A peer-reviewed version of this preprint was published in PeerJ on 15 September 2015.

Kato Y. 2015. An engineered bacterium auxotrophic for an unnatural amino acid: a novel biological containment system. PeerJ 3:e1247 https://doi.org/10.7717/peerj.1247

An engineered bacterium auxotrophic for an unnatural amino acid: a novel biological containment system

Yusuke Kato

Biological containment is a genetic technique to program dangerous organisms to grow only in the laboratory and to die in the natural environment. Auxotropy for a substance not found in the natural environment is an ideal biological containment. Here, we constructed an *Escherichia coli* strain that cannot survive in the absence of the unnatural amino acid 3-iodo-$_L$-tyrosine. This synthetic auxotrophy was achieved by conditional production of the antidote protein against the highly toxic enzyme colicin E3. An amber stop codon was inserted in the antidote gene. The translation of the antidote mRNA was controlled by a translational switch using amber-specific 3-iodo-$_L$-tyrosine incorporation. The antidote is synthesized only when 3-iodo-$_L$-tyrosine is present in the culture medium. The viability of this strain rapidly decreased with less than a 1 h half-life after removal of 3-iodo-$_L$-tyrosine, suggesting that the decay of the antidote causes the host killing by activated colicin E3 in the absence of this unnatural amino acid. This containment system can be constructed by only plasmid introduction without genome editing, suggesting that this system may be applicable to other microbes carrying toxin-antidote systems similar to that of colicin E3.

Author(s):

Yusuke Kato

Affiliation & Location:

Genetically Modified Organism Research Center,

National Institute of Agrobiological Sciences,

Tsukuba, Ibaraki, Japan.

*Address for correspondence:

Yusuke Kato, Ph.D.

Genetically Modified Organism Research Center

National Institute of Agrobiological Sciences

Oowashi 1-2

Tsukuba, Ibaraki, Japan

Phone/Fax: +81-29-838-6059

Email: kato@affrc.go.jp

Introduction

In Michael Crichton's novel "Jurassic Park", modern technology allowed ancient dinosaurs to come back into existence (Crichton, 1990). Those dinosaurs could not produce the amino acid Lys due to genomic manipulation. The scientists in this novel expected that the dinosaurs could survive only with Lys-feeding and never escape from the park because they would die without the supplement. However, the dinosaurs learned that eating Lys-rich foods, such as chickens or soya beans, allowed them to survive and then they escaped from the park. One could ask the question: if the auxotrophy was not for Lys but for an amino acid which does not exist in the natural environment, would the dinosaurs have been able to survive after escaping without the supplement?

Although "Jurassic Park" is a science-fiction story, similar problems have been proposed in "the real world" of environmental science. For instance, genetically modified organisms (GMOs) should not be allowed to proliferate in an open environment if their safety has not been certificated (Berg et al., 1975). Thus, such GMOs are strictly contained in the laboratory by both physical and biological methods. Harmful invasive species also need to be managed to prevent ecological damage (Marbuah, Gren & McKie, 2014). Pathogens for research use or for live vaccines may pose a serious health risk if the general public is exposed to them, so we must contain these organisms in controlled areas (Berns, 2014). We

are unable to control the proliferation of those organisms once they escape from the laboratory because they autonomously replicate and grow.

Biological containment is a technique used to contain such dangerous organisms in the laboratory (Berg et al., 1975; Curtiss III, 1979). For microbes, this technique genetically programs the organisms to grow only in the laboratory and to die in the natural environment. Biological containment has been investigated for genetically-engineered microbes since the initiation of biotechnology research because of their usefulness for environmental detoxification (Paul, Pandey & Jain, 2005; Ramos et al., 2011), biocontrol (Migheli, 2001), and live vaccines (Daniel et al., 2011).

Biological containment systems can be subdivided into active and passive forms (Steidler et al., 2003). Active containment provides control through the conditional expression of toxic genes. A classic example is the bacterium that is engineered to contain the membrane-disruptive toxin gene *hok* controlled by the *trp* promoter (Molin et al., 1987). This bacterium can grow in Trp-containing medium in the laboratory because *hok* expression is repressed. In contrast, the toxin is induced in the absence of Trp, resulting in the death of the bacterium. On the other hand, passive containment is achieved by eliminating essential genes. The contained microbes can grow by complementation of either an auxotrophy or intact gene. For instance, thymidylate synthase (*thyA)*-defective strains cannot grow in an

open environment because either thymine or a thymidine supplement is essential for their survival (Ross, O'Gara & Condon, 1990).

