

1 **Review for Special Issue on Synthetic Biology**

2 **Building-in Biosafety for Synthetic Biology**

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13 **Summary**

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

27 Introduction

28 Synthetic biology aims to design, model and apply modular whole-cell systems to provide solutions
29 to various challenges (Khalil & Collins, 2010). Real-world applications of synthetic biology range from
30 molecular biosynthesis in enclosed bioreactors (Martin *et al.*, 2003) through to sensing and acting
31 upon external cues during environmental release, such as for biosensors (French *et al.*, 2011),
32 bioremediation (Singh *et al.*, 2011) and biomining (Brune & Bayer, 2012). The majority of research
33 and development in synthetic biology has utilised microbes as the host cell, which, in comparison to
34 multicellular organisms, are more rapid to engineer and easier to understand. As synthetic biology
35 advances, however, concerns are being raised about adverse effects that synthetic microbes may
36 have if more broadly-used or released into the environment (Dana *et al.*, 2012; Boe-Behrens *et al.*,
37 2013). Could genetically modified microbes (GMMs) outcompete native species and disrupt
38 habitats? Could altered or synthetic genetic material escape its host and contaminate indigenous
39 organisms?

40 These concerns echo old questions raised previously by the introduction of recombinant DNA
41 technology (Berg & Singer, 1995). At the 1975 Asilomar conference, scientists agreed on a cautious
42 approach, incorporating both physical and biological containment into experimental design to
43 minimise environmental risks that *cis*- or *trans*-genics may pose (i.e. sequences native to the host, or
44 to another species, respectively) (Berg *et al.*, 1975). Four decades later, these principles have so far
45 ensured no significant disaster (Berg & Singer, 1995; Benner & Sismour, 2005). Following the recent
46 demonstration of a working synthetic genome (Gibson *et al.*, 2010), a high-profile review has
47 reaffirmed that the same caution applies to the use of “*syn-genic*” material, i.e. novel sequences not
48 found in nature (Presidential Commission for the Study of Bioethical Issues, 2010). Furthermore, the
49 success of the Asilomar approach may not primarily be due to stringent GMM regulation and a
50 subsequent limited number of environmental releases, but more because the effectiveness of
51 engineered microbes has been poor (Sayler & Ripp, 2000; de Lorenzo, 2010). Laboratory-
52 acclimatised cells are largely unable to establish themselves in the environment in a meaningful way,
53 and face a range of serious efficacy issues even during short-term retention by a habitat (Cases & de
54 Lorenzo, 2005). Synthetic biology, with a more holistic approach to cell engineering, appears poised
55 to change this. Asilomar concluded that assumptions on safety would need to be reviewed as new
56 data arise, and that research to improve and assess GMM containment was high-priority (Berg *et al.*,
57 1975). It therefore appears wise to revisit the efficacy of fastidious hosts, non-transmissible vectors
58 and other inbuilt biosafety mechanisms in light of their relevance to synthetic biology applications.

59

60 Horizontal gene transfer

61 Containment mechanisms built into GMMs, based on Asilomar’s recommendations, broadly perform
62 two tasks: (i) preventing the spread of recombinant and synthetic DNA to other organisms by
63 horizontal gene transfer (HGT), or (ii) preventing the engineered organism from overrunning or
64 polluting a habitat. The issue of HGT is especially relevant for microbial systems, as it is a common
65 and somewhat uncontrolled trait throughout the microbial biosphere (Dröge *et al.*, 1998). HGT
66 mainly occurs by transduction (active transfer via bacteriophages), conjugation (active transfer via
67 pili) and transformation (sequence-independent uptake of free DNA from the environment)

68 (Davison, 1999). While it is possible to engineer solutions to prevent the active mechanisms of HGT,
69 negating transformation is a more complicated challenge (Thomas & Nielsen, 2005). Natural cell
70 death and lysis mean there is a continual presence of free DNA in the environment, with estimates
71 of up to 1 µg nucleic acid per gram of soil and 80 µg per litre of marine water (Lorenz &
72 Wackernagel, 1994; Nielsen *et al.*, 2007). Depending on particular conditions, extracellular DNA can
73 be detected months after being placed in the environment (Nielsen *et al.*, 2007) and extracellular
74 DNA is actively assimilated (for nutritional or genetic utilisation) by many Gram-positive and -
75 negative bacteria (Lorenz & Wackernagel, 1994; Thomas & Nielsen, 2005) as well some unicellular
76 (Hall *et al.*, 2005) and multicellular eukaryotes (Boschetti *et al.*, 2012). Thus even GMMs
77 programmed to 'self-destruct' pose an environmental risk, as their genes can potentially be
78 scavenged by other cells after they have died.

