Microbiology (2013), **159**, 1221–1235 **DOI** 10.1099/mic.0.066308-0

Review

Building-in biosafety for synthetic biology

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As the field of synthetic biology develops, real-world applications are moving from the realms of ideas and laboratory-confined research towards implementation. A pressing concern, particularly with microbial systems, is that self-replicating re-engineered cells may produce undesired consequences if they escape or overwhelm their intended environment. To address this biosafety issue, multiple mechanisms for constraining microbial replication and horizontal gene transfer have been proposed. These include the use of host–construct dependencies such as toxin–antitoxin pairs, conditional plasmid replication or the requirement for a specific metabolite to be present for cellular function. While refactoring of the existing genetic code or tailoring of orthogonal systems, e.g. xeno nucleic acids, offers future promise of more stringent 'firewalls' between natural and synthetic cells, here we focus on what can be achieved using existing technology. The state-of-the-art in designing for biosafety is summarized and general recommendations are made (e.g. short environmental retention times) for current synthetic biology projects to better isolate themselves against potentially negative impacts.

Introduction

Synthetic biology aims to design, model and apply modular whole-cell systems to provide solutions to various challenges (Khalil & Collins, 2010). Real-world applications of synthetic biology range from molecular biosynthesis in enclosed bioreactors (Martin *et al.*, 2003) through to sensing and acting upon external cues during environmental release, such as for biosensors (French *et al.*, 2011), bioremediation (Singh *et al.*, 2011) and biomining (Brune & Bayer, 2012). The majority of research and development in synthetic biology has utilized microbes as the host cells, which, in comparison with multicellular organisms, are more rapid to engineer and easier to understand. As synthetic biology advances, however, concerns are being raised about adverse effects that synthetic microbes may have if more broadly used or released into the environment (Dana *et al.*, 2012; Moe-Behrens et al., 2013). Could genetically modified microbes (GMMs) outcompete native species and disrupt habitats? Could altered or synthetic genetic material escape its host and contaminate indigenous organisms?

These concerns echo old questions raised previously by the introduction of recombinant DNA technology (Berg & Singer, 1995). At the 1975 Asilomar conference, scientists agreed on a cautious approach, incorporating both physical and biological containment into experimental design to minimize environmental risks that cisgenics or transgenics may pose (i.e. sequences native to the host, or to another species, respectively) (Berg *et al.*, 1975). Four decades later, these principles have so far ensured no significant disaster (Berg & Singer, 1995; Benner & Sismour, 2005). Following the recent demonstration of a working synthetic genome (Gibson *et al.*, 2010), a high-profile review has reaffirmed that the same caution applies to the use of 'syngenic' material, i.e. novel sequences not found in nature (Presidential Commission for the Study of Bioethical Issues, 2010). Furthermore, the success of the Asilomar approach may not only be due to stringent GMM regulation and a subsequent limited number of environmental releases, but more because the effectiveness of engineered microbes has been poor (Sayler & Ripp, 2000; de Lorenzo, 2010). Laboratory-acclimatized cells are largely unable to establish themselves in the environment in a meaningful way and face a range of serious efficacy issues even during short-term retention by a habitat (Cases & de Lorenzo, 2005). Synthetic biology, with a more holistic approach to cell engineering, appears poised to change this. Asilomar concluded that assumptions on safety would need to be reviewed as new data arise and that research to improve and assess GMM containment was high-priority (Berg *et al.*, 1975). It therefore appears wise to revisit the efficacy of fastidious hosts, non-transmissible vectors and other inbuilt biosafety mechanisms in light of their relevance to synthetic biology applications.

Horizontal gene transfer

Containment mechanisms built into GMMs, based on the Asilomar recommendations, broadly perform two tasks: (i) preventing the spread of recombinant and synthetic DNA to other organisms by horizontal gene transfer (HGT) or (ii) preventing the engineered organism from overrunning or

polluting a habitat. The issue of HGT is especially relevant for microbial systems, as it is a common and somewhat uncontrolled trait throughout the microbial biosphere (Dröge *et al.*, 1998). HGT mainly occurs by transduction (active transfer via bacteriophages), conjugation (active transfer via pili) and transformation (sequence-independent uptake of free DNA from the environment) (Davison, 1999). While it is possible to engineer solutions to prevent the active mechanisms of HGT, negating transformation is a more complicated challenge (Thomas & Nielsen, 2005). Natural cell death and lysis mean there is a continual presence of free DNA in the environment, with estimates of up to 1 μg nucleic acid per gram of soil and 80 μg per litre of marine water (Lorenz & Wackernagel, 1994; Nielsen *et al.*, 2007). Depending on particular conditions, extracellular DNA can be detected months after being placed in the environment (Nielsen *et al.*, 2007) and extracellular DNA is actively assimilated (for nutritional or genetic utilization) by many Gram-positive and -negative bacteria (Lorenz & Wackernagel, 1994; Thomas & Nielsen, 2005) as well as some unicellular (Hall *et al.*, 2005) and multicellular eukaryotes (Boschetti *et al.*, 2012). Thus, even GMMs programmed to 'self-destruct' pose an environmental risk, as their genes can potentially be scavenged by other cells after they have died.

Monitoring rates of HGT in the field is challenging due to the large sample sizes or long time frames required for rare transformants to reach assayable populations (Townsend *et al.*, 2012). From studies so far the good news is that HGT events from GMMs are nearly always deleterious and that the natural transformation frequency of microbes in soil is itself less than 1×10^{-7} per bacterium exposed (Nielsen & Townsend, 2004). Moreover, extracellular DNA only seems to be capable of transformation for a matter of hours to days post-release into the environment despite longer retention times (Nielsen *et al.*, 2007). Despite these data it is clear that some DNA elements, such as antibiotic-resistance genes, can still propagate through large ecosystems (Pruden *et al.*, 2012). As antibiotic-resistance genes are commonly used as markers during plasmid construction, there is therefore major concern that their presence in environmentally released GMMs could contribute to the generation of antibiotic-resistant 'superbugs' (Mulvey & Simor, 2009). As antibiotic resistance serves no purpose for the intended function of most GMMs (the genes are merely a legacy of construction), a primary design consideration for synthetic microbes for real-world applications should be not to incorporate antibiotic-resistance genes unless truly necessary.