In both active and passive containment systems, the life-or-death of the microbes is usually controlled by a substance which occurs in extremely low amounts or is absent in the natural environment. These organisms can survive only by artificially-providing the substance in the laboratory. Outside of the controlled areas, they should die because the substance does not exist or is not present in high enough amounts. Contrary to this expectation, however, some papers have reported that some stably-contained bacteria can survive in the natural environment (Ramos et al., 2011). For example, a passively-contained strain of the bacterium *Rhyzobium meliloti* (*thyA*$^{-}$) became colonized in the presence of alfalfa which could supply some thymine or thymidine as root exudates (O'Flaherty et al., 1995). A strain of the bacterium *Pseudomonas putida* whose death is induced by proline starvation can proliferate where maize plants exude proline (van Dillewijn et al., 2004). These reports suggest that the substances which we believe to be rare in the natural environment sometimes exist unexpectedly in specific microenvironments. Thus, the biological containment systems which are controlled by natural substances have a risk of failure similar to the Lys-auxotrophy of dinosaurs in Jurassic Park. In other words, an ideal containment system is where the auxotrophy is for a substance not found in the natural environment.

Toxin-antidote systems have been reported in many bacteria and in archaea (Yamguchi, Park & Inouye, 2011). One example is colicin E3 (ColE3) and its cognate antidote (ImmE3) encoded in a plasmid (Jakes & Zinder, 1974; Masaki & Ohta, 1985). Although the free ColE3 kills the host bacterium *Escherichia coli*, ImmE3 neutralizes the toxicity by ColE3-ImmE3 complex formation. When the plasmid is lost from the bacterium, ColE3 is released from the existing complex because the ImmE3 is more unstable than ColE3, resulting in cell growth inhibition and eventual cell death. Thus, the ColE3-ImmE3 complex is believed to play a role in plasmid maintenance (Thisted et al., 1994).

Sequences of ribosomally-synthesized proteins are encoded in the nucleotide sequences of their genes. A sequence of either three DNA or RNA nucleotides (codon) assigns an amino acid. The codon-amino acid correspondence is highly conserved among organisms. Each codon encodes one of twenty amino acids except for the three specific codons mentioned below (Ambrogelly, Palioura & Söll, 2007). The three specific codons (amber, opal, and ochre) do not code for any amino acids with a few exceptions, and terminate protein translation by binding to peptide chain release factors. Since early in this century, a technique has been developed to incorporate unnatural amino acids other than the twenty standard amino acids in ribosomally-synthesized proteins *in vivo* (Wang et al., 2001; Sakamoto et al., 2002). This technique uses an engineered aminoacyl-tRNA synthetase

(aaRS) that specifically recognizes the unnatural amino acid and its cognate amber suppressor tRNA ($tRNA_{CUA}$). The unnatural amino acid is incorporated into proteins at a position encoded by the UAG amber codon in the cells expressing the engineered aaRS/$tRNA_{CUA}$ pair. This unnatural amino acid incorporation system was originally developed for structural and functional analyses, including labeling and functional alteration of proteins (Liu & Schultz, 2010). We focused on the switching function of this system to control translation by the presence/absence of an unnatural amino acid (Minaba & Kato, 2014). The translational switch regulates only the translation of target gene transcripts in which an amber stop codon is inserted next to the AUG translational start codon. In the presence of the unnatural amino acid, translation proceeds beyond the inserted amber stop codon, resulting in the expression of the functional target proteins. In contrast, absence of the unnatural amino acid causes translational termination at the N-terminus, and no functional proteins are obtained. Although leaky expression is often detected in the absence of the unnatural amino acid, such leakage can be reduced by optimization of expression intensity and balance of the aaRS/tRNA, multiplication of amber stop codons, or double-regulation with a transcriptional-controlling system. In addition to the all-or-none switching, the translational switch can control the translational efficiency at any intermediate magnitude by adjustment of the unnatural amino acid concentration (Kato, 2015).

Here, we constructed an *Escherichia coli* strain that cannot survive in the absence of unnatural amino acid which is a substance not found in the natural environment. This study is part of our research project “Construction of engineered organisms auxotrophic for unnatural substances (2013-2015)”. This strain has a synthetic essential gene that is expressed only in the presence of the unnatural amino acid which is a synthetic essential nutrient. A modified toxin-antidote system is introduced in this bacterium. The antidote is a protein and a unnatural amino acid translational switch controls the antidote expression. The bacterium can survive only when the antidote protein is produced in the presence of the unnatural amino acid in the laboratory. In the natural environment, the bacterium should die due to the absence of the unnatural amino acid and accumulation of the toxin. In this paper, we performed a proof-of-concept study for the novel biological containment system using this unnatural amino acid-auxotrophic *E. coli* strain.

Materials and Methods

Strains, culture conditions, and transformation

E. coli BL21-AI [F^- *ompT gal dcm lon hsdS*$_B$(r_B^- m_B^-) *araB::T7RNAP-tetA*] was used throughout this study. *E. coli* XL1-blue (carrying an amber suppressor mutation, *supE44*) was also used for plasmid construction. Liquid cultures were grown with rotary shaking at 200 rpm in LB medium (1% bacto tryptone, 0.5% yeast extract, and 1% NaCl). LB agar medium (LB + 20% agar) was used for solid medium cultures. All cultures were performed at 37°C in the presence or absence of the unnatural amino acid, 3-iodo-$_L$-tyrosine (3×10^{-4} M) (IY). Carbenicillin (100 μg/ml) and/or chloramphenicol (50 μg/ml) were added as appropriate. Plasmid-transformation was performed by electroporation using a Gene Pulser (BioRad).

