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Protecting Linear DNA Templates in Cell-Free Expression Systems from Diverse Bacteria

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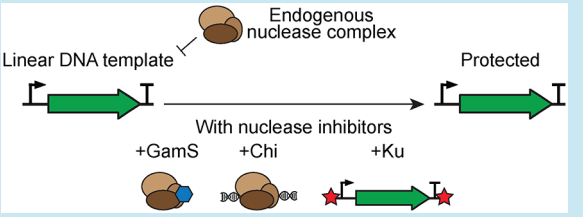
ABSTRACT: Recent advances in cell-free systems have opened up new capabilities in synthetic biology from rapid prototyping of genetic circuits and metabolic pathways to portable diagnostics and biomanufacturing. A current bottleneck in cell-free systems, especially those employing non-*E. coli* bacterial species, is the required use of plasmid DNA, which can be laborious to construct, clone, and verify. Linear DNA templates offer a faster and more direct route for many cell-free applications, but they are often rapidly degraded in cell-free reactions. In this study, we evaluated GamS from λ-phage, DNA fragments containing Chi-sites, and Ku from *Mycobacterium tuberculosis* for their ability to protect linear DNA templates in diverse bacterial cell-free systems. We show that these nuclease inhibitors exhibit differential protective activities against endogenous exonucleases in five different cell-free lysates, highlighting their utility for diverse bacterial species. We expect these linear DNA protection strategies will accelerate high-throughput approaches in cell-free synthetic biology.



KEYWORDS: *cell-free expression systems, linear DNA, RecBCD, GamS, Chi, Ku*

Cell-free expression systems greatly simplify the characterization and engineering of various biological processes *in vitro*.[1] Recent advances in cell-free synthetic biology are enabling rapid prototyping of genetic parts/circuits,[2−4] biosynthetic metabolic pathways,[5,6] and even gut microbiota and host biomarkers.[7] The simplicity and stability of cell-free systems (CFSs) have offered new capabilities to portably execute biological sensing and synthesis reactions for point-of-care diagnostics[8,9] and on-demand biomanufacturing.[10] CFSs have also become one of the major platforms for attempts to build and program minimal synthetic cell systems.[11,12]

While the vast majority of cell-free studies have used *Escherichia coli* cell lysates, new CFSs are being developed from diverse bacterial species to take advantage of the diversity of host properties and to prototype genetic systems for *in vivo* use.[3,13−17] Direct characterization and engineering of non-model bacterial species is often laborious and slow, primarily due to a lack of both efficient transformation methods and genetic tools. Cell-free systems can avoid these obstacles, thus greatly expediting the design−build−test cycle for developing genetic circuits and metabolic pathways in nonmodel microbes. Furthermore, the use of linear DNA templates in CFSs could dramatically shorten upfront preparation time for cell-free reactions by eliminating rate-limiting steps of plasmid construction, validation, and extraction. However, CFS applications have traditionally relied upon cloned plasmid DNA due to the poor stability of linear DNA which is a result of endogenous nuclease activities.

Two strategies have recently emerged to improve linear DNA stability in *E. coli* CFSs[18−20] that involve supplementation of reactions with either the bacteriophage protein GamS or the short DNA sequences containing Chi (crossover hotspot instigator) sites. However, there are relatively fewer reports of using linear DNA templates in non-*E. coli* bacterial CFSs,[14,17,21] which currently lack strategies for protection of linear DNA templates. To address this challenge, we evaluated the capacity of different established and new nuclease inhibitors to protect linear DNA in diverse bacterial CFSs and their potential to enable robust direct gene expression measurements.