General design considerations for biosafety

To avoid the use of antibiotic markers and to limit HGT from GMMs, it is tempting to consider abandoning the use of plasmids as vectors for synthetic DNA and disregarding bacterial cells as suitable hosts. Model bacteria such as *Escherichia coli* and *Bacillus subtilis* are, however, the cells we understand the most and have had the greatest successes in engineering so far. State-of-the-art applications of synthetic biology are routinely bacterial and almost always their encoding DNA is held on plasmids (e.g. *E. coli*) rather than introduced into the genome (e.g. *B. subtilis*) (Khalil & Collins, 2010). This is because plasmid manipulation and iteration is well described and simpler than genomic editing, while also offering larger gene dosage effects due to multiple copies per cell. Plasmids are modular, quick to design and build and, in the absence of selection, can be discarded by the host cell (Silva-Rocha *et al.*, 2013). This latter undervalued biosafety advantage contrasts with directly integrating engineered sequences into genomes, as such constructs are long-lasting without selection and therefore have a greater potential for 'genetic pollution', i.e. a long-term presence of unnatural genes, innocuous or otherwise, in the wider environment. This review therefore focuses on plasmid-based systems, although the points herein are generally applicable to a genomic integration strategy.

So what design considerations are of importance to maximize the safety of a plasmid system and in particular to prevent HGT of this naturally mobile element? A clear requirement is to ensure the absence of mobilization genes or origin sequences involved in conjugation or transduction (Davison, 1999). Furthermore, optimization of the vector backbone to minimize homology with mobile elements or the host genome is important in order to discourage sequence recombination (Bensasson *et al.*, 2004). Although these measures will alleviate the predominant sources of HGT amongst microbes, uptake of plasmid by indigenous organisms will still be possible through natural transformation (Thomas & Nielsen, 2005). To further guard against this we describe below a variety of the safety mechanisms and genetic devices that can be used to link a plasmid exclusively to its intended host. These typically require small changes to the host genome, as well as gross changes to the plasmid vector (illustrated by several examples in Fig. 1). The current consensus in the synthetic biology research community is that multiple biosafety mechanisms will be needed to ensure system redundancy in case of component inactivation (Presidential Commission for the Study of Bioethical Issues, 2010; see http://bioethics.gov/cms/node/172). However, the higher the complexity of a safety device, the more prone it may be to disturbance and failure. It is therefore important to understand the expression 'cost' of each component, as several in tandem will place an undesirable physiological burden on the host (Glick, 1995) and in turn act as a selective pressure to eject the system (Benner & Sismour, 2005). Counter intuitively, a lack of evolutionary stability of synthetic DNA can serve as a biosafety benefit over time, as system rejection can lead to a pseudo-restoration of a synthetic microbe to a near wild-type state. Clearly, evolutionary pressures are a crucial consideration in design.

While this review focuses solely on the biological mechanisms that can be used to contain GMMs, it should be noted that physical containment is also a powerful biosafety approach. Physical containment is the principal means that currently allows real-world handling of GMMs, either

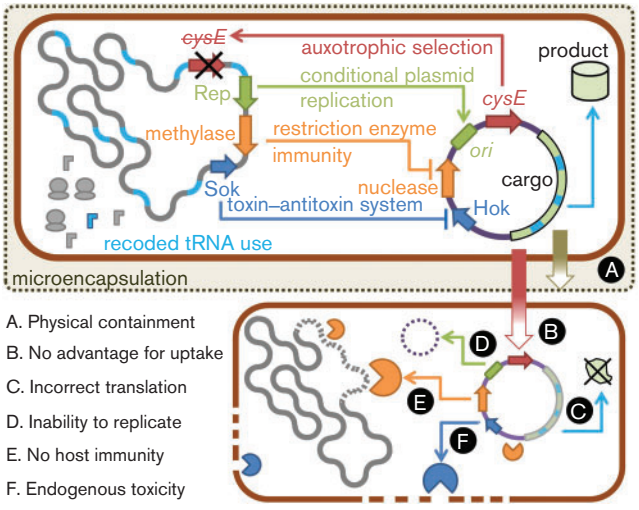

Fig. 1. Examples of safety mechanisms used to prevent transfer of a plasmid from engineered host bacteria to wild-type cells. In this example, the bacteria are physically confined in a material (e.g. alginate gel) to prevent contact with surrounding cells (A). The plasmid selection system is auxotrophy for an amino acid (cysteine) whose biosynthesis gene has been deleted from the host genome and placed on the plasmid. This selection marker, if transferred, confers no advantage to other cells as they can already perform the necessary biosynthesis (B). The synthetic DNA cargo contains sequences that can only be correctly translated by engineered bacterial hosts with refactored translation machinery (C). Replication of the plasmid origin requires a protein (Rep) that is not expressed in wild-type cells (D). In the engineered host, the Rep gene is co-integrated into the genome along with a DNA methylase and an antitoxin (Sok), which respectively suppress the lethal effects of expression of a restriction endonuclease (E) and of a membrane-depolarizing toxin (Hok) (F).

by confining cells to bioreactors (e.g. for biosynthesis applications) or using other methods such as cell micro-encapsulation (e.g. in alginate or silica beads) (Chang & Prakash, 2001; Nassif *et al.*, 2002; Papi *et al.*, 2005). Biology can achieve a lot in a contained environment; however, physical containment alone offers no guarantees. For example, no matter how ingenious a protective device or material may be for a GMM field application, an inventive way will eventually be found by an operator to compromise it. Failure in this case is a matter of when, not if. Although some form of physical containment is obviously prudent, inbuilt biological mechanisms remain crucial to biosafety.