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

93

94 **General design considerations for biosafety**

95 To avoid the use of antibiotic markers and to limit HGT from GMMs, it is tempting to consider
96 abandoning the use of plasmids as vectors for synthetic DNA and disregarding bacterial cells as
97 suitable hosts. Model bacteria such as *Escherichia coli* and *Bacillus subtilis* are, however, the cells we
98 understand the most and have had the greatest successes in engineering so far. State-of-the-art
99 applications of synthetic biology are routinely bacterial and almost always their encoding DNA is
100 held on plasmids (e.g. *E. coli*) rather than introduced into the genome (e.g. *B. subtilis*) (Khalil &
101 Collins, 2010). This is because plasmid manipulation and iteration is well-described and simpler than
102 genomic editing, while also offering larger gene dosage effects due to multiple copies per cell.
103 Plasmids are modular, quick to design and build, and, in the absence of selection, can be discarded
104 by the host cell (Silva-Rocha *et al.*, 2012). This undervalued biosafety advantage contrasts with
105 directly integrating engineered sequences into genomes, as these constructs are long-lasting without
106 selection and therefore have a greater potential for "genetic pollution", i.e. a long-term presence of
107 unnatural genes, innocuous or otherwise, in the wider environment. This review therefore focuses
108 on plasmid-based systems, although the points herein are generally applicable to a genomic
109 integration strategy.

110 So what design considerations are of importance to maximise the safety of a plasmid system, and in
111 particular to prevent HGT of this naturally mobile element? A clear requirement is to ensure the
112 absence of mobilisation genes or origin sequences involved in conjugation or transduction (Davison,
113 1999). Furthermore, optimisation of the vector backbone to minimise homology with mobile
114 elements or the host genome is important in order to discourage sequence recombination
115 (Bensasson *et al.*, 2004). Although these measures will alleviate the predominant causes of HGT
116 amongst microbes, uptake of plasmid by indigenous organisms will still be possible through natural
117 transformation (Thomas & Nielsen, 2005). To further guard against this we describe below a variety
118 of the safety mechanisms and genetic devices that can be used to link a plasmid exclusively to its
119 intended host. These typically require making small changes to the host genome, as well as gross
120 changes to the plasmid vector (illustrated by several examples in **Figure 1**). The current consensus in
121 the synthetic biology research community is that multiple biosafety mechanisms will be needed to
122 ensure system redundancy in case of component inactivation (Presidential Commission for the Study
123 of Bioethical Issues, 2010; see <http://bioethics.gov/cms/node/172>). However, the higher the
124 complexity of a safety device, the more prone it may be to disturbance and failure. It is therefore
125 important to understand the expression 'cost' of each component, as several in tandem will place an
126 undesirable physiological burden on the host (Glick, 1995) and in turn act as a selective pressure to
127 eject the system (Benner & Sismour, 2005). Counter intuitively, a lack of evolutionary stability of
128 synthetic DNA can serve as a biosafety benefit over time, as system rejection can lead to a pseudo-
129 restoration of a synthetic microbe to a near wild-type state. Clearly, evolutionary pressures are a
130 crucial consideration in design.

131 While this review focuses solely on the biological mechanisms that can be used to contain GMMs, it
132 should be noted that **physical containment** is also a powerful biosafety approach. Physical
133 containment is the principal means that currently allows real-world handling of GMMs, either by
134 confining cells to bioreactors (e.g. for biosynthesis applications) or using other methods such as cell
135 microencapsulation (e.g. in alginate or silica beads) (Chang & Prakash, 2001; Nassif *et al.*, 2002; Papi
136 *et al.*, 2005). Biology can achieve a lot in a contained environment; however, physical containment
137 alone offers no guarantees. For example, no matter how ingenious a protective device or material
138 may be for a GMM field application, an inventive way will eventually be found by an operator to
139 compromise it. Failure in this case is a matter of when, not if. Although some form of physical
140 containment is obviously prudent, inbuilt biological mechanisms remain crucial to biosafety.

141

142 **Dependency devices to reduce the probability of GMM proliferation and HGT**

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

150 For **toxin-antitoxin pairs**, the activity of a small toxin (<15 kDa) is abrogated by a short-lived, *cis*-
151 encoded antitoxin (Hayes & Van Melder, 2011; Yamaguchi *et al.*, 2011). In the type I class, anti-
152 sense RNA inhibits toxin translation; in type II, the antitoxin is proteinaceous (typically <10 kDa); and
153 in type III systems, the antitoxin is an RNA that directly binds and inhibits the toxin. A large number
154 of type I and type II systems are known, and a database of various type II systems is available (Shao
155 *et al.*, 2011). Alongside classic toxin-antitoxin pairs, a variety of other systems can be viewed as
156 toxins with countering antitoxins. Restriction endonucleases are often viewed as the immune system
157 of bacteria, being DNA-cutting toxins whose activity is blocked by immunity-providing DNA
158 methylases (Roberts *et al.*, 2007). Likewise, bacteriocins (Hammami *et al.*, 2010) and bacteriophage
159 lytic systems (Catalão *et al.*, 2012) are also toxic to bacteria with the activity of each of being
160 suppressed by their own antidotes.

