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Article

An Unnatural Amino Acid-Regulated Growth Controller Based on Informational Disturbance

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Abstract: We designed a novel growth controller regulated by feeding of an unnatural amino acid, N^{ε}-benzyloxycarbonyl-L-lysine (ZK), using a specific incorporation system at a sense codon. This system is constructed by a pair of modified pyrrolisyl-tRNA synthetase (PylRS) and its cognate tRNA ($tRNA^{pyl}$). Although ZK is non-toxic for normal organisms, the growth of *Escherichia coli* carrying the ZK incorporation system was inhibited in a ZK concentration-dependent manner without causing rapid bacterial death, presumably due to generation of non-functional or toxic proteins. The extent of growth inhibition strongly depended on the anticodon sequence of the $tRNA^{pyl}$ gene. Taking advantage of the low selectivity of PylRS for $tRNA^{pyl}$ anticodons, we experimentally determined the most effective anticodon sequence among all 64 nucleotide sequences in the anticodon region of $tRNA^{pyl}$ gene. The results suggest that the ZK-regulated growth controller is a simple, target-specific, environmental noise-resistant and titratable system. This technique may be applicable to a wide variety of organisms because the growth inhibitory effects are caused by "informational disturbance", in which the highly conserved system for transmission of information from DNA to proteins is perturbed.

Keywords: synthetic biology; genetic parts; kill-switch; growth control; unnatural amino acids; codon reassignment; pyrrolysine

1. Introduction

Growth control is a crucial technique for applications in microbial biotechnology [1]. Bacterial cells have a limited number of resources and there is a trade-off between microbial growth and all other cellular functions, including production of useful proteins or metabolites. This suggests that precise growth control is necessary to achieve maximum efficiency of microbial production. Therefore, there is ongoing development of methods to control growth. Classically, antibiotics, nutritional restriction, and temperature control have been used [2-5].

Multi-cell systems have many advantages over traditional clonal population systems, but each cell population must be regulated to optimize its percentage in the total population [6,7]. This challenge has prompted studies of more precise growth control for a specific population. Several methods for growth control have been described, including synthetic growth switches based on controlled expression of RNA polymerase or methionine synthetase [8,9]. These methods are effective, but also have some inherent drawbacks, such as non-titratable regulation and use of dropout media [9,10]. Engineered cell-cell signaling systems have been proposed to enable autonomous coordination of subpopulation densities, but these sophisticated systems require construction of complex artificial genetic circuits [10-14].

We previously reported a tight and titratable translational controller for toxic protein production and biological containment (Figure 1a) [15-17]. This technique is based on conditional translation of target proteins using site-specific unnatural amino acid (Uaa) incorporation [15,18-21]. In this system, a Uaa-specific tRNA synthetase (UaaRS) and its cognate tRNA, which incorporates the Uaa at the UAG stop codon, are expressed in the cells

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of a target organism. In addition, a single or several UAG codons are inserted into the coding region of target genes. Translation of the UAG-inserted target genes is interrupted in the absence of Uaa, but functional proteins are produced in the presence of Uaa by UAG stop codon readthrough.