RESULTS AND DISCUSSION

Cell-free systems rely upon either individually reconstituted cellular machineries or crude cell lysates.[1] Owing to their relatively simple and easy preparation pipeline, crude cell lysate-based CFSs have gained increasing popularity for many synthetic biology applications. These lysate-based systems benefit from constituent cellular machineries and metabolic capabilities that can approximate their corresponding cellular conditions *in vitro*. However, endogenous nucleases and proteases in cell lysates can negatively interfere with cell-free reactions, especially for linear DNA templates due to exonuclease activity of the RecBCD complex.[18−20] Currently available strategies using GamS or Chi-site stabilize linear

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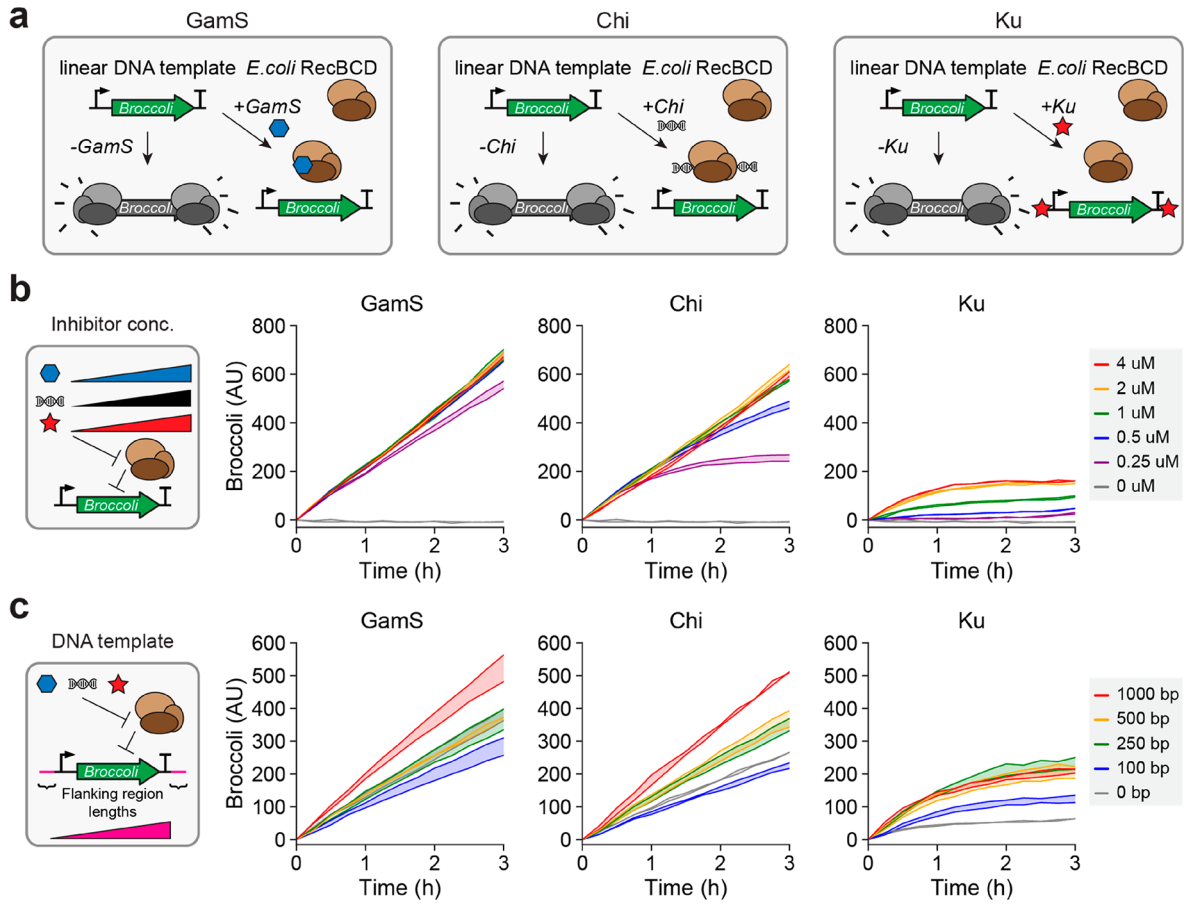

Figure 1. Nuclease complex inhibitors enhance the stability and activity of linear DNA in *E. coli* cell-free expression system. (a) Nuclease inhibiting mechanisms of GamS, Chi-sites, and Ku. (b) Various concentrations of the inhibitors and (c) lengths of flanking region were examined. A linear DNA template (12 nM) encoding RNA fluorescence aptamer Broccoli under the control of a strong broad-host-range constitutive promoter (P_{Gen_18145}) was used to quantify the level of protection from exonuclease activity using excitation and emission wavelengths of 482 and 505 nm, respectively. Background signals at 0 h time point were subtracted for normalization. All measurements are based on two biological replicates. Line represents each biological replicate, and shaded region represents range between the two biological replicates.