161 First generation dependency devices consisted of a toxin alone placed under the control of a
162 repressible promoter as the output of a genetic circuit (Molin *et al.*, 1993). Upon repressor removal
163 by the addition of an inducer (e.g. IPTG (Bej *et al.*, 1988)), or by depletion of a contaminant (e.g. 3-
164 methylbenzoate) or key metabolite (e.g. phosphate), toxin expression commences and host death
165 follows (Contreras *et al.*, 1991; Schweder *et al.*, 1992). Unfortunately, this type of system, often
166 called a 'kill switch', is prone to low but significant rates of failure (Molin *et al.*, 1993). Random
167 mutation of the constantly repressed toxin gene can lead to toxin inactivation and hence mutant
168 outgrowth when the kill switch is thrown. The probability of such escape has been estimated at
169 1×10^{-6} or greater per cell generation in *E. coli* when tested in small batch cultures (Knudsen &
170 Karlström, 1991; Moe-Behrens *et al.*, 2013). The addition of a second toxin should theoretically
171 increase this to 1×10^{-12} , but survival frequencies of 1×10^{-8} per cell generation are more the norm and
172 are independent of toxin type (Knudsen *et al.*, 1995; Pecota *et al.*, 1997). This is due to there being
173 no requirement for inactivating mutations to happen simultaneously; sequential inactivation of each
174 toxin over time is sufficient.

175 Second generation devices make toxin expression constitutive, with expression of an antitoxin being
176 the controlled output (Paul *et al.*, 2005; Peubez *et al.*, 2010). This arrangement may improve
177 evolutionary stability: a recent comparison of *E. coli* genomes found that highly expressed genes are
178 less prone to mutation (Martincorena *et al.*, 2012). However, in studies so far, mutations in second
179 generation devices still occur at frequencies similar to first generation devices (Pecota *et al.*, 1997;
180 Pasotti *et al.*, 2011). Splitting the pair so that the antitoxin resides on the host chromosome while
181 the toxin is plasmid-encoded does not abate this (Torres *et al.*, 2003), but has the added benefit
182 that, if HGT of a plasmid occurs, a recipient organism finds itself expressing toxin without the
183 antidote (as in **Figure 1**). In this arrangement, the probability of biocontainment failure is reduced, as
184 it becomes the product of the toxin inactivation rate multiplied by the frequency of plasmid uptake
185 by a wild-type cell. Third generation devices may involve coupling synthetic counting circuits to the
186 induction of toxin components (Friedland *et al.*, 2009; Callura *et al.*, 2010). Although intriguing as a
187 route to creating microbes that commit suicide after a defined environmental retention time, this
188 elegant approach would still suffer from the same drawbacks as above. Regardless of the intricacy of
189 the circuit design, when a kill switch needs to be activated, a lack of selection against mutations in its
190 regulatory or toxin sequences can lead to microbial escape. Lastly, it has recently become clear that
191 chromosomally-encoded toxin-antitoxin systems play a role in persister formation (Gerdes &
192 Maisonneuve, 2012), in which a fraction of cells survive physiochemical insult (Lewis, 2010). Care

193 should therefore be taken with re-engineered toxin-antitoxin systems that they do not promote this
194 effect and thus negate efforts to induce the elimination of a GMM.

195 As the examples above indicate, dependency devices based solely on toxins seem destined for
196 failure due to their inability to withstand mutation over time (Schmidt & de Lorenzo, 2012). Indeed,
197 losing genetic information is a common problem for some synthetic biology circuits (Sleight *et al.*,
198 2010). A more robust approach relies on complementation of deleted or mutated chromosomal
199 genes as a plasmid selection system, i.e. **auxotrophy**. Mutations that overcome auxotrophic
200 selection in bacteria are unlikely as it is very difficult for a microbe to quickly evolve to re-acquire a
201 gene or its function (Benner & Sismour, 2005). For this reason it should be noted that merely
202 mutating a chromosomal gene promoter to be inactive, rather than entirely deleting the gene,
203 allows for the possibility of reversion mutations (Cranenburgh *et al.*, 2001; Pfaffenzeller *et al.*,
204 2006a). Auxotrophic selection is also preferential for mitigating the potential harm of successful
205 HGT, as an auxotrophic marker (e.g. a biosynthesis gene) is unlikely to provide any evolutionary
206 benefit to a receiving cell, which likely already possesses it.

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

217 Auxotrophic selection does, however, suffer drawbacks. Expression levels of the plasmid-borne
218 complementation gene need to be optimised, as over-expression can lead to toxic effects (Vidal *et al.*,
219 2008). Laboratory-based auxotrophies also typically rely on defined, minimal media that lack the
220 key natural metabolite; however, in deployment beyond a lab, heterogeneous environments may
221 remove this selection pressure. This is also exhibited during metabolic cross-feeding, where plasmid-
222 free cells are able to parasitically rely on metabolite supply from neighbouring prototrophs.
223 Alternative systems impervious to cross-feeding exist but their host strains are difficult to culture
224 prior to plasmid introduction, as their supplements cannot enter the cells (Hägg *et al.*, 2004). Such
225 an approach could still prove useful if used as a final construction step for a pre-optimised synthetic
226 biology system.