Dependency devices to reduce the probability of GMM proliferation and HGT

A useful selection of simple genetic systems that could be employed for biological containment is described in Table 1. These are either natural systems that could be reformatted for use in synthetic biology (e.g. toxin–antitoxin pairs or auxotrophies) or existing engineered devices developed previously by others. By splitting the genetic material so that essential components are expressed *in trans* from both the plasmid and host genome, these systems become dependency devices that either make host viability dependent on maintaining the plasmid or, conversely, ensure that plasmid propagation is dependent upon staying in a specified host.

For toxin–antitoxin pairs, the activity of a small toxin (<15 kDa) is abrogated by a short-lived, *cis*-encoded antitoxin (Hayes & Van Melderen, 2011; Yamaguchi *et al.*, 2011). In the type I class, antisense RNA inhibits toxin translation; in type II, the antitoxin is proteinaceous (typically <10 kDa); and in type III systems, the antitoxin is an RNA that directly binds and inhibits the toxin. A large number of type I and type II systems are known, and a database of various type II systems is available (Shao *et al.*, 2011). Alongside classic toxin–antitoxin pairs, a variety of other systems can be viewed as toxins with countering antitoxins. Restriction endonucleases are often viewed as the immune system of bacteria, being DNA-cutting toxins whose activity is blocked by immunity-providing DNA methylases (Roberts *et al.*, 2007). Likewise, bacteriocins (Hammami *et al.*, 2010) and bacteriophage lytic systems (Catalão *et al.*, 2013) are also toxic, with the activity of each being suppressed by their own cis-encoded antidote.

First-generation dependency devices consisted of a toxin alone placed under the control of a repressible promoter as the output of a genetic circuit (Molin *et al.*, 1993). Upon repressor removal by the addition of an inducer [e.g. IPTG (Bej *et al.*, 1988)], or by depletion of a targeted contaminant (e.g. 3-methylbenzoate) or key metabolite (e.g. phosphate), toxin expression commences and host death follows (Contreras *et al.*, 1991; Schweder *et al.*, 1992). Unfortunately, this type of system, often called a 'kill switch', is prone to low but non-negligible rates of failure (Molin *et al.*, 1993). Random mutation of the constantly repressed toxin gene can lead to toxin inactivation and hence mutant outgrowth when the kill switch is thrown. The probability of such escape has been estimated at 1×10^{-6} or greater per cell generation in *E. coli* when tested in small batch cultures (Knudsen & Karlström, 1991; Moe-Behrens *et al.*, 2013). The addition of a second toxin should theoretically decrease this failure rate to 1×10^{-12}, but survival frequencies of 1×10^{-8} per cell generation are more the norm and are independent of toxin type (Knudsen *et al.*, 1995; Pecota *et al.*, 1997). This is due to there being no requirement for inactivating mutations to happen simultaneously; sequential inactivation of each toxin over time is sufficient.

Second-generation devices make toxin expression constitutive, with expression of an antitoxin being the controlled output (Paul *et al.*, 2005; Peubez *et al.*, 2010). This arrangement may improve evolutionary stability: a recent comparison of *E. coli* genomes found that highly expressed genes are less prone to mutation (Martincorena *et al.*, 2012). However, in studies so far, mutations in second-generation devices still occur at frequencies similar to those

Table 1. A selection of dependency devices that could be split *in trans* to make cell viability dependent on maintaining a plasmid or to make plasmid propagation dependent on a specific host

Strengths and weaknesses are given for using each type of device as a biosafety mechanism to limit successful HGT. Examples for each type of device include component molecular masses, which give an indication of the burden they may impose on a host cell.

System name	Brief description	Strengths (+) and weaknesses (−) for use	References
Toxin–antitoxin systems			
Type I e.g. Hok/*sok*, Fst/RNAII, TxpA/*ratA*	Type I toxin–antitoxin systems consist of a membrane-active protein toxin whose expression is repressed by a short RNA antitoxin; the *E. coli* Hok (6.1 kDa)/*sok* (65 nt) system is the best characterized of these, originating from the *parB* locus of *E. coli* plasmid R1; Fst (3.7 kDa)/RNAII (66 nt) is a Gram-positive equivalent from *Enterococcus faecalis* plasmid pAD1 and TxpA (6.7 kDa)/*ratA* (222 nt) is a homologue found on the *B. subtilis* chromosome	+ Low metabolic load due to post-transcriptional regulation − Type I systems appear to be specific for Gram-negative or -positive cells + Hok is a well-described post-segregation killing system for Gram-negative bacteria, several homologues available (e.g. HokC [*gef* gene]) − Hok-resistant *E. coli* persisters can result from overexpression of Hok − TxpA will require genomic knockout for use in *B. subtilis*	Gerdes *et al.* (1997), Gerdes & Wagner (2007), Brantl (2012), Weaver (2012), Durand *et al.* (2012)
Type II (RNase) e.g. Kid/Kis, Txe/Axe, MazF/MazE, RelE/RelB, HicA/HicB	The majority of described type II toxin–antitoxin systems consist of an endoribonuclease paired with an inhibitor antitoxin protein; Kid (14.8 kDa)/Kis (9.3 kDa) is from the *parD* locus of *E. coli* plasmid R1 and Txe (10.2 kDa)/Axe (13.6 kDa) is from the *axetxe* locus of *Enterococcus faecium* plasmid pRUM; further well-characterized examples that are native to the *E. coli* genome include MazF (12.1 kDa)/MazE (9.4 kDa) from the *rel* operon, RelE (11.2 kDa)/RelB (9.1 kDa) from the *relBE* locus and HicA (6.8 kDa)/HicB (15.2 kDa) from the *hicAB* locus	+ Type II endoribonuclease toxin–antitoxin systems are well described, with many homologues available in diverse bacteria − Type II endoribonucleases are bacteriostatic rather than bactericidal + Kid, Txe, MazF and RelE and HicA are broadly active in both Gram-positives and -negatives ± Kid, MazF and RelE are known to be toxic when expressed in eukaryotes − Txe/Axe is not well characterized − MazF, RelE and HicA all require genomic knockout for use in *E. coli*	Pedersen *et al.* (2002), Grady & Hayes (2003), Pedersen *et al.* (2003), Kamphuis *et al.* (2007), Neubauer *et al.* (2009), Diago-Navarro *et al.* (2010), Halvorsen *et al.* (2011)
Type II (gyrase inhibitor) e.g. CcdB/CcdA	CcdB (11.7 kDa) poisons DNA gyrase to inhibit replication, while CcdA (8.4 kDa) acts as the labile antitoxin; sourced from the *ccd* locus of *E. coli* plasmid F	+ Well described, analogues available (e.g. ParE/ParD from plasmid R2K) − Only active in Enterobacteriacae − Intellectual property is claimed on this system	Bernard & Couturier (1992), Bernard *et al.* (1994), Jiang *et al.* (2002)
Type II (translation inhibitor) e.g. Doc/Phd	Doc (13.6 kDa) stabilizes bound mRNA at the 30S ribosomal subunit to block translation; this is relieved by Phd (8.1 kDa); sourced from the prophage plasmid of bacteriophage P1	− Bacteriostatic rather than bactericidal ± Unknown if Doc is toxic in eukaryotes, but expected to be given that the site of action is the same as the aminoglycoside hygromycin B	Brodersen *et al.* (2000), Liu *et al.* (2008)