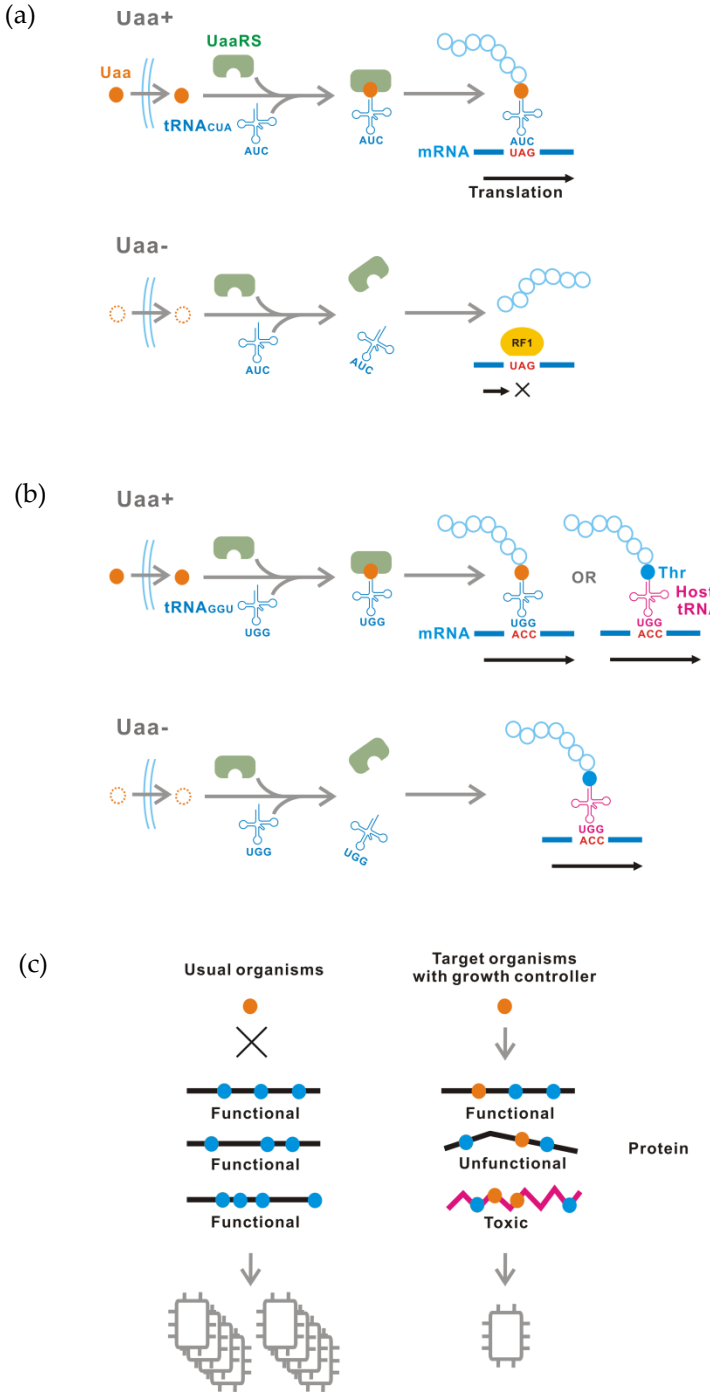

Figure 1. Principles of the Uaa-regulated growth controller. (a) Schematic of a Uaa-controlled translational switch using conditional UAG stop codon readthrough to construct synthetic auxotrophy for Uaa. (b, c) Schematic of a Uaa-regulated growth controller using Uaa incorporation at a sense codon. Uaa incorporation at the ACC codon assigned to Thr is shown as an example.

Here, we developed a simple growth control device based on a Uaa-regulated growth controller, which is conceptually similar to the Uaa-regulated translational controller. This

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growth controller incorporates the Uaa at a sense codon that is assigned to a normal proteinous amino acid (Figure 1b). The Uaa is competitively incorporated into many proteins against the originally assigned amino acid, resulting in production of abnormal proteins that may be non-functional or toxic (Figure 1c) [22]. Finally, growth of the target organism is inhibited by disturbance of a wide range of biological process. In this study, we examined the underlying principles of the Uaa-regulated growth controller.

The Uaa-regulated growth controller was designed to satisfy four conditions of an ideal growth control device. First, non-target organisms should not be affected. The site-specific Uaa incorporation system generally uses less cytotoxic Uaa's because the Uaa's are incorporated into proteins using the translation machinery in viable cells, suggesting that such Uaa's are not harmful to non-target organisms [23]. Second, growth inhibition should be resistant to environmental noise. Uaa's are not present in the natural environment or in living organisms, suggesting that accidental contamination from these sources will not cause unexpected growth inhibition. Thus, intentional feeding of a Uaa is the only way to inhibit the growth of the target organisms. Third, the device should be as simple as possible genetically. This is achieved because the Uaa-regulated growth controller includes only two genes: a Uaa-specific tRNA synthetase and its cognate tRNA. Any versatile genetic elements such as inducible promoters are not needed. Fourth, excellent future scalability is required. The codon-amino acid assignment is stably conserved, with some exceptions [24]. A limited assignment change is likely to alter expression of many genes in the cell and inhibit a wide range of biological process [25]. The harmful effects of the Uaa-regulated growth controller are caused by "informational disturbance", through perturbance of the highly conserved system of transmission of information from DNA to proteins. Therefore, a Uaa-regulated growth controller should be effective in a wide range of organisms. In addition, several hundred site-specific Uaa incorporation systems have already been established that can be used to build growth control devices [26].

2. Materials and Methods

2.1. Strains and growth conditions

BL21-AI[*F ompT gal dcm lon hsdSB(rB mB) araB*::*T7RNAP tetA*] was used throughout the study [27]. Bacteria were grown in Luria-Bertani (LB) medium at 37°C. For preparation of solid medium, an aliquot of agar (2%) was added.