227 Another method of introducing auxotrophies is the use of the **amber suppressor** system (Kleina *et al.*,
228 1990). Traditional use involves the introduction of a single amber stop codon (UAG) into an
229 auxotrophy gene, ensuring premature termination of translation unless supplemented with an
230 aminoacyl-charged tRNA carrying the requisite anticodon (CUG). Although the end circuit is still
231 susceptible to interference by metabolic cross-feeding as described above, non-canonical amino acid
232 (ncAAs) systems can also be utilised (Hoesl & Budisa, 2012). For example, a mutant aminoacyl-tRNA
233 synthetase/tRNA pair was developed in *E. coli* to only recognise the nCAA *O*-methyl-L-tyrosine and
234 exclude any natural amino acid (Wang *et al.*, 2001). When incorporated into amber suppression,

235 host growth only occurs when this ncAA is supplied. Such a system is therefore ‘**orthogonal**’ to the
236 host translation machinery in that the two are mutually independent (Liu & Schultz, 2010), and cell
237 growth becomes dependent on a synthetic metabolite being provided, thus allowing for control of
238 cell proliferation. Given, however, that *E. coli* uses the amber codon for termination in over 300
239 open reading frames (Blattner *et al.*, 1997), this system is far from ideal. Inappropriate read-through
240 of native genes can lead to deleterious effects and amber suppression is never fully effective in *E.*
241 *coli*. Recent work on engineering modified cells for amber suppression has alleviated some of these
242 problems, allowing read-through of several amber codons in a single gene (Hoesl & Budisa, 2012).
243 The use of amber codons within a synthetic DNA cargo is also an attractive mechanism against HGT,
244 as translation of such a gene would be prematurely halted in wild-type cells. A potential issue,
245 however, is that the yield from translation of this cargo may be diminished due to inefficiencies in
246 amber suppression, perhaps to the extent that the circuit is no longer fit-for-purpose.

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

256 Some of the devices described above have been used in tandem. A gene therapy vector developed in
257 1999 used a dual-dependency device where an amber suppressor tRNA gene and a COR (R6K *ori-γ*)
258 were supplied on a plasmid transformed into *E. coli* (Soubrier *et al.*, 1999). The host was genomically
259 modified to contain the R6K plasmid replication initiator, π , and the *argE* gene was mutated to
260 include an amber codon near the beginning of its open reading frame. In the absence of the plasmid-
261 supplied suppressor tRNA, the *E. coli* was an arginine auxotroph in minimal media, and the plasmid
262 itself was unable to replicate in wild-type *E. coli*. Ramos and colleagues also updated the
263 aforementioned 3-methylbenzoate-responsive kill switch (Contreras *et al.*, 1991) so that this
264 compound both repressed expression of a plasmid-bound toxin (HokC) as before, but additionally
265 induced expression of a modified genomic copy of *asd*, necessary for peptidoglycan synthesis
266 (Ronchel & Ramos, 2001). During experiments in 3-methylbenzoate-contaminated soil their modified
267 *Pseudomonas putida* strain survived and maintained its plasmid. Upon contaminant depletion,
268 however, this GMM dropped below detectable levels after 25 days. Mutant escape was below their
269 detection limit, and was therefore estimated to occur at a probability of 1×10^{-9} or less per cell
270 generation.

271

272 **DNA barcodes to trace synthetic biology designs**

273 Assessing the efficacy of biosafety systems by measuring GMM spread and HGT events in sample
274 environments is a complex task. However, with DNA sequencing becoming rapid and affordable,
275 direct sequencing of environmental samples can now be used to identify contaminating synthetic

276 DNA. Synthetic operons can be designed to contain genetic ‘barcodes’, which, if indexed to a pre-
277 release database, could be used to identify their origin and particulars. Others have embedded ‘DNA
278 watermarks’ in multiple genomic locations to aid in identifying their engineered cells. (Gibson *et al.*,
279 2010). Barcodes not only aid in identifying GMMs in the environment but can be used commercially
280 to mark proprietary strains that may be stolen during industrial espionage. To guard against stolen
281 strains simply having their DNA re-coded (removing evidence of theft), cryptography approaches
282 have also been applied that introduce cryptic ‘DNA watermarks’ either into multiple genomic
283 locations in engineered cells (Gibson *et al.*, 2010) or directly into synthetic genes via manipulation of
284 their codon usage (Liss *et al.*, 2012). This latter approach, presuming such codon changes are
285 functionally neutral, would be especially suitable to exploit for tracing synthetic DNA in the
286 environment. Embedding watermarks directly within genes likely to experience positive selection in
287 the environment will help ensure their incorporation during instances of HGT, whereas up- or
288 downstream barcode elements may be lost during recombination events (Thomas & Nielsen, 2005).