Plasmid design

The BL21-AI(IY) strain, which incorporates IY into proteins at a position encoded by the UAG amber codon, was generated by introduction of the plasmid pTYR MjIYRS2-1(D286) MJR1×3 (p15A replicon, chloramphenicol resistant) constitutively expressing IYRS and MJR1, originally constructed by K. Sakamoto, RIKEN (Sakamoto et al., 2009). The plasmid pSH350(1amb-immE3) (pME1 amplicon, ampicillin resistant) encoding *colE3* and an amber-inserted *immE3* was generated from pSH350 which had been originally constructed by H.

Masaki (Masaki & Ohta, 1985). An amber stop codon was inserted next to the start codon ATG of *immE3* by inverse PCR using the primers immE3-1amb-s and inv-immE3-as. The primers used in this study are summarized in Table S1. The inverse PCR product was circularized by self-ligation and was transformed into *E. coli* XL1-blue. The transformants were selected on a solid medium containing both chloramphenicol and carbenicillin. Correctly constructed plasmids were selected by colony PCR using the primers amb-immE3-confirm-s and immE3-confirm-as. The sequences of selected plasmids were confirmed by nucleotide sequencing. The confirmed plasmid was transformed into BL21-AI(IY) to construct BL21AI(IY,1amb-immE3).

Determination of mutation rates

Mutation rates were estimated by a fluctuation assay (Luria & Delbrück, 1943). A frozen stock of BL21-AI(IY,1amb-immE3) was diluted to prepare an approximately 10 cfu/ml bacterial suspension in a liquid medium containing IY, chloramphenicol, and carbenicillin. Nine parallel cultures (1 ml each) were incubated for 16 h (OD_{590} = 0.03 to 0.1). The bacteria were collected by centrifugation (10,000 rpm for 1 min). The bacterial pellets were resuspended in an IY-free liquid medium and centrifuged again for washing. This wash was repeated four times to remove IY completely. After washing, the bacterial pellets were resuspended in 1 ml of IY-free liquid medium again, and an aliquot (250 µl) was inoculated

onto an IY-free solid medium to detect the “escapers”. Another aliquot was diluted 10^3-fold, and inoculated onto an IY-containing solid medium to estimate the total number of bacteria. After a 30 h incubation, the number of colonies was counted. The mutation rate was calculated by the web tool FALCOR using the Ma-Sandri-Sarkar Maximum Likelihood Estimator (MSS-MLE) method (Hall et al., 2009; Ma, Sandri & Sarkar, 1992). The applicability of this assay was evaluated from the detected mutation number per culture and the number of parallel cultures (Rosche & Foster, 2000).

Rate of cell death

BL21-AI(IY,1amb-immE3) was grown to OD_{590} = 0.03 to 0.1 in an IY-containing liquid medium. After washing as described above, the culture was diluted 10^3-fold in an IY-free medium. An aliquot (250 µl) was withdrawn every hour and inoculated onto an IY-containing solid medium. After a 30 h culture, the number of colonies was counted. A single fitted curve was generated using Origin7.

Growth curve

All tested bacterial strains were grown to approximately OD_{590} = 0.05 in an IY-containing liquid medium. We then measured the change of OD_{590} every 20 min for 2 h. A single fitted curve was generated for each strain using Origin7.

Results

Construction of an unnatural amino acid-auxotrophic bacterium

An unnatural amino acid-auxotrophic *E. coli* strain was constructed by introduction of 2 plasmids (Fig. 1A). One plasmid encoded the toxin-antidote pair ColE3-ImmE3. ColE3 is a highly toxic RNase that can kill the host bacterium with only a few molecules (Lazzaroni, Dubuisson & Vianney, 2002; Bowers et al., 2004). ImmE3 forms a complex with ColE3 and inhibits the RNase activity (Yajima et al., 1992). In this plasmid, an amber stop codon was inserted next to the translation start codon ATG of *immE3* (Fig.1B). Toxin-antidote systems, such as ColE3-ImmE3, are good examples of selfish genetic elements (Inglis et al., 2013). We speculate that the relative expression levels of those genes may be optimally adjusted to maintain themselves for many generations with minimum consequences to the host. The gene organization and sequences of *colE3-immE3* were unchanged, except for the amber insertion in *immE3*. Another plasmid expresses the aaRS/tRNA pair (IYRS/MJR1) for amber-specific incorporation of the unnatural amino acid 3-iodo-$_L$-tyrosine (IY) (Sakamoto et al., 2009). We constructed the BL21-AI strain carrying these 2 plasmids and designated it BL21-AI(IY,1amb-immE3). IY from the culture medium is taken up into the intracellular space of the bacteria. Intracellular IY is ribosomally incorporated in ImmE3 at the position of the inserted

amber codon, resulting in the functional expression of ImmE3 that represses the ColE3 toxicity (Fig. 1C & 1D). BL21-AI(IY,1amb-immE3) therefore can survive in an IY-containing medium. In contrast, the absence of IY interrupts the production of ImmE3, resulting in the death of BL21-AI(IY,1amb-immE3) due to ColE3 toxicity.