2.2. Plasmid construction

The plasmid pTK2-1 ZLysRS1 encoding the parent ZKRS and $tRNA^{Pyl}$ was provided by Kensaku Sakamoto and Shigeyuki Yokoyama (RIKEN) [28]. A plasmid library containing $tRNA^{Pyl}$ genes with randomized anticodon sequences was constructed as shown in Figure 2a. The anticodon sequence was randomized using inverse PCR with a random 3-nucleotide sequence in the anticodon region and 16 bp overlapped primers, followed by circularization using enzymatic recombination (In-Fusion HD cloning kit, Takara). PCR was performed using a high fidelity DNA polymerase (KOD-plus-Neo, Toyobo). Circularized PCR products were transfected into electrocompetent *E. coli* cells by electroporation using a Gene Pulser II electroporator (Bio-Rad). A total of 191 strains were isolated and subjected to a preliminary ZK-sensitivity test. Plasmids were purified from these strains and the anticodon region of $tRNA^{Pyl}$ genes was sequenced. Anticodon sequences that were not found in the 191 clones were synthesized as described above, using a primer containing the missing anticodon sequence.

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2.3. ZK-sensitivity test

A preliminary test was performed using a LB-agar plate. Tested strains carrying pTK2-1 ZLysRS1 variants containing various anticodon sequences were cultured in 1 ml of LB medium containing chloramphenicol (50 μg/mL). The overnight (about 16 h) culture of bacteria was 10^3-fold diluted in fresh LB medium. An aliquot of diluted bacterial suspension (250 μL) was inoculated on LB-agar plates containing 3 mM ZK (Bachem). After an overnight culture, growth inhibition was evaluated by comparing bacterial growth on the ZK-containing plate with that on the ZK-free plate. For a more precise test, a liquid culture was used. An overnight culture of tested strains was 10^3-fold diluted in LB medium without chloramphenicol and incubated for 5 h. After the incubation, the bacterial culture was 10^2-fold diluted in fresh LB medium containing ZK without chloramphenicol. After a 3 h culture, the OD_{590} was measured. Growth inhibition was evaluated by comparing the OD_{590} in ZK-containing medium with that of ZK-free medium. To determine the mode of action, the time course of cell viability was measured. An overnight culture of tested strains was 10^4-fold diluted in LB medium containing 3 mM ZK with or without chloramphenicol. An aliquot (10 μl) was withdrawn at specified time points. After a 10^2-fold dilution, the bacterial suspension was inoculated onto a ZK-free LB-agar plate. After an overnight culture, the number of colonies was counted to evaluate cell viability. As a preliminary test, the viability was measured only at times 0 and 6 h.