Table 1. cont.

System name	Brief description	Strengths (+) and weaknesses (−) for use	References
Type II (other) e.g. Zeta/Epsilon	Zeta (32.4 kDa) phosphorylates UDP-GlcNAc, preventing its use by MurA in cell wall synthesis; epsilon (10.7 kDa) acts as the antitoxin; sourced from *Streptococcus pyogenes* plasmid pSM19035	+ Active in Gram-positives and -negatives − Not as toxic to Gram-negatives as Gram-positives ± Only mildly toxic in eukaryotes (e.g. *Saccharomyces cerevisiae*)	Zielenkiewicz & Ceglowski (2005), Mutschler & Meinhart (2011)
Type III e.g. ToxN/ToxI	ToxN (19.7 kDa) is an endoribonuclease that is directly inhibited by pseudoknots encoded at the 5′ end of its transcript (ToxI region; 5.5 repeats of 36 nt); sourced from *Pectobacterium atrosepticum* plasmid pECA1039	+ Broad presence in both Gram-negatives and Gram-positives + The *cis*-encoded RNA antitoxin reduces metabolic load on cell − Bacteriostatic rather than bactericidal	Fineran *et al.* (2009), Blower *et al.* (2011, 2012)
Other toxin systems Restriction enzymes e.g. *Eco*RI	Site-specific endonucleases used by bacteria to neutralize foreign DNA, with host sequence being protected by methylation; *Eco*RI (31.1 kDa) cleaves GAATTC, while *Eco*RI methyltransferase (38.0 kDa) methylates this sequence to provide immunity; sourced from *E. coli* plasmid pMB1	+ Highly specific + Well characterized, many analogues available − Large proteins and therefore an increased metabolic load − Other organisms may carry immunity − Requires host genome to be methylated at hundreds of sites	Greene *et al.* (1981), Williams (2003), Roberts *et al.* (2003), Roberts (2005)
Bacteriocins e.g. Colicin-E3	Protein toxins used by bacteria to control growth of surrounding cells; colicin-E3 (58.0 kDa) is a RNase that specifically cleaves 16S rRNA, with ImmE3 (9.9 kDa) as an antidote; sourced from *E. coli* plasmid ColE3-CA38	+ Bacteriocins possess a large range of targets, e.g. DNA, RNA, cell wall − Some bacteriocins require post-translational modification − Many bacteriocins exhibit a relatively narrow killing spectrum due to targeting a species-specific cell receptor for uptake	Soelaiman *et al.* (2001), Riley & Wertz (2002), Cotter *et al.* (2005), Cascales *et al.* (2007)
Bacteriophage lytic systems e.g. T4 phage	Prophage-encoded system to rapidly induce host lysis for release of new virions; the formation of large inner-membrane pores by holin (*t* gene; 25.2 kDa) is inhibited by antiholin (*rI* gene; 11.1 kDa); lethality is increased by the cell wall glycosylase endolysin (*e* gene; 18.6 kDa)	+ Several heterologous systems are available to trial, large diversity − Complete system is three genes, therefore an increased metabolic load − T4 lytic systems exhibit toxicity in yeast − Fundamental triggers for holin pore formation still not understood	Garrett *et al.* (1990), Young *et al.* (2000), Dewey *et al.* (2010), White *et al.* (2011), Catalão *et al.* (2013)
Essential gene complementation			
Auxotrophy e.g. Amino acid biosynthesis	An essential gene for metabolism is knocked out and instead expressed from a plasmid; genes involved in amino acid synthesis are commonly utilized, e.g. *proA* (proline; 44.6 kDa product), *glyA* (glycine; 45.3 kDa product), *cysE* (cysteine; 29.3 kDa product), but others could be considered	+ Host strain can be cultivated in absence of plasmid by use of rich media − Overproduction from plasmid-based auxotrophic genes can have deleterious effects (e.g. *glyA* leads to acetic acid production) − Metabolic cross-feeding from cell debris can negate selection pressure − The use of (costly) minimal media is usually required	Fiedler & Skerra (2001), Baba *et al.* (2006), Vidal *et al.* (2008)

Table 1. cont.