The IY-auxotrophy of BL21-AI(IY,1amb-immE3) was experimentally evaluated (Fig. 2A). The bacterium was inoculated onto either an IY-free or IY-containing solid medium, after a stringent wash to remove IY completely. Several thousand viable colonies were observed on the IY-containing medium. In contrast, no colonies were detected on the IY-free medium, indicating that IY is essential for the survival of BL21-AI(IY,1amb-immE3).

Rate of killing after IY removal

Contained bacteria may make an impact on the natural environment if they can survive for a significantly long time after removal of their auxotropic substances. We determined the rate of killing after IY-removal for BL21-AI(IY,1amb-immE3) (Fig. 2B and Data S1). The number of viable bacteria increased until 1 h after IY-removal. At 2 h after the removal of IY, the viability rapidly decreased. The half-life was estimated to be 49.2 $\pm$ 7.2 min (Fig. S1).

Growth rate

The growth rate is an important characteristic related to competitiveness for survival.

The growth rates of BL21-AI(IY,1amb-immE3) and related strains were determined and compared with each other (Fig. 2C and Data S2). BL21-AI(IY,1amb-immE3) grew at a slower rate (doubling time = 59.8 ± 9.6 min) than that of the parent strain carrying no amber-inserted *immE3* (40.5 ± 2.6 min) and of a vector control strain (37.9 ± 1.6 min) (Fig. S2).

Escape frequency

An important problem in biological containment is emergence of escapers by genetic mutations. Only a few escapers can lead to an uncontrolled proliferation of contained microbes. Therefore, we estimated the frequency of escaper emergence for BL21-AI(IY,1amb-immE3) using a fluctuation assay (Data S3). The frequency was estimated to be 1.4 mutations (95% highest posterior density 1.1 - 1.8) per 10^5 cell divisions. This means that 71 thousand cell divisions will generate one escaper.

Discussion

In this study, we constructed the *E. coli* strain BL21-AI(IY,1amb-immE3) which cannot survive in the absence of the unnatural amino acid IY. The biological containment system used for this strain involves a ColE3-dependent killing mechanism, indicating that this system is an active containment system. ImmE3 is produced in the presence of IY and keeps this strain alive although its gene is supplied from outside of the bacterium, suggesting that *immE3* is a synthetic essential gene. IY is not only a signaling molecule, such as isopropyl β-$_{D}$-1-thiogalactopyranoside (IPTG) for the *lac* promoter control, but it is also a building block which forms an aminoacyl-tRNA and target proteins, indicating that IY is a synthetic essential "nutrient" (Minaba & Kato, 2014). This containment system therefore involves complementation of an auxotrophy, suggesting that this system is also a passive containment system.

In usual passive containment systems, an auxotrophy is conferred by disruption of genes in the synthetic pathway for an essential metabolite (Steidler et al., 2003). Such auxotrophy is limited to natural metabolites, and is not possible for substances that do not occur in the natural environment. The unnatural amino acid IY does not exist in the natural environment, suggesting that the auxotrophy for the unnatural amino acid cannot be

238 constructed using classic methods. The IY-auxotrophy was constructed by the introduction of
239 an IY-specific aaRS/$tRNA_{CUA}$ pair and an insertion of an amber codon into a target gene. This
240 engineered auxotrophy is a promising method to generate organisms that require for their
241 survival substances that do not naturally occur, and thus is a robust biological containment
242 system.

243 BL21-AI(IY,1amb-immE3) was constructed only by the introduction of 2 plasmids and
244 without genome editing. Although plasmids are useful for easy gene introduction, their use
245 has a problem for environmental applications because of possible horizontal gene transfer to
246 other bacteria (Ramos et al., 2011). In the case of BL21-AI(IY,1amb-immE3), the plasmid
247 carrying *colE3-immE3* is not transmittable because the *immE3* in this plasmid is a loss-of-
248 function mutant if the recipient strains are not amber-suppressor mutants. Even if an amber-
249 suppressor strain receives the plasmid, the *colE3-immE3* is a native *E.coli* plasmid gene
250 cluster in which the natural sequences are kept except for an insertion of the amber codon. In
251 addition, the substrates of IY-specific aaRS/$tRNA_{CUA}$, which is encoded in another plasmid,
252 do not exist in the natural environment with a few exceptions (Kato, 2015), suggesting that
253 the genetic and ecological impact may be minimal if the those genetic parts are released into
254 the natural environment. Although the selection markers (chloramphenicol- and ampicillin-
255 resistant genes) are definitively problematic, we can avoid those genes by alternative

methods (Wright et al., 2014).