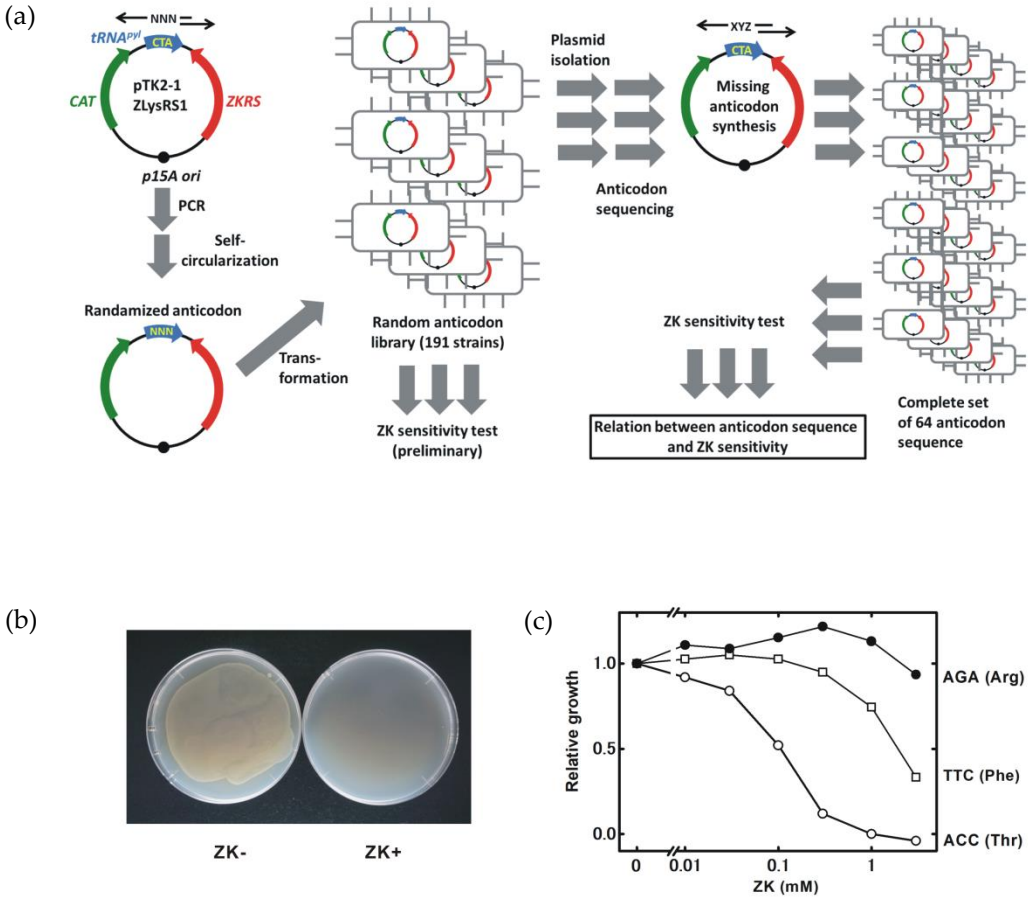

Figure 2. Growth inhibition of *E. coli* cells by activation of the ZK-regulated growth controller. (a) Construction of a $tRNA^{Pyl}$ gene library containing all 64 anticodons. Black arrows indicate PCR primers. N, non-specific base (A, T, G or C). X-Z, specific bases defined in the study. (b) ZK-induced growth inhibition. *E. coli* carrying the ZK-regulated growth controller containing the $tRNA^{Pyl}$ gene with a CGC anticodon, complementary to the GCG codon for Ala, was cultured with or without 3 mM ZK. (c) Relationship between ZK dose and bacterial growth inhibition. Very sensitive, moderately sensitive and resistant strains are shown. The complementary sequence of the anticodon and the assigned amino acid are indicated to the right. Growth of bacteria was normalized to that in the absence of ZK.

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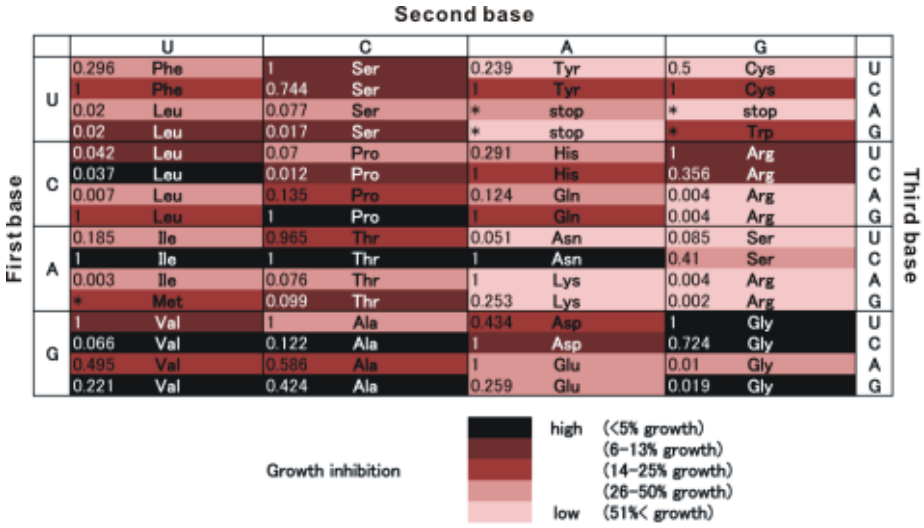
3. Results

The Uaa-regulated growth controller was constructed and characterized in *Escherichia coli* bacteria. Pyrrolysyl-tRNA synthetase (PylRS) of the archaeon *Methanosarcina mazei* aminoacylates a unique proteinous amino acid pyrrolysine onto its cognate tRNA ($tRNA^{pyl}$) [29-31]. PylRS-$tRNA^{pyl}$ is orthogonal to both prokaryotic and eukaryotic cells; i.e., PylRS does not aminoacylate any host tRNAs and host aminoacyl-tRNA synthetases (aaRSs) do not aminoacylate $tRNA^{pyl}$, and pyrrolysine is not catalyzed by host aaRSs and no other canonical proteinous amino acids are catalyzed by PylRS [32]. Many modified PylRSs that specifically recognize Uaa's have been generated to incorporate the Uaa into ribosomally synthesized proteins in various host cells [26]. We used a modified PylRS that specifically charges $tRNA^{pyl}$ with the unnatural amino acid N^{ε}-benzyloxycarbonyl-L-lysine (ZK) to construct the Uaa-regulated growth controller [19,28].

