used to seed 200 μL of fresh LB (no antibiotic) and incubated at 37 °C, ∼850 rpm, for ∼6 h to achieve culture saturation (approximately 10 generations). Passaging was repeated until ∼100 generations were achieved, at which point 20 μL of a 1×10^{-5} dilution was added to 50 μL fresh LB and plated on LB agar with or without antibiotic. Resultant colonies (from the nonselective plates) were examined for the presence of mRFP1 fluorescence as an indicator of plasmid retention using a hand-held 532 nm laser pointer in combination with the bandpass emission filter of a Visi-Blue transilluminator (Ultra-Violet Products Ltd.). For ColE2 CORs, where fluorescence in the low-copy DIAL strain AB was too faint to reliably assess, a combination of colony PCR, streak testing on selective media, and comparison of colony numbers on selective/nonselective media were instead made.

Soil Assay. One g of fresh or autoclaved garden soil (London, postcode SW10 0QP) was added to 3 mL of SOB medium (no antibiotic) and inoculated with 1 μL of overnight culture (grown in the presence of supplement where necessary). Cultures were grown overnight in 15 mL tubes at 30 °C, ∼225 rpm, and soil allowed to sediment before serial dilutions (1×10^{-2} to 1×10^{-7}) were made using a 96-well plate (final volumes of 180 μL). Each dilution (10 μL) was dropped onto LB agar containing kanamycin to select for auxotrophic *E. coli* strain growth, allowed to dry, and incubated overnight at 30 °C. Only ColE2 CORs in medium copy (EI) DIAL strain auxotrophs were assessed; the wild-type control

(i.e., prototroph) harbored an intermediary construction plasmid containing a R6K COR and *kanR* cassette.

Growth Curves. 0.2 μL of overnight culture was diluted $^{1}/_{9000}$ in 1.8 mL LB (~5 × 10^4 colony forming units/mL[53]), with 200 μL subsequently aliquoted into a 96-well plate. Experiments were performed *in situ* using a POLARstar Omega microplate reader (BMG Labtech GMBH), at 37 °C, ~700 rpm, with OD_{600} read every 30 min. Data was collected using Omega (v1.02) and MARS Data Analysis software (v1.10; BMG Labtech GMBH), and growth curves manually shifted along the *X*-axis to compensate for any initial lag phase.

Western Blotting. 500 μL aliquots of mid log phase cultures ($^{1}/_{100}$ overnight culture dilution, grown for 3 h at 37 °C, ~225 rpm) were pelleted and resuspended in sufficient Laemmli sample buffer[54] to normalize OD_{600} to 0.4 (in an assumed 50 μL volume), boiled for ~2 min and then allowed to cool. For ζ-His/ε-His, 7 μL of prepared sample was loaded onto an Any kD Mini-PROTEAN TGX precast gel (Bio-Rad Laboratories Ltd.); for fainter Kid-His/Kis-His samples, 14 μL was used. The gel was run in a Mini-PROTEAN Tetra Cell (Bio-Rad Laboratories Ltd.) with 5 μL of PageRuler Prestained Protein Ladder (Fisher Scientific U.K. Ltd.), transferred to a PVDF membrane using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad Laboratories Ltd.), and visualized with a WesternBreeze chromogenic immunodetection kit using Novex histidine tag (6×His) monoclonal mouse antibody (Life Technologies Ltd.) as the primary antibody at a concentration of 0.33 μg/mL ($^{1}/_{1500}$ dilution) (all as per the manufacturer's instructions).

Transformation Assay. For *E. coli* strains, 1 μL of 50 ng/μL plasmid stock was electroporated (1.8 kV, 0.1 cm electrocuvettes) into 50 μL aliquots of prepared cells (harvested mid log phase, washed and concentrated ~133x in 20% glycerol, stored at −80 °C) using a MicroPulser electroporator (Bio-Rad Laboratories Ltd.) as per the manufacturer's instructions, and recovered in 300 μL of LB for 1 h at 37 °C, ~225 rpm. 100 μL was then plated on selective LB agar. For *B. subtilis* SCK6 (integrated P_{xylA}-*comK*[12]), 1 mL of overnight culture was diluted with 2 mL fresh LB, and 105 μL of filter-sterilized 30% D-xylose added to induce competence (1% final conc.). After 2 h at 37 °C, ~225 rpm, 100 μL aliquots were taken, 5 μL of 20 ng/μL plasmid added and incubation continued for 90 min, after which all was plated on LB agar (with chloramphenicol). All plates were incubated overnight at 37 °C.

Biosensor Assay. Overnight culture (15 μL) was diluted $^{1}/_{100}$ in 1.5 mL LB, with 180 μL subsequently added to wells containing 20 μL of serially diluted heavy-metal (32 μM to 0.5 μM Na_2HAsO_4; 6 to 0.5 μM $HgCl_2$; 2,000 to 31 μM $CuSO_4$) in a 96-well plate, mixing well. Sealed plates were incubated at 30 °C, ~710 rpm, for 6 h prior to OD_{600} and GFPmut3b fluorescence (485 nm excitation, 520 nm emission, with the gain set to 1000 and bottom reading optics used) being read on a POLARstar Omega microplate reader (BMG Labtech GMBH; see previous). Antibiotic (kanamycin) was only present in samples containing the original pSB3K3 plasmids.

■ ASSOCIATED CONTENT

Supporting Information

Supplementary figures, sequence tables, GenBank accession numbers, and annotated plasmid ApE files (ApE can be downloaded for free at http://biologylabs.utah.edu/jorgensen/wayned/ape/). This material is available free of charge via the Internet at http://pubs.acs.org. In addition, selected GeneGuard plasmids may be obtained from the SEVA repository by request (see http://seva.cnb.csic.es/).

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Author Contributions

O.W. designed, constructed and tested the various devices and GeneGuard plasmids. M.D. assisted with construction and testing. O.W. and T.E. wrote the manuscript, analyzed data and created figures. T.E. and G.-B.S. designed the project, supervised and coordinated the research.

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Notes

The authors declare no competing financial interest.

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