The target gene products that are controlled by the IY-controlling translational switch necessitate the incorporation of IY. IY-incorporation may cause functional alterations in some target proteins. The IY-incorporated ImmE3 functions to neutralize the ColE3 toxicity. The chimeric ImmE3 whose N-terminal region is replaced by that of ImmE6 maintains its protection against ColE3, suggesting that the N-terminal region is not essential (Masaki et al., 1991). This report agrees with our observation. However, the growth rate of BL21-AI(IY,1amb-immE3) was slower than that of its parent strain, suggesting that either the expression level of or immunity to amber-inserted *immE3* might be partially reduced.

BL21-AI(IY,1amb-immE3) continued to proliferate even 1 h after IY removal. This could be due to intracellular IY accumulation. The termination of synthesis and degradation of ImmE3 are assumed to be the cause of the rapid decline in viability within 2 h after the removal of IY. The leakage translation which is several percent of the maximum translation was observed in the IY-controlling translational switch (Minaba & Kato, 2014; Kato, 2015), suggesting that such incomplete repression of ImmE3 is sufficient to induce the host killing by ColE3 in this system.

The frequency of emergence of escapers was 1.4×10^{-5} mutations/cell/generation. This value is one order higher than that reported for an early plasmid containment system

using the conditional expression of *relF* controlled by the inducible *lac* promoter (Knudsen & Karlström, 1991). The biological containment system reported here cannot therefore be used as a practical system in the present form (Schmidt & de Lorenzo, 2012). However, the efficacy of biological containment systems using a single containment gene is highly insufficient. Higher containment efficacy is usually achieved by multi-layer combination of containment systems (Ronchel & Ramos, 2001; Torres et al., 2003; Rovner et al., 2015). Our system should also be improved by such a combination use to repress the emergence of escapers completely. In addition, the mechanisms of escaper emergence should be also analyzed for system improvement.

Early this year, passive containment systems using conditional expression of genomic essential genes depending on unnatural amino acids were reported (Mandell et al., 2015; Rovner et al., 2015). These systems have achieved an excellent containment efficacy by controlling multiple containment genes. They used the genomically-recoded *E. coli* strain C321.AA (GRO) as a host (Lajoie et al., 2013). GRO has a largely edited genome whose amber codons (TAG) were all substituted by ochre codons (TAA). Moreover, the peptide chain release factor gene *prfA* was deleted for efficient incorporation of unnatural amino acids. These systems could therefore not be used for other microbes in which the techniques for genome editing have not been adequately developed. In contrast, the containment system

shown here was constructed by introduction of only 2 plasmids with a minimum alteration of the natural sequence, i.e. only an amber insertion into *immE3*, suggesting that this system could be easily used for other bacteria and for archaea in which toxin-antidote systems with protein antidotes (type II, type IV, or type V) have been found (Schuster & Bertram, 2013).

296 **Acknowledgements**

297

298 We thank the following researchers: Kensaku Sakamoto and Shigeyuki Yokoyama
299 (RIKEN) for IYRS-MJR1 expression plasmids. Haruhiko Masaki (Tokyo University) for the
300 *colE3* clone.

References

Ambrogelly, A., Palioura, S., Söll, D. 2007. Natural expansion of the genetic code. Nat. Chem. Biol. 3, 29-35

Berg, P., Baltimore, D., Brenner, S., Roblin, R. O., Singer, M. F. 1975. Summary statement of the Asilomar conference on recombinant DNA molecules. Proc. Natl. Acad. Sci. U. S. A. 72, 1981-1984.

Berns K. I. 2014. Grand challegnes for biosafety and biosecurity. Front. Bioeng. Biotechnol. 2, 35

Bowers, L. B., LaPoint, K., Anthony, L., Pluciennik, A., Filutowicz, M. 2004. Bacterial expression system with tightly regulated gene expression and plasmid copy number. Gene 340, 11-18.

Crichton, M. 1990. Jurassic park. Alfred A. Knopf.

Curtiss III, R. 1978. Biological containment and cloning vector transmissibility. J. Infect. Dis. 137, 668-675.

Daniel, C., Roussel, Y., Kleerebezem, M., Pot, B. 2011. Recombinant lactic acid bacteria as mucosal biotherapeutic agents. Trends Biotechnol. 29, 499-508.

Hall, B. M., Ma, C.-X., Liang, P., Singh, K. K. 2009. Fluctuation AnaLysis CalculatOR: a web tool for the determination of mutation rate using Luria-Delbrück fluctuation analysis. Bioinformatics 25, 1564-1565.

Inglis, R. F., Bayramoglu, B., Gillor, O., Ackermann, M. 2013. The role of bacteriocins as selfish genetic elements. Biol. Lett. 9, 20121173.

Jakes, K. S., Zinder, N. D. 1974. Highly purified colicin E3 cantains immunity protein. Proc. Natl. Acad. Sci. USA. 71, 3380-3384.

Kato, Y. 2015. Tunable translational control using site-specific unnatural amino acid incorporation in *Escherichia coli*. PeerJ 3, e904.