DNA by directly interacting with the *E. coli* RecBCD complex and effectively titrating out its nuclease activity[18−20] (Figure 1a). While those methods are highly effective in *E. coli* CFSs, the RecBCD inhibitors may not work in non-*E. coli* CFSs due to evolutionary divergence of the exonuclease complex or existence of alternative DNA processing enzymes in diverse bacterial species.[22] Therefore, we sought to explore an alternative, potentially universal, strategy to protect linear DNA in cell lysates by binding to 5′ and 3′ ends of linear DNA and thus blocking access to any exonucleases.

Ku is a highly conserved protein from bacteria to humans and is a part of the nonhomologous end joining (NHEJ) DNA repair pathway.[23,24] Ku protects damaged DNA from endogenous exonucleases by binding to exposed dsDNA ends in the host[25] and has played a key role in NHEJ-mediated genome editing applications.[25−27] Previously, it was experimentally demonstrated that mycobacterial Ku protects dsDNA ends from mycobacterial AdnAB (an ortholog of RecBCD in *E. coli* or AddAB in *B. subtilis*) *in vitro*.[25] Therefore, we assessed the capacity of Ku from *Mycobacterium tuberculosis* to protect linear DNA in cell-free reactions in comparison to GamS and Chi-site strategies. We first examined the interaction between the nuclease inhibitors and linear DNA, confirming direct binding of Ku to linear DNA (Figure S1). To determine whether Ku can provide linear dsDNA protection from exonuclease activity, we then used a PCR product encoding RNA fluorescence aptamer Broccoli[28] in *E. coli* CFS (Figure 1a). As expected, linear DNA could not activate gene expression unless the nuclease inhibitors were added into the reactions, but with lower yields compared to plasmid DNA, which is resistant to exonuclease activity (Figure S2). As shown previously,[18−20] GamS and Chi-oligos provided strong protection against *E. coli* RecBCD, rapidly titrating out its exonuclease activity even at the lowest concentrations tested (0.25−0.5 μM) (Figure 1b). We found that Ku is also able to protect linear DNA templates, leading to increased gene expression in a concentration-dependent manner in *E. coli* cell-free reactions, although at a lower efficiency and earlier saturation compared to GamS and Chi-oligos. Given that Ku could in theory interfere with gene expression by blocking the binding of RNA polymerase to a promoter sequence near the end of a linear fragment, we further tested linear DNA templates with varying lengths of flanking regions. GamS did not provide higher level of protection for flanking regions below 1000 bp, while Chi provided increased protection with flanking regions longer than 250 bp. In contrast, Ku provided protection with flanking regions as short as 100 bp, with a 250 bp flanking region achieving the highest protective activity using the Broccoli RNA expression reporter (Figure 1c). We also examined the translation level from linear DNA templates,

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ACS Synth. Biol. XXXX, XXX, XXX−XXX