(a)



(b)

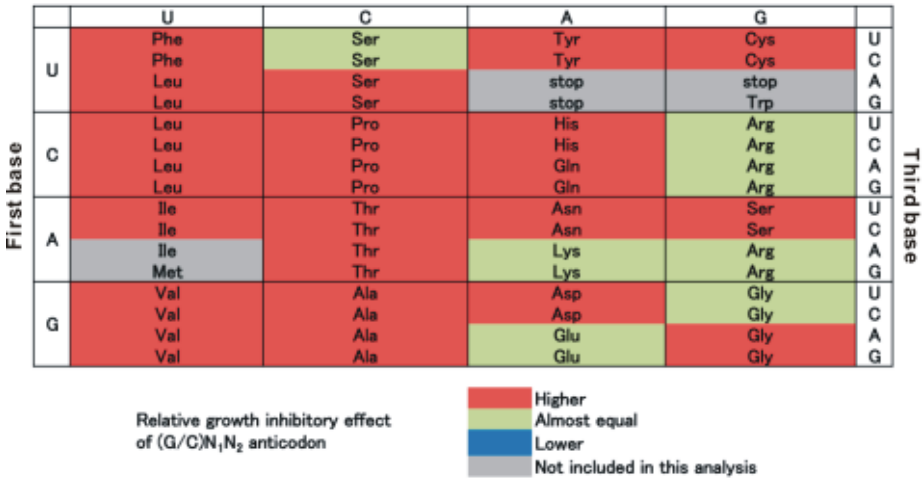


Figure 3. Anticodon sequence dependency of growth inhibition. (a) Relationship between anticodon sequence and growth inhibition. Anticodon sequences are shown as corresponding codon sequences. Amino acids assigned to the codons are also shown. The experiment was performed in the absence of chloramphenicol as a selection marker antibiotic because ZK incorporation at sense codons may also affect chloramphenicol sensitivity due to modification of the chloramphenicol acetyl transferase transgene product. The value to the left of the amino acid name shows the frequency of codon usage in highly expressed genes as a codon adaptation index [35,36]. (b) Higher growth inhibitory effects of GN_1N_2 and CN_1N_2 anticodons. Effects of GN_1N_2 anticodons, corresponding to (complementary N_2N_1)C codons, were compared with those of AN_1N_2, corresponding to (complementary N_2N_1)U codons (purine pairs). Similarly, effects of CN_1N_2 anticodons, corresponding to (complementary N_2N_1)G codons, were compared with those of UN_1N_2, corresponding to (complementary N_2N_1)A codons (pyrimidine pairs).

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The natural tRNA^{Pyl} incorporates the Uaa's at UAG because the anticodon sequence is CUA [33]. Fortunately, PylRS does not use the anticodon sequence of tRNA^{Pyl} for substrate tRNA recognition, which suggests that the anticodon sequence can be modified to incorporate Uaa's at other codons [32,34]. The anticodon sequence of the tRNA^{Pyl} gene was randomized to incorporate ZK at various sense and nonsense codons to give 191 strains of *E. coli* BL21-AI carrying the plasmid containing the ZK-specific modified pylRS (ZKRS) gene and the tRNA^{Pyl} gene with a randomized anticodon sequence (Fig 2a). In a preliminary test, the growth of some of these strains was markedly inhibited in the presence of ZK, although others were relatively resistant (Fig 2b).

The plasmid was isolated from each strain and the anticodon sequence of the modified tRNA^{Pyl} gene was determined. The relationship between growth inhibition and ZK concentration was also determined to evaluate the ZK sensitivity precisely. The ZK sensitivity was clearly affected by the anticodon sequence (Fig 2c). A submilimolar concentration of ZK suppressed growth of some sensitive strains, and growth inhibition became more severe at a higher concentration. The concentration-inhibition curve was shifted to a higher concentration range in less-sensitive strains.

Anticodon sequences not found in the 191 strains were newly synthesized and transfected into bacterial cells to reveal the complete relationship between ZK sensitivity and anticodon sequence. Finally, a complete collection of anticodon sequences complementary to all 64 codons was prepared and the ZK sensitivity was determined (Fig 3a). The magnitude of growth inhibition was confirmed to be dependent on the anticodon sequence of the tRNA^{Pyl} gene.