System name	Brief description	Strengths (+) and weaknesses (−) for use	References
Non-metabolic gene knockout e.g. *infA*	An essential gene not directly involved in metabolism is expressed from a plasmid and its native genomic copy subsequently knocked out; translation initiation factor 1 (IF-1; 8.3 kDa, product of *infA* gene) is essential to *E. coli* and has been used in this manner	+ Growth rate of wild-type vs *infA*- strain with *infA*+ plasmid is the same + Cells are impermeable to IF-1, residual release from cells cannot support neighbouring plasmid-free cells − Requirement to transform plasmid into host prior to knocking out endogenous *infA* limits downstream modification	Cummings & Hershey (1994), Hägg *et al.* (2004)
Amber suppression e.g. tRNA-Phe$_{CUA}$	An amber stop codon (UAG) is placed within the ORF of an essential gene, creating an auxotrophy, e.g. amino acid biosynthesis; suppression of premature translational termination occurs via plasmid-supplied suppressor tRNA (~100 nt), charged by a natural or mutated aminoacyl-tRNA synthetase (e.g. TyrRS from *Methanocaldococcus jannaschii*; 35.0 kDa)	+ Semantic containment possible through amber codon incorporation into plasmid cargo gene(s) + Non-canonical amino acids can be used to introduce synthetic auxotrophy, i.e. dependence on chemicals not found in nature − Amber suppression can be inefficient due to suppressor tRNA competition with translational termination protein release factor 1	Kleina *et al.* (1990), Wang *et al.* (2001), Soubrier *et al.* (1999), Liu & Schultz (2010), Hoesl & Budisa (2012)
Control of non-metabolic gene e.g. TetR control system on *murA*	Endogenous promoter of an essential gene is replaced with a promoter dependent on plasmid presence; e.g. promoter of genomic *murA* (cell wall formation) is replaced by *pLtetO*; repression by co-integrated TetR (23.3 kDa), whose own promoter includes part of the ColE1 *ori* RNA II sequence (550 nt), is alleviated by ColE1-type plasmids expressing their native copy control transcript RNA I (108 nt)	+ Reduced load on the plasmid (only origin of replication required) + Host strain can be cultivated without plasmid by addition of tetracycline − Essential gene retained on chromosome, so reversion mutants likely − Only compatible with ColE1-type plasmids (e.g. pBR322, pUC and pET)	del Solar *et al.* (1998), Pfaffenzeller *et al.* (2006a, b), Mairhofer *et al.* (2008)
Operator–repressor titration e.g. LacI titration derepressing *dapD*	The *lac* promoter/operator is used to replace the native promoter of an essential gene and *lacI* (38.6 kDa product) is integrated into the genome; multiple *lac* operator sites (~60 bp) on an introduced plasmid compete for LacI, relieving repression of the essential gene (E.g. *dapD*, involved in peptidoglycan and lysine biosynthesis)	+ Reduced metabolic load on the plasmid (only *lac* operator repeats required) + Host strain can be cultivated without plasmid by addition of IPTG − Leaky expression from *lac* promoter relieves dependence − Mutation to *lac* operator region will lead to escape from dependence − *dapD* knockout can lead to increased DNA damage in *recA*$^-$strains − Intellectual property is claimed on this system	Degryse (1991), Williams *et al.* (1998), Cranenburgh *et al.* (2001, 2004), Garmory *et al.* (2005)
Miscellaneous systems			

Table 1. cont.

System name	Brief description	Strengths (+) and weaknesses (−) for use	References
RNA-OUT e.g. Levansucrase (*sacB*)	Levansucrase (53.0 kDa; *sacB* gene) is chromosomally integrated and constitutively expressed with RNA-IN leader (35 nt) placed in 5′ UTR; an RNA-OUT transcript (150 nt; plasmid-expressed) binds the RNA-IN sequence to prevent *sacB* translation, relieving the host of sensitivity to sucrose (SacB metabolizes sucrose into toxic fructose polymers)	+ Not reliant on RNA that is specific for any plasmid type + Presence of sucrose is required for toxicity, so *sacB* integrants can easily be grown in sucrose-minus conditions − Not suitable for environmental release or most industrial uses, as continued presence of sucrose is required to maintain selection	Kittle *et al.* (1989), Metcalf *et al.* (1994), Mutalik *et al.* (2012)
Overexpression of endogenous gene e.g. *fabI*	Plasmid-based overexpression of native *E. coli* enoyl ACP reductase (*fabI*; 27.9 kDa product) reduces susceptibility of *E. coli* to the biocide triclosan, which inhibits *fabI* action in fatty acid biosynthesis	+ No editing of host genome is required + Triclosan is inexpensive and wide environmental use has not led to general antibiotic-resistance (i.e. cross-resistance) − Overexpression of *fabI* in the absence of triclosan is mildly toxic − Spontaneous mutants resistant to triclosan readily occur − Cannot be used in environment as continued presence of triclosan required	McMurry *et al.* (1998), Heath *et al.* (1998), Cole *et al.* (2003), Goh & Good (2008)
Conditional origins of plasmid replication			
Tunable replication e.g. R6K *ori*-γ/π, ColE2 *ori*/Rep	Replication initiation proteins for *E. coli* plasmids R6K (π; 35.0 kDa) or ColE2-P9 (Rep; 33.6 kDa) can be relocated to the host genome and control replication of the relevant plasmid *in trans* (R6K *ori*- γ, ~380 bp; ColE2 *ori*, ~470 bp); altering the expression level of these proteins (or mutating them, e.g. π116) results in different plasmid copy numbers	+ Both Rep and π have been shown to work *in trans* with their *ori* without the need for other regulatory components + Can have a tuneable range of plasmid copy number (up to 250 per cell) − If high-copy mutant π used, additional *cer* element (~400 bp) recommended in plasmids to allow *E. coli*-mediated resolution of plasmid multimers	Filutowicz *et al.* (1986), Hiraga *et al.* (1994), Metcalf *et al.* (1994), del Solar *et al.* (1998), Kittleson *et al.* (2011), Yagura *et al.* (2006)
Host-independent replication e.g. *oriV*/RepCAB	*E. coli* plasmid RSF1010 encodes replication initiation protein RepC (31.1 kDa), DNA helicase RepA (29.9 kDa) and DNA primase RepB (35.9 kDa), which *in trans* initiate replication at the *oriV* site (~420 bp)	+ Broad host range over many Gram-negative bacteria as no reliance on host-encoded primosome − A low-copy-number replicon (10–15 per cell) − Multiple replicon components required results in an increased metabolic load	Scherzinger *et al.* (1991), del Solar *et al.* (1998)