289

290 **Orthogonal systems for semantic containment**

291 While the biosafety systems outlined above can presently be incorporated into synthetic biology
292 designs, more radical solutions to containment are on the horizon. Researchers are now pursuing
293 biosafety through **semantic containment**, whereby a ‘genetic firewall’ is erected between synthetic
294 microbes and natural organisms much like a linguistic barrier (Schmidt, 2010). This process involves
295 *refactoring*, in which the composition or order of the basic genetic material of a GMM is changed
296 without altering its encoded output (i.e. polypeptide sequence). In this manner a synthetic gene may
297 no longer be meaningfully read by a natural organism. Such orthogonality is possible in a number of
298 ways, and has been recently reviewed by Schmidt & de Lorenzo (2012); highlights, including
299 subsequent work, are briefly mentioned below. One approach involves refactoring a cell’s codon
300 usage, and has now led to the substitution of all 314 amber codons in the *E. coli* genome with an
301 alternative stop codon (Wang *et al.*, 2009; Isaacs *et al.*, 2011). In these cells, the liberated UAG
302 codon is freed to encode ncAA auxotrophies at will (Chin, 2012; Lajoie *et al.*, 2013). Genome
303 modification *en masse* could also be conceivably used to shuffle codon assignments in a synthetic
304 microbial genome, resulting in the same protein products as wild-type cells but via a different
305 genetic code. Translation of an altered synthetic gene from such a system by a natural organism
306 would give an effectively mistranslated product. Evolved ribosomes, which recognise non-natural
307 ribosome binding sites for translation or translate recognising a quadruple base pair code, are
308 another way of obtaining genetic code orthogonality (Neumann *et al.*, 2010). While both of these
309 orthogonal approaches utilise natural nucleic acids, others are pursuing synthetic versions (Kwok,
310 2012). An *E. coli* incorporating 5-chlorouracil into its DNA was recently created by weaning the cell
311 off the thymine nucleotide over 25 weeks (Marlière *et al.*, 2011). At the end of this directed
312 evolution, descendant microbes grew only in the presence of this synthetic nucleotide. Beyond this,
313 efforts to expand the genetic code beyond four bases are advancing. Alternative base pair
314 combinations accepted by natural DNA polymerases *in vitro* have been found (Leconte *et al.*, 2008;
315 Yang *et al.*, 2011), and work to prove these synthetic bases work with plasmids *in vivo* is on-going.
316 Xeno nucleic acids (XNA), where the backbone sugars of DNA are changed, seem more problematic
317 as natural DNA and RNA polymerases do not recognise them. Although this currently limits their
318 utility *in vivo*, recent *in vitro* work has taken a first step towards solving this problem by creating

319 polymerase mutants that can use DNA as a template for XNA synthesis, and vice-versa (Pinheiro *et*
320 *al.*, 2012). The use of XNA *in vivo* is, however, many years away. XNA to XNA replication needs to be
321 established, and a xenobiotic host would require re-engineered RNA polymerases, as well as other
322 XNA-compatible replication and transcription components (Herdewijn & Marlière, 2009; Schmidt,
323 2010).

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

336

337 **Now and the future**

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

355 A further important mechanism that can be implemented is *imperfect retention*, i.e. cells that
356 survive for months but not years in the environment, or plasmid-based constructs that are gradually
357 lost after their hosts are deployed. Asilomar recommended attenuated microbial strains, and those
358 deficient in cell maintenance (Schweder *et al.*, 1995) so far seem unable to establish themselves in
359 tested environments (Benner & Sismour, 2005; Cases & de Lorenzo, 2005; de Lorenzo, 2010). As

360 short-term environmental retention times are more palatable (Church, 2005), it seems prudent for
361 synthetic biology to be intentionally designing GMMs with half-lives of days or weeks where the
362 intended application permits. Analysis of evolution tells us that losing a genetic circuit is much easier
363 than obtaining it anew (Benner & Sismour, 2005). As most engineered cells are being made to
364 perform work superfluous to their critical functions, they are likely to be out-competed beyond the
365 comforts of the lab and therefore either die out or eject their synthetic circuits.

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

375 Whether any GMM application will be approved in the near future for real-world use outside
376 controlled premises is not clear. Realistically, if risk assessment primarily hinges on what effect a
377 synthetic gene may have if accidentally established in the wild, then mitigating biosafety
378 mechanisms are a secondary consideration (Molin *et al.*, 1993). However, the various regulatory
379 bodies operating in biosecurity, healthcare, agriculture, etc. take differing paths to their decisions
380 (Rodemeyer, 2009), and may show a variety of sensitivities to the use of *cis*-, *trans*- or *syn*-genic
381 material (i.e. potential sub-classes of GMMs). Scientists must realise that for some proposed real-
382 world synthetic biology applications, the benefits of their deployment may never outweigh the
383 perceived risks, which range from genetic pollution via HGT of innocuous synthetic genes through to
384 the dual-use of technologies by those intent on causing harm (Dana *et al.*, 2012; Hoffman *et al.*,
385 2012). In working towards future applications of GMMs, researchers should therefore not only aim
386 to incorporate biosafety mechanisms into their designs to help alleviate potential risks, but should
387 also seek to engage stakeholders and regulators, which will ultimately decide how safe is safe-
388 enough (Bhattachary *et al.*, 2010; Presidential Commission for the Study of Bioethical Issues, 2010).

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

399 Ultimately, the ideal safety strategy for a particular GMM depends not only on biosafety
400 mechanisms but also the genetic cargo the cell contains, the task this performs, the intended end-
401 user and the environment in which it will be used. While biosafety is an under-reported aspect of

402 synthetic biology research, it is clear that it needs to be better addressed if the full potential of
403 synthetic biology is to be realised.