337

338 **Kunden, S. M., Karström, O. H.** 1991. Development of efficient suicide mechanisms for
339 biological containment of bacteria. Appl. Environ. Microbiol. 57, 85-92.

340

341 **Lajoie, M. J., Rovner, A. J., Goodman, D. B., Aerni, H.-R., Haimovich, A. D., Kuznetsov,**
342 **G., Mercer, J., Wang, H. H., Carr, P. A., Mosberg, J. A., Rohland, N., Schultz, P. G.,**
343 **Jacobson, J. M., Rinehart, J., Church, G. M., Isaacs, F.** 2013. Genomically recoded
344 organisms expand biological functions. Scinece 342, 357-360.

345

346 **Lazzaroni, J.-C., Dubuisson, J.-F., Vianney, A.** 2002. The Tol proteins of *Escherichia coli*
347 and their involvement in the translocation of group A colicins. Biochimie 84, 391-397.

348

349 **Liu, C. C., Schultz, P. G.** 2010. Adding new chemistries to the genetic code. Annu. Rev.
350 Biochem. 79, 413-444.

351

352 **Ma, W. T., Sandri, G. v. H., Sarkar, S.** 1992. Analysis of the Luria-Delbrück distribution using
353 discrete convolution powers. J. Appl. Prob. 29, 255-267.

354

Marbuah, G., Gren, I.-M., McKie, B. 2014. Economics of harmful invasive species: a review. Diversity 6, 500-523.

Masaki, H., Akutsu, A., Uozumi, T., Ohta, T. 1991. Identification of a unique specificity determinant of the colicin E3 immunity protein. Gene 107, 133-138.

Masaki, H., Ohta, T. 1985. Colicin E3 and its immunity genes. J. Mol. Biol. 182, 217-227.

Migheli, Q. 2001. Genetically modified biocontrol agents: environmental impact and risk analysis. J. Plant Pathol. 83, 47-56.

Minaba, M., Kato, Y. 2014. High-yield, zero-leakage expression system with a translational switch using site-specific unnatural amino acid incorporation. Appl. Environ. Microbiol. 80, 1718-1725.

Molin, S., Klemm, P., Poulsen, L. K., Biehl, H., Gerdes, K., Andersson, P. 1987. Conditional suicidal system for containment of bacteria and plasmids. Nat. Biotechnol. 5, 1315–1318.

O'Flaherty, S., Moënne-Loccoz, Y., Boesten, B., Higgins, P., Dowling, D. N., Condon, S., O'Gara, F. 1995. Greenhouse and field evaluations of an autoselective system based on an essential thymidylate synthase gene for improved maintenance of plasmid vectors in modified *Rhizobium meliloti*. Appl. Environ. Microbiol. 61, 4051-4056.

Paul, D., Pandey, G., Jain R. K. 2005. Suicidal genetically engineered microorganisms for bioremediation: need and perspectives. BioEssays 27, 563-573.

Ramos, H. J. O., Yates, M. G., Pedrosa, F. O., Souza, E. M. 2011. Strategies for bacterial tagging and gene expression in plant-host colonization studies. Soil Biol. Biochem. 43, 1626-1638.

Ronchel, M. C., Ramos, J. L. 2001. Dual system to reinforce biological containment of recombinant bacteria designed for rhizoremediation. Appl. Environ. Microbiol. 67, 2649-2656.

Rosche, W. A., Foster, P. L. 2000. Determining mutation rates in bacterial populations. Methods 20, 4-17.

Ross, P., O'Gara, F., Condon, S. 1990. Thymidylate synthase gene from *Lactococcus lactis* as a genetic marker: an alternative to antibiotic resistance genes. Appl. Environ. Microbiol. 56, 2164-2169.

Rovner, A. J., Haimovich, A. D., Katz, S. R., Li, Z., Grome, M. W., Gassaway, B. M., Amiram, M., Patel, J. R., Gallagher, R. R., Rinehart, J., Isaacs, F. J. 2015. Recoded organisms engineered to depend on synthetic amino acids. Nature 518, 89-93.

Sakamoto, K., Hayashi, A., Sakamoto, A., Kiga, D., Nakayama, H., Soma, A., Kobayashi, T., Kitabatake, M., Takio, K., Saito, K., Shirouzu, M., Hirao, I., Yokoyama, S. 2002. Site-specific incorporation of an unnatural amino acid into proteins in mammalian cells. Nucleic Acids Res. 30, 4692-4699.

Sakamoto, K., Maruyama, K., Oki, K., Iraha, F., Kato-Maruyama, M., Takahashi, M., Ohtake, K., Kobayashi, T., Kuramitsu, S., Shirouzu, M., Yokoyama, S. 2009. Genetic encoding of 3-iodo-$_L$-tyrosine in *Escherichia coli* for single-wavelength anomalous dispersion phasing in protein crystallography. Structure 17, 335-344.

Schmidt, M., de Lorenzo, V. 2012. Synthetic constructs in/for the environment: managing the interplay between natural and engineered biology. FEBS Lett. 586, 2199-2206.