for first-generation devices (Pecota *et al.*, 1997; Pasotti *et al.*, 2011). Splitting the pair so that the antitoxin resides on the host chromosome while the toxin is plasmid-encoded does not abate this (Torres *et al.*, 2003), but has the added benefit that, if HGT of a plasmid occurs, a recipient organism may find itself expressing toxin without the antidote (as in Fig. 1). In this arrangement, the probability of biocontainment failure is reduced, as it becomes the product of the toxin inactivation rate multiplied by the frequency of plasmid uptake by a wild-type cell. Third-generation devices may involve coupling synthetic counting circuits to the induction of toxin components (Friedland *et al.*, 2009; Callura *et al.*, 2010). Although intriguing as a route to creating microbes that commit suicide after a defined environmental retention time, this elegant approach would still suffer from the same drawbacks as described above. Regardless of the intricacy of the circuit design, when a kill switch needs to be activated, a lack of selection against mutations in its regulatory or coding sequences can lead to microbial escape. Lastly, it has recently become clear that chromosomally encoded toxin–antitoxin systems play a role in persister formation (Gerdes & Maisonneuve, 2012), in which a fraction of cells survive physicochemical insult (Lewis, 2010). Care should therefore be taken with re-engineered toxin–antitoxin systems that they do not promote this effect and thus negate efforts to induce the elimination of a GMM.

As the examples above indicate, dependency devices based solely on toxins seem destined for failure due to their inability to withstand mutation over time (Schmidt & de Lorenzo, 2012). Indeed, losing genetic information is a common problem for some synthetic biology circuits (Sleight *et al.*, 2010). A more robust approach relies on complementation of deleted or mutated chromosomal genes as a plasmid selection system, i.e. auxotrophy. Mutations that overcome auxotrophic selection in bacteria are unlikely as it is very difficult for a microbe to quickly evolve to reacquire a lost gene's function (Benner & Sismour, 2005). For this reason it should be noted that merely mutating a chromosomal gene promoter to be inactive, rather than entirely deleting the gene, allows for the possibility of reversion mutations (Cranenburgh *et al.*, 2001; Pfaffenzeller *et al.*, 2006a). Auxotrophic selection is also preferential for mitigating the potential harm of successful HGT, as an auxotrophic marker (e.g. a biosynthesis gene) is unlikely to provide any evolutionary benefit to a receiving cell, which is likely to already possess it.

Examples of several auxotrophies are outlined in Table 1 and a list of commonly used auxotrophic bacterial strains can be found elsewhere (e.g. http://cgsc.biology.yale.edu). For *E. coli*, the Keio collection of single-gene knockouts indicates potential targets for exploitation (Baba *et al.*, 2006; Yamamoto *et al.*, 2009). While amino acid auxotrophy is usually used, other auxotrophies involving genes in carbohydrate and lipid metabolism are also worth consideration (Baba *et al.*, 2006). For knockouts unable to grow in standard rich growth media (e.g. LB), *thiL* (cofactor biosynthesis), *dapA* (peptidoglycan biosynthesis) and *thyA* (nucleotide biosynthesis) are attractive targets, as supplementation with relatively inexpensive thiamine pyrophosphate, diaminopimelic acid and thymidine (or thymine), respectively can restore growth (Imamura & Nakayama, 1982; Acord & Masters, 2004; Wong *et al.*, 2005).

Auxotrophic selection does, however, suffer drawbacks. Expression levels of the plasmid-borne complementation gene need to be optimized, as overexpression can lead to toxic effects (Vidal *et al.*, 2008). Laboratory-based auxotrophies also typically rely on defined minimal media that lack the key natural metabolite; however, in deployment beyond a lab, heterogeneous environments may remove this selection pressure. This is also exhibited during metabolic cross-feeding, where plasmid-free cells are able to parasitically rely on key metabolite supply from neighbouring prototrophs. Alternative systems impervious to cross-feeding exist but their host strains are difficult to culture prior to plasmid introduction, as their supplements cannot enter the cells (Hägg *et al.*, 2004). Such an approach could still prove useful if used as a final construction step for a pre-optimized synthetic biology system.

Another method of introducing auxotrophies is the use of the amber suppressor system (Kleina *et al.*, 1990). Traditional use involves the introduction of a single amber stop codon (UAG) into an auxotrophy gene, ensuring premature termination of translation unless supplemented with an aminoacyl-charged tRNA carrying the requisite anticodon (CUA). Although the end circuit is still susceptible to interference by metabolic cross-feeding as described above, non-canonical amino acid (ncAA) systems can be utilised in tandem (Hoesl & Budisa, 2012). For example, a mutant aminoacyl-tRNA synthetase–tRNA pair was developed in *E. coli* to only recognize the ncAA *O*-methyl-L-tyrosine and exclude any natural amino acid (Wang *et al.*, 2001). When incorporated into amber suppression, host growth only occurs when this ncAA is supplied. Such a system is therefore 'orthogonal' to the host translation machinery in that the two are mutually independent (Liu & Schultz, 2010). Cell growth becomes dependent on a synthetic metabolite being provided, thus allowing for control of cell proliferation. Given, however, that *E. coli* uses the amber codon for termination in over 300 ORFs (Blattner *et al.*, 1997), this system is far from ideal. Inappropriate read-through of native genes can lead to deleterious effects and amber suppression is never fully effective in *E. coli*. Recent work on engineering modified cells for amber suppression has alleviated some of these problems, allowing read-through of several amber codons in a single gene (Hoesl & Budisa, 2012). The use of amber codons within a synthetic DNA cargo is also an attractive mechanism against HGT, as translation of such a gene would be prematurely halted in wild-type cells. A potential issue, however, is that the yield from translation of this cargo may be diminished due to inefficiencies in amber suppression, perhaps to the extent that the circuit is no longer fit-for-purpose.

While loss-of-function auxotrophies are evolutionarily hardier than toxin–antitoxin systems, they do not prevent a plasmid establishing itself in a wild-type microbe, especially if the synthetic DNA 'cargo' of the vector provides an evolutionary advantage. A further way of enforcing plasmid biocontainment is to make its replication dependent on a specific host using a system known as conditional origin of replication [COR, (Soubrier *et al.*, 1999)]. Conditional plasmid origins use a *cis*-encoded replication initiation protein (del Solar *et al.*, 1998), which, if relocated to the host chromosome, can still perform its function *in trans* in modified cells (Kittleson *et al.*, 2011). In this split replication machinery scenario, any uptake of a COR plasmid by a wild-type microbe would only be transient due to an absence of the requisite replication initiation protein.