Several interesting correlations were observed in these results. Anticodons complementary to codons for small amino acids, such as Gly, Val, Ala and Pro, were frequently highly toxic. In contrast, those for larger amino acids, such as Arg, Lys, Trp, Tyr and Phe, were mostly less toxic. However, these tendencies were not completely consistent and some exceptions were observed, such as the high toxicity of an anticodon corresponding to a codon for CGU(Arg) and CGC(Arg). Anticodons complementary to stop codons were weak or non-toxic. The relative growth was 1.04, 0.48 and 0.93 for UGA, UAA and UAG stop codons, respectively.

It is noteworthy that the toxicity of tRNA^{Pyl}s containing GN_1N_2 and CN_1N_2 anticodons, corresponding to (complementary N_2N_1)C and G codons, were relatively higher than those of AN_1N_2 and TN_1N_2 anticodons, corresponding to (complementary N_2N_1)U and A codons, in many N_1N_2 pairs assigned to identical amino acids (Fig 3b). The toxicities of some GN_1N_2-AN_1N_2 (purine) pairs and CN_1N_2-UN_1N_2 (pyrimidine) pairs were almost equal, but no AN_1N_2 or UN_1N_2 anticodons were more toxic than GN_1N_2 or CN_1N_2 anticodons in any pairs.

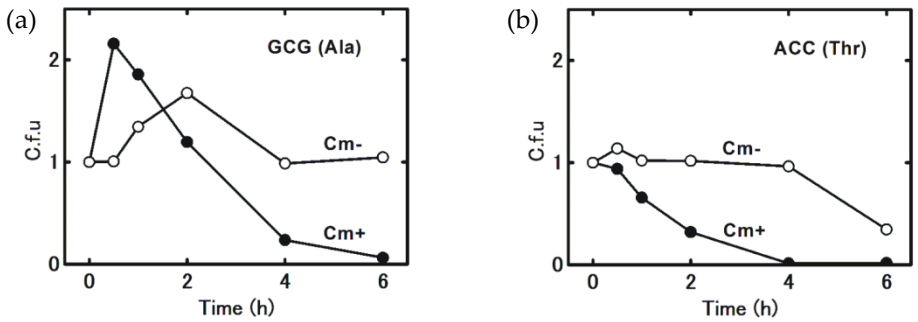

Figure 4. Mode of action. Time courses of viability are shown. The tested bacterial strains were incubated in the presence of 3 mM ZK for the indicated time. Tests were performed for strains carrying tRNA^{Pyl} containing CGC (a) and GGU (b), which have anticodon sequences complementary to the codons of GCG(Ala) and ACC(Thr), respectively. Cm- and Cm+: absence and presence of chloramphenicol, respectively.

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Having identified several strong growth inhibitory anticodon sequences, we next examined the mode of growth inhibition. The mode of antimicrobial action is usually categorized as bacteriostatic or bactericidal. Bacteriostatic antimicrobials suppress growth and proliferation, but bacteria do not die and growth is re-established after return to a permissive condition. In contrast, antimicrobials with a bactericidal action kill bacteria irreversibly. Preliminarily tests of the mode of action were performed against strains carrying $tRNA^{pyl}$ genes with strong growth inhibitory anticodon sequences, corresponding to CUC(Leu), AUC(Ile), GUC(Val), GUG(Val), CCG(Pro), ACC(Thr), GCC(Ala), GCG(Ala), AAC(Asn), GGU(Gly), GGC(Gly) and GGG(Gly), based on measurement of the change in viability over time in the presence of ZK. The mode of action was bacteriostatic for all tested strains.

The detailed time courses of viability were determined for selected strains carrying $tRNA^{pyl}$ genes containing anticodons GGT and CGC, corresponding to ACC(Thr) and GCG(Ala), respectively. The number of viable *E. coli* carrying a $tRNA^{pyl}$ gene with a CGC anticodon sequence was well maintained for 6 h, suggesting that bacteria survived without dying off quickly, despite severe growth inhibition (Fig 4a). For the GGT anticodon, the number of surviving bacteria was also well maintained until 4 h, but gradually decreased in 4 to 6 h (Fig 4b). These results confirm the bacteriostatic effect of the Uaa-regulated growth controller, although prolonged ZK treatment gradually kills the bacteria.