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Tables

System name	Brief description	Strengths (+) and Weaknesses (-) for use	References
Toxin-Antitoxin Systems			
Type I e.g. Hok/ <i>sok</i> Fst/RNAII TxpA/ <i>ratA</i>	Type I toxin-antitoxin systems consist of a membrane-active protein toxin whose expression is repressed by a short RNA antitoxin. The <i>E. coli</i> Hok (6.1 kDa)/ <i>sok</i> (65 nt) system is the best-characterised of these, originating from the <i>parB</i> locus of <i>E. coli</i> plasmid R1. Fst (3.7 kDa)/RNAII (66 nt) is a Gram-positive equivalent from <i>E. faecalis</i> plasmid pAD1, and TxpA (6.7 kDa)/ <i>ratA</i> (222 nt) is a homolog found on the <i>B. subtilis</i> chromosome.	<ul style="list-style-type: none"> + 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 [<i>gef</i> gene]) - Hok-resistant <i>E. coli</i> persisters can result from overexpression of Hok - TxpA will require genomic knockout for use in <i>B. subtilis</i> 	<p>(Gerdes <i>et al.</i>, 1997) (Gerdes & Wagner, 2007) (Brantl, 2012) (Weaver, 2012) (Durand <i>et al.</i>, 2012)</p>
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 <i>parD</i> locus of <i>E. coli</i> plasmid R1 and Txe (10.2 kDa)/Axe (13.6 kDa) is from the <i>axetxe</i> locus of <i>E. faecium</i> plasmid pRUM. Further well-characterised examples that are native to the <i>E. coli</i> genome include MazF (12.1 kDa)/MazE (9.4 kDa) from the <i>rel</i> operon, RelE (11.2 kDa)/RelB (9.1 kDa) from the <i>relBE</i> locus, and HicA (6.8 kDa)/HicB (15.2 kDa) from the <i>hicAB</i> locus.	<ul style="list-style-type: none"> + 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 characterised - MazF, RelE and HicA all require genomic knockout for use in <i>E. coli</i> 	<p>(Pedersen <i>et al.</i>, 2002) (Grady & Hayes, 2003) (Pedersen <i>et al.</i>, 2003) (Kamphuis <i>et al.</i>, 2007) (Neubauer <i>et al.</i>, 2009) (Diago-Navarro <i>et al.</i>, 2010) (Halvorsen <i>et al.</i>, 2011)</p>
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 <i>ccd</i> locus of <i>E. coli</i> plasmid F.	<ul style="list-style-type: none"> + Well described, analogues available (e.g. ParE/ParD from plasmid R2K) - Only active in Enterobacteriaceae - Intellectual property is claimed on this system 	<p>(Bernard & Couturier, 1992) (Bernard <i>et al.</i>, 1994) (Jiang <i>et al.</i>, 2002)</p>
Type II (translation inhibitor) e.g. Doc/Phd	Doc (13.6 kDa) stabilises 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.	<ul style="list-style-type: none"> - 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 	<p>(Brodersen <i>et al.</i>, 2000) (Liu <i>et al.</i>, 2008)</p>
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 <i>S. pyogenes</i> plasmid pSM19035.	<ul style="list-style-type: none"> + Active in Gram-positives and -negatives - Not as toxic to Gram-negatives as Gram-positives ± Only mildly toxic in eukaryotes (e.g. <i>S. cerevisiae</i>) 	<p>(Zielenkiewicz & Ceglowski, 2005) (Mutschler & Meinhart, 2011)</p>

Type III e.g. ToxN/ToxI	ToxN (19.7 kDa) is an endoribonuclease that is directly inhibited by pseudoknots encoded at the 5' of its transcript (ToxI region; 5.5 repeats of 36 nt). Sourced from <i>P. atrosepticum</i> plasmid pECA1039.	<ul style="list-style-type: none"> + Broad presence in both Gram-negatives and Gram-positives + The <i>cis</i>-encoded RNA antitoxin reduces metabolic load on cell - Bacteriostatic rather than bactericidal 	(Fineran <i>et al.</i> , 2009) (Blower <i>et al.</i> , 2011) (Blower <i>et al.</i> , 2012)
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Other Toxin Systems

Restriction enzymes e.g. EcoRI	Site-specific endonucleases used by bacteria to neutralise foreign DNA, with host sequence being protected by methylation. EcoRI (31.1 kDa) cleaves GAATTC, while EcoRI methyltransferase (38.0 kDa) methylates this sequence to provide immunity. Sourced from <i>E. coli</i> plasmid pMB1.	<ul style="list-style-type: none"> + Highly specific + Well characterised, 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 <i>et al.</i> , 1981) (Williams, 2003) (Roberts <i>et al.</i> , 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 ribonuclease that specifically cleaves 16S ribosomal RNA, with ImmE3 (9.9 kDa) as an antidote. Sourced from <i>E. coli</i> plasmid ColE3-CA38.	<ul style="list-style-type: none"> + 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 <i>et al.</i> , 2001) (Riley & Wertz, 2002) (Cotter <i>et al.</i> , 2005) (Cascales <i>et al.</i> , 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 (<i>t</i> gene; 25.2 kDa) is inhibited by antiholin (<i>rl</i> gene; 11.1 kDa). Lethality is increased by the cell wall glycosylase endolysin (<i>e</i> gene; 18.6 kDa).	<ul style="list-style-type: none"> + 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 <i>et al.</i> , 1990) (Young <i>et al.</i> , 2000) (Dewey <i>et al.</i> , 2010) (White <i>et al.</i> , 2011) (Catalão <i>et al.</i> , 2012)