Some of the devices described above have been used in tandem. A gene therapy vector developed in 1999 used a dual-dependency device where an amber suppressor tRNA gene and a COR (R6K *ori-*γ) were supplied on a plasmid transformed into *E. coli* (Soubrier *et al.*, 1999). The host was genomically modified to contain the R6K plasmid replication initiator (π), and the *argE* gene was mutated to include an amber codon near the beginning of its ORF. In the absence of the plasmid-supplied suppressor tRNA, the GMM was an arginine auxotroph in minimal media and the plasmid itself was unable to replicate in wild-type *E. coli*. Ramos and colleagues also updated the aforementioned 3-methylbenzoate-responsive kill switch (Contreras *et al.*, 1991) so that this compound repressed expression of a plasmid-bound toxin (HokC) as before, but additionally induced expression of a modified genomic copy of *asd*, necessary for peptidoglycan synthesis (Ronchel & Ramos, 2001). During experiments in 3-methylbenzoate-contaminated soil their modified *Pseudomonas putida* strain survived and maintained its plasmid. Upon contaminant depletion, however, this GMM dropped below detectable levels after 25 days. Mutant escape was below their detection limit and was therefore estimated to occur at a probability of 1×10^{-9} or less per cell generation.

DNA barcodes to trace synthetic biology designs

Assessing the efficacy of biosafety systems by measuring GMM spread and HGT events in sample environments is a complex task. However, with DNA sequencing becoming rapid and affordable, direct sequencing of environmental samples can now be used to identify contaminating synthetic DNA. Synthetic operons can be designed to contain genetic 'barcodes', which, if indexed to a pre-release database, could be used to identify their origin and particulars. Others have embedded 'DNA watermarks' in multiple genomic locations to aid in identifying their engineered cells (Gibson *et al.*, 2010). Barcodes not only aid in identifying GMMs in the environment, but can be used commercially to mark proprietary strains that may be stolen during industrial espionage. To guard against stolen strains simply having their DNA recoded (removing evidence of theft), cryptography approaches have also been applied that introduce cryptic 'DNA watermarks' either into multiple genomic locations in engineered cells (Gibson *et al.*, 2010) or directly into synthetic genes via manipulation of their codon usage (Liss *et al.*, 2012). This latter approach, presuming such codon changes are functionally neutral, would be especially suitable to exploit for tracing synthetic DNA in the environment. Embedding watermarks directly within genes likely to experience positive selection in the environment will help ensure their incorporation during instances of HGT, whereas upstream or downstream barcode elements may be lost during recombination events (Thomas & Nielsen, 2005).

Orthogonal systems for semantic containment

While the biosafety systems outlined above can presently be incorporated into synthetic biology designs, more radical solutions to containment are on the horizon. Researchers are now pursuing biosafety through semantic containment, whereby a 'genetic firewall' is erected between synthetic microbes and natural organisms much like a linguistic barrier (Schmidt, 2010). This process involves refactoring, in which the composition or order of the basic genetic material of a GMM is changed without altering its encoded output (i.e. polypeptide sequence). In this manner a synthetic gene may no longer be meaningfully read by a natural organism. Such orthogonality is possible in a number of ways and has been recently reviewed by Schmidt & de Lorenzo (2012); highlights, including subsequent work, are briefly mentioned below. One approach involves refactoring a cell's codon usage, and has now led to the substitution of all 314 amber codons in the *E. coli* genome with an alternative stop codon (Wang *et al.*, 2009; Isaacs *et al.*, 2011). In these cells, the liberated UAG codon is freed to encode ncAA auxotrophies at will (Chin, 2012; Church lab, unpublished data). Genome modification en masse could also be conceivably used to shuffle codon assignments in a synthetic microbial genome, resulting in the same protein products as wild-type cells but via a different genetic code. Translation of an altered synthetic gene from such a system by a natural organism would give an effectively mistranslated product. Evolved ribosomes, which recognize non-natural ribosome-binding sites for translation or translate recognizing a quadruple-base-pair code, are another way of obtaining genetic code orthogonality (Neumann *et al.*, 2010). While both of these orthogonal approaches utilize natural nucleic acids, others are pursuing synthetic versions (Kwok, 2012). An *E. coli* incorporating 5-chlorouracil into its DNA was recently created by weaning cells off the thymine nucleotide over 25 weeks (Marlière *et al.*, 2011). At the end of this directed evolution, descendant microbes grew only in the presence of the synthetic nucleotide. Beyond this, efforts to expand the genetic code beyond four bases are advancing. Alternative base pair combinations accepted by natural DNA polymerases *in vitro* have been found (Leconte *et al.*,

2008; Yang *et al.*, 2011), and work to prove these synthetic bases work with plasmids *in vivo* is ongoing. Xeno nucleic acids (XNA), where the backbone sugars of DNA are changed, seem more problematic as natural DNA and RNA polymerases do not recognize them. Although this currently limits their utility *in vivo*, recent *in vitro* work has taken a first step towards solving this problem by creating polymerase mutants that can use DNA as a template for XNA synthesis and vice-versa (Pinheiro *et al.*, 2012). The use of XNA *in vivo* is, however, many years away. XNA to XNA replication needs to be established, and a xenobiotic host would require re-engineered RNA polymerases, as well as other XNA-compatible replication and transcription components (Herdewijn & Marlière, 2009; Schmidt, 2010).