Shuster, C. F., Bertram, R. 2013. Toxin-antitoxin systems are ubiquitous and versatile modulators of prokaryotic cell fate. FEMS Microbiol. Lett. 340, 73-85.

Steidler, L., Neirynck, S., Huyghebaert, N., Snoeck, V., Vermeire, A., Goddeeris, B., Cox, E., Remon, J. P., Remaut, E. 2003. Biological containment of genetically modified *Lactococcus lactis* for intestinal delivery of human interleukin 10. Nat. Biotechnol. 21, 785-789.

Thisted, T., Sorensen, N. S., Wagner, E. G., Gerdes, K. 1994. Mechanism of post-segregational killing: Sok antisense RNA interacts with Hok mRNA via its 5'-end single-stranded leader and competes with the 3'end of Hok mRNA for binding to the *mok* translational initiation region. EMBO J. 13, 1960-1968.

Torres, B., Jaenecke, S., Timmis, K. N., García, J. L., Díaz, E. 2003. A dual lethal system to enhance containment of recombinant micro-organisms. Microbiol. 149, 3595-3601.

van Dillewijn, P., Víchez, S., Paz. J. A., Ramos, J. L. 2004. Plant-dependent active biological containment system for recombinant rhizobacteria. Environ. Microbiol. 6, 88-92.

Wang, L., Brock, A., Herberich, B., Schultz, P. G. 2001. Expanding the genetic code of *Escherichia coli.* Science 292, 498-500.

Wright, O., Delmans, M., Stan, G.-B., Ellis, T. 2015. GeneGuard: a modular plasmid system designed for biosafety. ACS Synth. Biol. 4, 307-316.

Yajima, S., Muto, Y., Yokoyama, S., Masaki, H., Uozumi, T. 1992. The secondary structure of the colicin E3 immunity protein as studied by proton-proton and proton-nitrogen-15 two-dimensional NMR spectroscopy. Biochemistry 31, 5578-5586.

Yamaguchi, Y., Park, J.-H., Inouye, M. 2011. Toxin-antitoxin systems in bacteria and archaea. Annu. Rev. Genet. 45, 61-79.