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 utilised, e.g. <i>proA</i> (proline; 44.6 kDa product), <i>glyA</i> (glycine; 45.3 kDa product), <i>cysE</i> (cysteine; 29.3 kDa product), but others could be considered.	<ul style="list-style-type: none"> + Host strain can be cultivated in absence of plasmid by use of rich media - Overproduction from plasmid-based auxotrophic gene can have deleterious effects (e.g. <i>glyA</i> 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 <i>et al.</i> , 2006) (Vidal <i>et al.</i> , 2008)
Non-metabolic gene knockout e.g. <i>infA</i>	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 <i>infA</i> gene) is essential to <i>E. coli</i> and has been used in this manner.	<ul style="list-style-type: none"> + Growth rate of wild-type vs. <i>infA</i>- strain with <i>infA</i>+ 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 <i>infA</i> limits downstream modification 	(Cummings & Hershey, 1994) (Hägg <i>et al.</i> , 2004)
Amber suppression e.g. tRNA-Phe _{CUA}	An amber stop codon (UAG) is placed within the open reading frame 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 <i>M. jannaschii</i> ; 35.0 kDa).	<ul style="list-style-type: none"> + 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 <i>et al.</i> , 1990) (Wang <i>et al.</i> , 2001) (Soubrier <i>et al.</i> , 1999) (Liu & Schultz, 2010) (Hoesl & Budisa, 2012)