The above approaches could lead to effective semantic containment within decades; however, this would not stop a refactored microbe from competing at the physiological level with natural flora and fauna during environmental release. As per Asilomar, short-lived microbes should be utilized (Berg *et al.*, 1975). For alternative base pair and XNA systems, this is easily achievable without requiring attenuating knockouts, as the requirement for exogenous synthetic components would make them auxotrophic by definition. Supplementation at the site of release with the required synthetic compound would be required, and xeno-synthetic microbial death would ensue upon xeno-metabolite withdrawal. To stringently guard against evolution around this auxotrophy (i.e. removing synthetic-compound dependence), the xeno-metabolite would be at least two steps of synthesis away from any natural compound (Schmidt, 2010). Although only theoretical at this stage, such a system should represent the safest biocontainment mechanism possible through the incorporation of both trophic and semantic containment (Marlière, 2009).

Now and the future

Orthogonal biological systems and xenobiology offer significant hope for microbial cells designed to have minimal genetic interaction with nature, and further development of these will surely proceed. However, while truly orthogonal, environmentally relevant synthetic microbes remain years away, the repurposing of natural components remains our best arsenal for inbuilt biological safety. With no single perfect mechanism, the current consensus is that the bare minimum of safety for a deployed GMM should consist of multiple devices of different types (Presidential Commission for the Study of Bioethical Issues, 2010). This redundancy would present a GMM with several evolutionary hurdles to overcome simultaneously in order for system failure to occur, therefore greatly safeguarding against 'life finding a way' (Benner & Sismour, 2005). Physical containment should be used where suitable, and microencapsulation systems already exist that can be implemented (Chang & Prakash, 2001; Nassif *et al.*, 2002). As it is prudent not to use antibiotic-resistance markers, auxotrophic selection appears wise to incorporate into design, although care must be taken that the auxotrophy chosen is appropriate for the environment into which the GMM will be applied. DNA barcodes or watermarks provide an efficient route for tracing synthetic microbes, and plasmids with conditional origins of replication can guard against plasmid establishment following instances of HGT. The use of toxin–antitoxin pairs to secure plasmids to hosts and vice-versa is more problematic due to a lack of evolutionary stability.

A further important mechanism that can be implemented is imperfect retention, i.e. cells that survive for months but not years in the environment, or plasmid-based constructs that are gradually lost after their hosts are deployed. Asilomar-recommended attenuated microbial strains and those deficient in cell maintenance (Schweder *et al.*, 1995) so far seem unable to establish themselves in tested environments (Benner & Sismour, 2005; Cases & de Lorenzo, 2005; de Lorenzo, 2010). As short-term environmental retention times are more palatable (Church, 2005), it seems prudent for synthetic biology to be intentionally designing GMMs with half-lives of days or weeks where the intended application permits. Analysis of evolution tells us that losing a genetic circuit is much easier than obtaining it anew (Benner & Sismour, 2005). As most engineered cells are being made to perform work superfluous to their critical functions, they are likely to be out-competed beyond the comforts of the lab and therefore either die out or eject their synthetic circuits.

Further thought is required on how to design synthetic constructs and microbes to be intentionally out-competed over time. For this research to progress, more quantitative data are needed for how GMMs perform in sample environments. The current lack of in-depth testing means that it is hard to accurately assess which safety mechanisms and designs are best at preventing ecological invasion and HGT. Only initial studies with toxin systems have so far been informative, showing that simple kill switches alone are not adequate. Priority should be given to future studies assessing the successes and failures of various containment mechanism combinations in appropriate environmental situations. If a successful case study were to be taken out of the lab and put into practice, it would greatly inform the field.

Whether any GMM application will be approved in the near future for real-world use outside controlled premises is not clear. Realistically, if risk assessment primarily hinges on what effect a synthetic gene may have if accidentally established in the wild, then mitigating biosafety mechanisms are a secondary consideration (Molin *et al.*, 1993). However, the various regulatory bodies operating in biosecurity, healthcare, agriculture, etc. take differing paths to their decisions (Rodemeyer, 2009) and may show a variety of sensitivities to the use of cisgenic, transgenic or syngenic material (i.e. potential subclasses of GMMs). Scientists must realize that for some proposed real-world synthetic biology applications, the benefits of their

deployment may never outweigh the perceived risks, which range from genetic pollution via HGT of innocuous synthetic genes through to the dual-use of technologies by those intent on causing harm (Dana *et al.*, 2012; Hoffman *et al.*, 2012). In working towards future applications of GMMs, researchers therefore should not only aim to incorporate biosafety mechanisms into their designs to help alleviate potential risks, but should also seek to engage stakeholders and regulators, who will ultimately decide how safe is safe-enough (Bhattachary *et al.*, 2010; Presidential Commission for the Study of Bioethical Issues, 2010).

Encouragingly, the synthetic biologists of the future are already showing a serious interest in interacting with society beyond the lab and also in incorporating biosafety considerations into their designs. In the undergraduate synthetic biology competition iGEM (http://igem.org), the Imperial College 2011 team discussed their project design with environmental scientists and incorporated a 'Gene Guard' device to minimize HGT by using the T4 bacteriophage holin/endolysin system *in trans*. Towards the same ends, the Paris Bettencourt 2012 team created 'bWARE', a system combining physical encapsulation, an endonuclease/bacteriocin-dependency device and semantic containment via an amber suppressor system. They also compiled information on the safety mechanisms attempted by other teams (available at http://2012.igem.org/Team:Paris_Bettencourt), which will serve as a useful compendium for others.

Ultimately, the ideal safety strategy for a particular GMM depends on not only biosafety mechanisms but also the genetic cargo the cell contains, the task this performs, the intended end-user and the environment in which it will be used. While biosafety is an under-reported aspect of synthetic biology research, it is clear that it needs to be better addressed if the full potential of synthetic biology is to be realized.

Acknowledgements

The authors thank Dr Claire Marris and all members of Imperial College's Centre for Synthetic Biology and Innovation for their useful conversations that have contributed to this manuscript. This work has been supported by a grant, awarded through the Biotechnology and Biological Sciences Research Council (BBSRC)/Defence Science and Technology Laboratory (DSTL)/Engineering and Physical Sciences Research Council (EPSRC)/Medical Research Council (MRC) Joint Synthetic Biology Initiative (BB/J019720/1). G. B. S. and T. E. are further supported by EPSRC Science and Innovation Award EP/G036004/1.

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