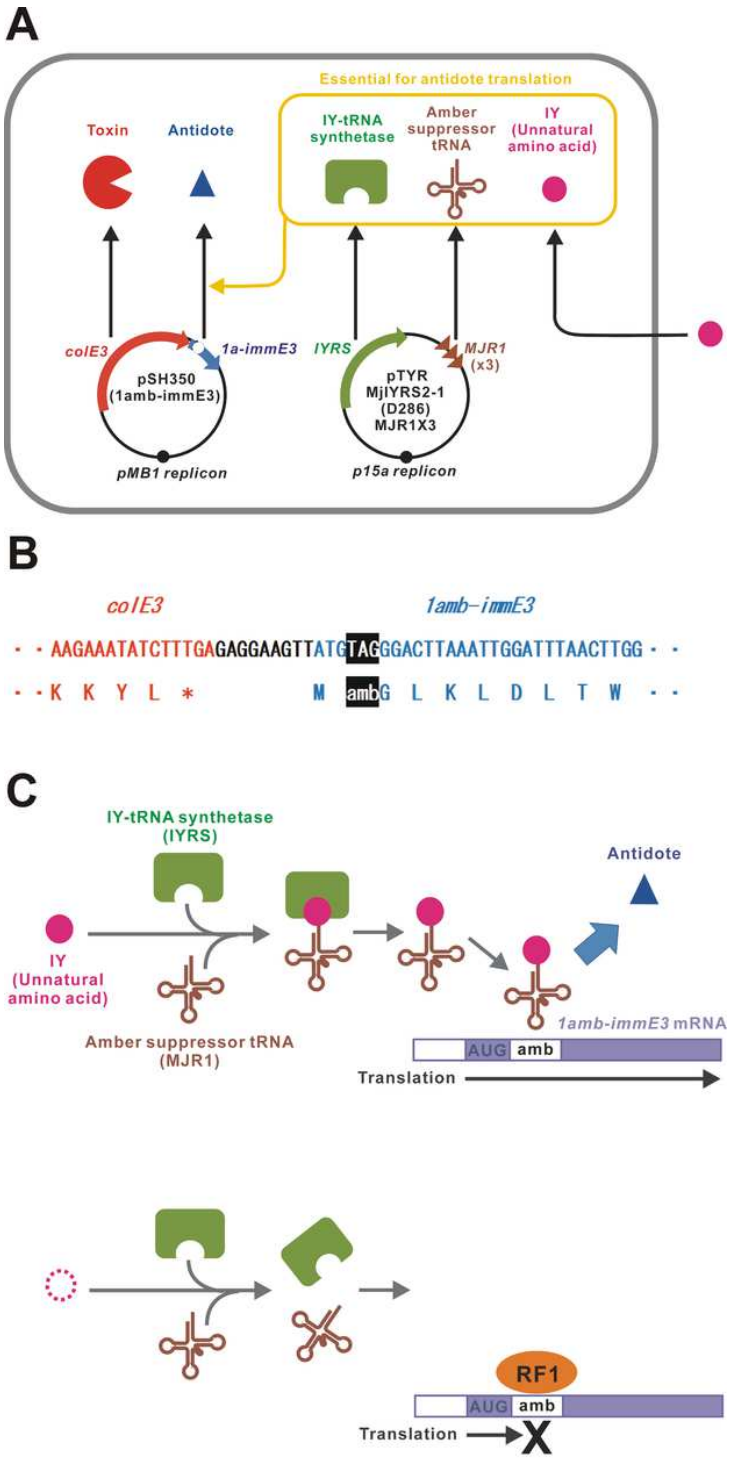
Figure legends

Fig. 1. Construction of a bacterium auxotrophic for IY. (A) Schematic of the gene circuit for a synthetic IY-auxotrophy. The IY-auxotrophic *E. coli* strain BL21-AI(IY,1amb-immE3) was constructed by introduction of 2 plasmids, pSH350(1amb-immE3) and pTYR MjIYRS2-1(D286)MJR1X3. The former plasmid encodes a highly toxic RNase ColE3 and an antidote ImmE3. *immE3* was modified as described below. IYRS and MJR1 genes are encoded in the latter plasmid. The translation of ImmE3 is controlled by the IY-controlling translational switch. (B) Alteration of *immE3*. An amber stop codon was inserted next to the translation start codon ATG. This inserted amber codon is the site of IY incorporation. (C) IY-dependent translation of ImmE3. IY is incorporated at the position of the inserted amber stop codon in the presence of IYRS and MJR1, resulting in the successful translation of ImmE3 encoded downstream of the amber codon. The translation is interrupted in the absence of IY. RF1, peptide chain release factor 1. (D) Expected responses. The antidote (ImmE3) is produced in the presence of IY (in the laboratory), thus neutralizing the toxin (ColE3), and the host bacterium survives. In contrast, the antidote is not produced in the absence of IY (in the natural environment), thus, the toxin is expressed, and the host bacterium is killed.

Fig.2. Characterization of the constructed bacterium. (A) Evaluation of IY-auxotrophy. Ten thousand viable BL21-AI(IY,1amb-immE3) bacterial cells were inoculated onto a solid medium. Left, an IY-containing medium; right, an IY-free medium. (B) Rate of killing. IY in the medium was removed at time 0. The number of viable cells is normalized (the value at time 0 = 1). n = 4 independent experiments using completely separate bacterial cultures. (C) Growth curves. BL21-AI(IY,1amb-immE3), the parent strain carrying pSH350, and a vector control carrying pBR322 instead of pSH350 were tested. The OD_{590} was normalized (the value at 60 min = 1). n = 3 independent experiments using completely separate bacterial cultures. Data (B and C) are shown as means ± SEM.

Fig. 1. Construction of a bacterium auxotrophic for IY.

(A) Schematic of the gene circuit for a synthetic IY-auxotrophy. The IY-auxotrophic *E. coli* strain BL21-AI(IY,1amb-immE3) was constructed by introduction of 2 plasmids, pSH350(1amb-immE3) and pTYR MjIYRS2-1(D286)MJR1X3. The former plasmid encodes a highly toxic RNase ColE3 and an antidote ImmE3. *immE3* was modified as described below. IYRS and MJR1 genes are encoded in the latter plasmid. The translation of ImmE3 is controlled by the IY-controlling translational switch. (B) Alteration of immE3. An amber stop codon was inserted next to the translation start codon ATG. This inserted amber codon is the site of IY incorporation. (C) IY-dependent translation of ImmE3. IY is incorporated at the position of inserted amber stop codon in the presence of IYRS and MJR1, resulting the successful translation of ImmE3 encoded the downstream of the amber codon. The translation is interrupted in the absence of IY. RF1, peptide chain release factor 1. (D) Expected responses. The antidote (ImmE3) is produced in the presence of IY (in the laboratory), resulting that the toxin (ColE3) is neutralized and the host bacterium survives. In contrast, the antidote is not produced in the absence of IY (in the natural environment), resulting that the toxin become to be free and the host bacterium is killed.



2

Fig.2. Characterization of the constructed bacterium.

(A) Evaluation of IY-auxotrophy. Ten thousand of viable BL21-AI(IY,1amb-immE3) bacterial cells were inoculated on a solid medium. Left, an IY-containing medium; right, an IY-free medium. (B) Time-killing curve. IY in the medium was removed at time 0. The number of viable cell is normalized (the value at time 0 = 1). n = 4 independent experiments using completely separated bacterial cultures. (C) Growth curves. BL21-AI(IY,1amb-immE3), the parent strain carrying pSH350 and a vector control carrying pBR322 instead of pSH350 were tested. The value of OD_{590} is normalized (the value at 60 min = 1). n = 3 independent experiments using completely separated bacterial cultures. Data (B and C) are shown as means ± SEM.