The ZK-regulated growth controller may also affect transgene products, including a selection marker: chloramphenicol acetyltransferase (CAT). To test the impact on CAT, we performed a similar experiment in the presence of chloramphenicol, and found a marked change in the results. Use of the CGC anticodon resulted in only 3% of bacteria remaining viable after 6h, although the viable cells initially increased (Fig 4a). With the GGT anticodon, the number of viable bacteria monotonically decreased and only 1% of inoculated cells survived after 4 h (Fig 4b). These results suggest that the Uaa-regulated growth controller caused a bactericidal effect in the presence of chloramphenicol by attenuating the total activity of CAT.

4. Discussion

A ZK-regulated growth controller in *E. coli* using ZKRS and $tRNA^{pyl}$ with an anticodon corresponding to a sense codon was constructed and characterized in this study. Growth of bacteria carrying this growth controller containing the $tRNA^{pyl}$ gene with an appropriate anticodon sequence was inhibited in the presence of ZK. The mode of action was mostly bacteriostatic. Several previous studies have shown that bacteria are broadly tolerant of proteome-wide missense substitutions with a normal proteinous amino acid, although with cellular growth rates that were uniformly slower than normal [37-40]. Our results show that bacterial growth can be perfectly suppressed without killing bacteria by choosing the appropriate Uaa incorporation system, Uaa concentration and anticodon sequence. In addition, growth inhibition could be titrated based on the ZK concentration. In contrast, ZK did not affect growth of bacteria carrying $tRNA^{pyl}$ with non-toxic anticodons such as UGA and UAG stop codons, which indicates that ZK did not have any harmful pharmacological effects. These results suggest that the ZK-regulated growth controller is a simple, target-specific and titratable growth control system.

Growth inhibition was strongly dependent on the anticodon sequence of the $tRNA^{pyl}$ gene. There may be several factors underlying this anticodon sequence dependency, but the precise causes were not examined. Theoretically, the inhibition mechanism can be divided into the following steps: efficient charging of $tRNA^{pyl}$ with ZK, efficient ZK incorporation from ZK-$tRNA^{pyl}$ into proteins, and growth inhibitory effects exerted by the ZK-containing proteins. All steps should be satisfied for strong growth inhibitory anticodons. In contrast, one or more of these steps may not be efficient for less toxic or non-toxic anticodons.

The first step, efficient charging of $tRNA^{pyl}$ with ZK, may be influenced by aminoacylation by a host aminoacyl-tRNA synthetase, which uses the anticodon sequence for

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substrate tRNA recognition. For instance, in *Mycoplasma capricolum*, $tRNA^{Pyl}_{CCG}$ is recognized well by the endogenous arginyl-tRNA synthetase and CGG codons are translated as Arg [41]. This type of mechanism may also interfere with ZK charging of $tRNA^{Pyl}$ containing other anticodon sequences because anticodons in tRNAs are typically used as recognition elements of most aaRSs in *E. coli* [42]. ZK incorporation into ZKRS is also should be taken into account because the efficiency and substrate specificity may be affected.

Post-transcriptional nucleotide modification may affect the second step, efficient ZK incorporation from ZK-$tRNA^{Pyl}$ into proteins, but modification of *M. mazei* $tRNA^{Pyl}$ has not been widely studied in *E. coli* [43]. Modification of nucleotides is critical for all core aspects of tRNA function, such as folding, stability, and decoding [44]. The hypermodified base N^6-threonylcarbamoyladenosine and its derivatives, which locate in the anticodon stem-loop at position 37 adjacent to the anticodon, is present in nearly all tRNAs that decode ANN codons [45,46]. This nucleotide modification stabilizes the anticodon loop, which promotes accurate decoding of ANN codons during protein synthesis [47,48]. The N^6-threonylcarbamoyladenosine modification was not found in *Methanosarcina barkeri* $tRNA^{Pyl}$, suggesting that $tRNA^{Pyl}_{NNU}$ is not optimized for decoding of ANN codons [49]. In addition, nucleotide modification at position 34, the first position of the anticodon in tRNA, modulates codon recognition, thereby promoting accurate decoding during protein synthesis. Thus, the nucleotide modification may complicate the relationship between anticodon sequences and growth inhibition [50].

The growth inhibitory effect of $tRNA^{Pyl}$ containing G or C at position 34 was higher than that containing A or U, respectively, in the box assigned to an identical amino acid. Previous reports suggest that $tRNA^{Pyl}$ is not optimized to incorporate amino acids in prokaryotes and eukaryotes [51,52]. Formation of a strong hydrogen bond between G and C at position 34, the first nucleotide of the anticodon and the third nucleotide of the codon, may contribute to efficient incorporation of ZK and partially optimize $tRNA^{Pyl}$ in the translation machinery of bacteria.