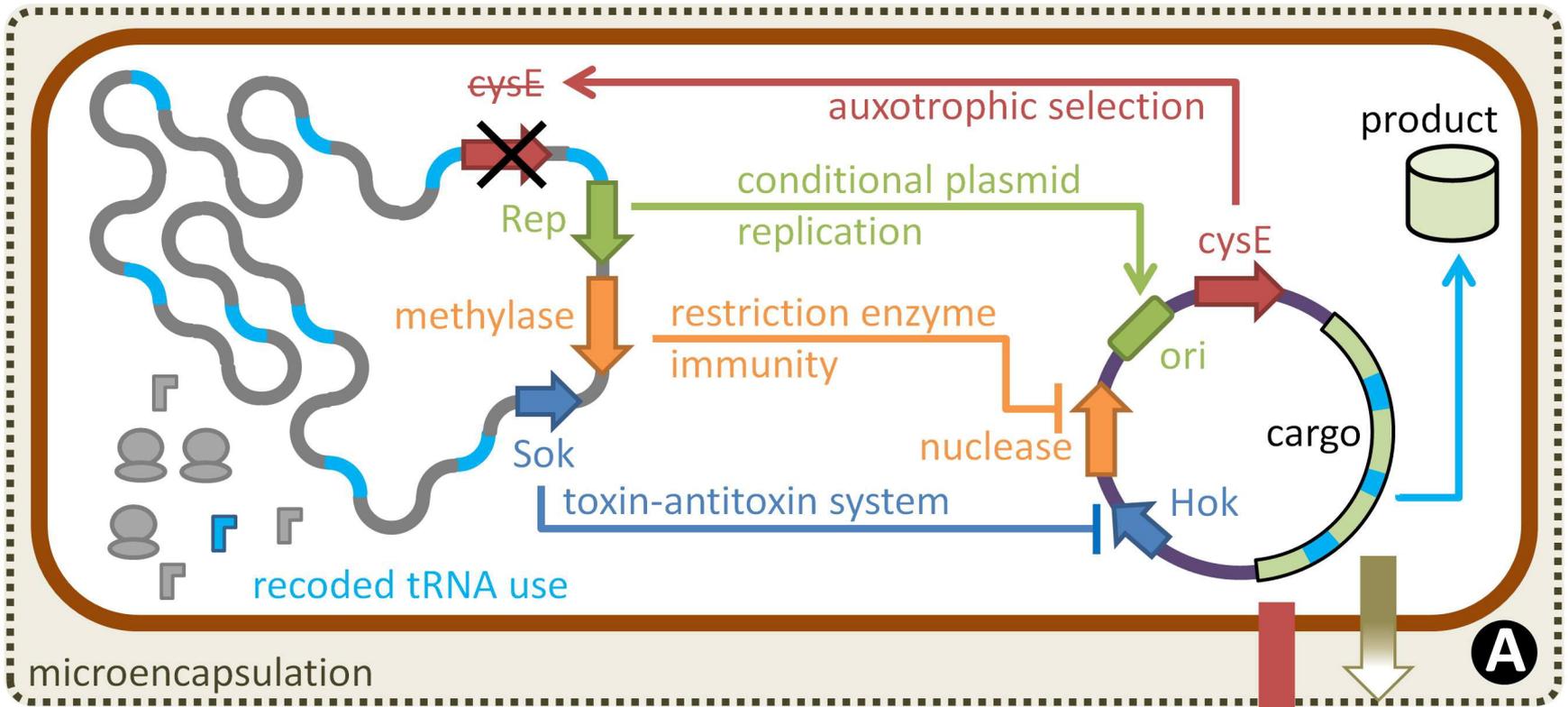
Control of non-metabolic gene e.g. TetR control system on <i>murA</i>	Endogenous promoter of an essential gene is replaced with a promoter dependent on plasmid presence. E.g. Promoter of genomic <i>murA</i> (cell wall formation) is replaced by <i>pLtetO</i> . Repression by co-integrated TetR (23.3 kDa), whose own promoter includes part of the ColE1 <i>ori</i> RNA II sequence (550 nt), is alleviated by ColE1-type plasmids expressing their native copy control transcript RNA I (108 nt).	<ul style="list-style-type: none"> + 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 <i>et al.</i> , 1998) (Pfaffenzeller <i>et al.</i> , 2006a) (Pfaffenzeller <i>et al.</i> , 2006b) (Mairhofer <i>et al.</i> , 2008)
Operator-repressor titration e.g. LacI titration derepressing <i>dapD</i>	The <i>lac</i> promoter/operator is used to replace the native promoter of an essential gene and <i>lacI</i> (38.6 kDa product) is integrated into the genome. Multiple <i>lac</i> operator sites (~60 bp) on an introduced plasmid compete for LacI, relieving repression of the essential gene (E.g. <i>dapD</i> , involved in peptidoglycan and lysine biosynthesis).	<ul style="list-style-type: none"> + Reduced load on the plasmid (only <i>lac</i> operator repeats required) + Host strain can be cultivated without plasmid by addition of IPTG - Leaky expression from <i>lac</i> promoter relieves dependence - Mutation to <i>lac</i> operator region will lead to escape from dependence - <i>dapD</i> knockout can lead to increased DNA damage in <i>recA</i>- strains - Intellectual property is claimed on this system 	(Degryse, 1991) (Williams <i>et al.</i> , 1998) (Cranenburgh <i>et al.</i> , 2001) (Cranenburgh <i>et al.</i> , 2004) (Garmory <i>et al.</i> , 2005)
Miscellaneous Systems			
RNA-OUT e.g. Levansucrase (<i>sacB</i>)	Levansucrase (53.0 kDa; <i>sacB</i> 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 <i>sacB</i> translation, relieving the host of sensitivity to sucrose (SacB metabolises sucrose into toxic fructose polymers).	<ul style="list-style-type: none"> + Not reliant on RNA that is specific for any plasmid type + Presence of sucrose is required for toxicity, so <i>sacB</i> integrants can easily be grown in sucrose-minus conditions - Not suitable for environmental release or most industrial uses, as continued presence of is sucrose required to maintain selection 	(Kittle <i>et al.</i> , 1989) (Metcalfe <i>et al.</i> , 1994) (Mutalik <i>et al.</i> , 2012)
Overexpression of endogenous gene e.g. <i>fabI</i>	Plasmid-based overexpression of native <i>E. coli</i> enoyl ACP reductase (<i>fabI</i> ; 27.9 kDa product) reduces susceptibility of <i>E. coli</i> to the biocide triclosan, which inhibits <i>fabI</i> action in fatty acid biosynthesis.	<ul style="list-style-type: none"> + No editing of host genome is required + Triclosan is inexpensive and wide environmental use has not lead to general antibiotic resistance (i.e. cross-resistance) - Over-expression of <i>fabI</i> in the absence of triclosan is mildly toxic - Spontaneous mutants resistant to triclosan readily occur - Can't use in environment as continued presence of triclosan required 	(McMurry <i>et al.</i> , 1998) (Heath <i>et al.</i> , 1998) (Cole <i>et al.</i> , 2003) (Goh & Good, 2008)
Conditional Origins of Plasmid Replication			
Tunable replication e.g. R6K <i>ori</i> - γ / π ColE2 <i>ori</i> /Rep	Replication initiation proteins for <i>E. coli</i> 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 <i>trans</i> (R6K <i>ori</i> - γ , ~380 bp; ColE2 <i>ori</i> , ~470 bp). Altering the expression level of these proteins (or mutating them, e.g. π 116) results in different plasmid copy numbers.	<ul style="list-style-type: none"> + Both Rep and π have been shown to work in <i>trans</i> with their <i>ori</i> 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 <i>cer</i> element (~400 bp) required in plasmids to allow <i>E. coli</i>-mediated resolution of plasmid multimers 	(Filutowicz <i>et al.</i> , 1986) (Hiraga <i>et al.</i> , 1994) (Metcalfe <i>et al.</i> , 1994) (del Solar <i>et al.</i> , 1998) (Kittleson <i>et al.</i> , 2011) (Yagura <i>et al.</i> , 2006)

Host-independent replication e.g. <i>oriV</i> /RepCAB	<i>E. coli</i> 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 <i>trans</i> initiate replication at the <i>oriV</i> site (~420 bp).	<ul style="list-style-type: none"> + Broad host range over many Gram-negative bacteria as no reliance on host-encoded primosome - A low-copy replicon (10-15 per cell) - Multiple replicon components required results in a metabolic load 	(Scherzinger <i>et al.</i> , 1991) (del Solar <i>et al.</i> , 1998)
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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 weakness 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 weights, which give an indication of the burden they may impose on a host cell.

Figures

Figure 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 a membrane-depolarizing toxin (Hok) (**F**).



A. Physical containment

B. No advantage for uptake

C. Incorrect translation

D. Inability to replicate

E. No host immunity

F. Endogenous toxicity