We predicted that the second step was hampered by competitive incorporation of the natural proteinous amino acid that was originally assigned to the identical sense codon and charged to the host tRNA. Endogenous tRNA numbers were moderately inversely correlated with the sense codon reassignment efficiency in *E. coli* carrying a sense codon targeting a Tyr incorporation system using the *Methanocaldococcus jannaschii* TyrRS-$tRNA^{tyr}$ [38]. In *E. coli*, the frequency of codon usage in highly expressed genes is directly proportional to the corresponding tRNA population [53,54]. However, contrary to expectations, $tRNA^{Pyl}$s corresponding to frequently appearing codons were often more growth inhibitory, including for codons assigned to Phe, Ile, Tyr, His, Gln, Asn, Asp, Cys and Arg. This suggests that the competition between $tRNA^{Pyl}$ and endogenous tRNA is not a dominant determinant of toxicity (Fig 3a).

It is reasonable to expect that substitutions of amino acids with other amino acids that are clearly physicochemically distant are highly toxic because these substitutions are likely to destroy the original conformation of a protein [55]. Therefore, the third step, a growth inhibitory effect exerted by ZK-containing proteins, is predicted to depend on which amino acid is substituted with ZK. This may explain the observation that anticodons corresponding to codons assigned to amino acids that are physicochemically distant from the bulky ZK, such as Gly, Val, Ala and Pro, were highly toxic, whereas bulky amino acids that are similar to ZK, such as Arg, Lys, Trp, Tyr and Phe, were less toxic or non-toxic. ZK incorporation at stop codons mostly results only in production of longer polypeptide chains. Moreover, the proteins in which Uaa's were incorporated at endogenous stop codons were detected only at low levels [56]. These observations agree well with the result that anticodons corresponding to stop codons were less toxic or non-toxic. However, species-specific toxicity of stop codon suppression still needs to be considered carefully [57].

Overall, the relationship between growth inhibitory effect and anticodon sequence is difficult to predict because many factors may interact and affect the relationship, as

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described above. An advantage of our system is that strong growth inhibitory anticodon sequences can be identified experimentally in each target organism because of the low selectivity toward the $tRNA^{Pyl}$ anticodon of PylRS, which can be used as an orthogonal enzyme in both prokaryotes and eukaryotes [26]. In addition, incorporation of Uaa's with physicochemical properties that are distinct from those of ZK may lead to a different spectrum of toxicity. Genetic incorporation of many Uaa's has been reported using the PylRS-$tRNA^{Pyl}$-based system [32]. Therefore, the Uaa-regulated growth controller using a modified PylRS-$tRNA^{Pyl}$ is an excellent flexible platform to select the most effective Uaa and anticodon sequence for a target organism. Moreover, it will be possible to control multiple populations using several modified PylRS-$tRNA^{Pyl}$ pairs that recognize distinct Uaa's orthogonally.

The Uaa-regulated growth controller also affected transgene products. This side effect could be prevented by removing the target codon from transgenes by synonymous codon substitution. On the other hand, target bacteria carrying a highly growth inhibitory $tRNA^{Pyl}$ were killed irreversibly in the additional presence of chloramphenicol, presumably due to reduction of total CAT activity. This procedure can be used to eliminate specific sub-populations from microbial consortia. In the case of $tRNA^{Pyl}_{CGC}$, the bacteria initially grew and then started to die. This delayed toxic effect can be explained by a time lag in adequate uptake of ZK into the bacteria [17]. The lack of a similar time lag with $tRNA^{Pyl}_{GGT}$ suggests more effective inactivation of CAT, which results in a lower intracellular concentration of ZK being sufficient to express toxicity.

The site-specific Uaa incorporation system can be controlled not only by a Uaa, but also by expression control of UaaRS and/or its cognate tRNA [15,16]. This control of UaaRS and tRNA gene expression may be useful in connection of the Uaa-controlling kill-switch to a gene circuit.

5. Conclusions

The ZK-regulated growth controller is a simple, target-specific, environmental noise-resistant and titratable growth control device. The PylRS-$tRNA^{Pyl}$-based Uaa incorporation system is an ideal platform to construct an effective Uaa-regulated growth controller because this system allows experimental identification of the most effective anticodon sequence and Uaa. This system is also applicable in various organisms because PylRS-$tRNA^{Pyl}$ can be used in both prokaryotes and eukaryotes and the basis of growth inhibition is "informational disturbance".

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