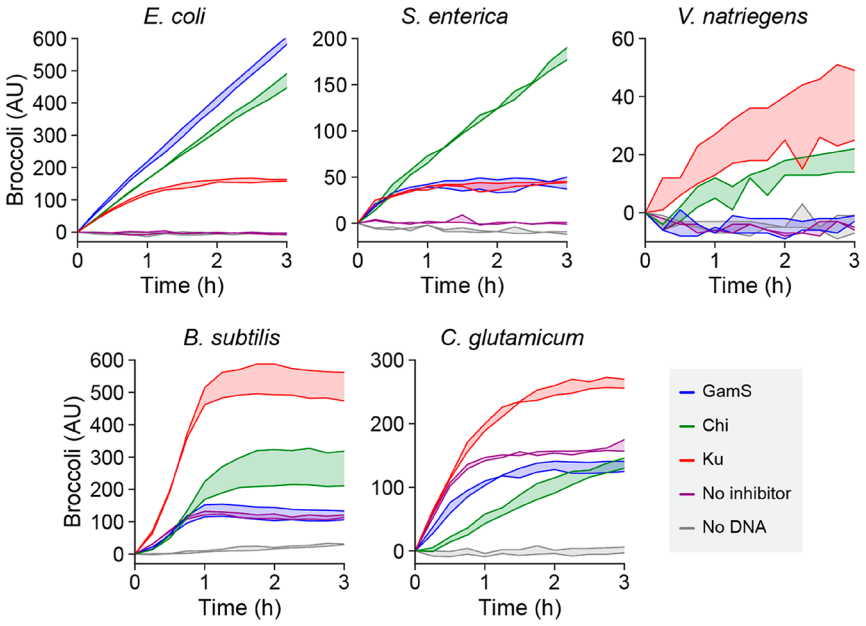

Figure 2. Transcription from linear DNA templates in diverse bacterial cell-free systems. A linear DNA template (12 nM) encoding RNA fluorescence aptamer Broccoli under the control of a strong broad-host-range constitutive promoter (P_{Gen_18145}) was used to quantify the level of protection from exonuclease activity using excitation and emission wavelengths of 482 and 505 nm, respectively. 2 μM of each nuclease inhibitor was used. Background signals at 0 h time point were subtracted for normalization. All measurements are based on two biological replicates. Line represents each biological replicate, and shaded region represents range between the two biological replicates.

which showed similar trends of protective activities by the nuclease inhibitors (Figure S3). Taken together, these results demonstrate that Ku stabilizes linear DNA against exonucleases in cell-free reactions by a different mechanism of protection.

Although Ku was not as effective as GamS and Chi in *E. coli* CFS, we hypothesized that Ku could have utility in non-*E. coli* CFSs with its alternative protection mechanism. To investigate the feasibility of this idea, we performed Broccoli transcription reactions in cell-free expression systems from five diverse bacterial species (*Escherichia coli, Salmonella enterica, Vibrio natriegens, Bacillus subtilis, Corynebacterium glutamicum*) spanning three phyla (Proteobacteria, Firmicutes, Actinobacteria).[3] We used a linear DNA template encoding Broccoli as a reporter under the control of a strong broad-host-range sigma70-dependent promoter (Gen_18145, Table S4),[29] which was sufficiently active on a plasmid DNA in all tested bacterial CFSs in our previous study.[3] Interestingly, the inhibitors exhibited different levels of DNA protection in different CFSs (Figure 2). In *S. enterica* CFS, while all three inhibitors could facilitate gene expression from linear DNA, Chi-oligos showed the highest protective activity. High similarities between each protein of the RecBCD complex to *E. coli* (85%, 88.7%, and 84% pairwise identities for RecB, RecC and RecD, respectively) could explain the Chi-oligos compatibility between *E. coli* and *S. enterica* CFSs. Chi-oligos (with *E. coli* Chi sequence) moderately improved Broccoli expression also in *V. natriegens* and *B. subtilis* CFSs, despite the difference in Chi sequences of the hosts. This unexpected protection could be due to excess Chi DNA substrates present in the reaction slowing the exonuclease complex from acting on the Broccoli-expressing DNA template through competition. In addition, GamS did not further stabilize our linear reporter construct in *V. natriegens*, *B. subtilis*, and *C. glutamicum* CFSs. The incompatibility of GamS for linear DNA protection in *V. natriegens* CFS was previously reported.[21] *B. subtilis* has an AddAB exonuclease complex that is a functional analogue of RecBCD but with a distinctly different structure,[22] and *C. glutamicum* is known to lack the RecBCD pathway.[30] In contrast, Ku's alternative mechanism of protection was able to improve gene expression from linear DNA in a broader range of CFSs, especially the Gram-positive strains *B. subtilis* and *C. glutamicum* CFSs with 4.43- and 1.58-fold improvement, respectively, in comparison to the control reactions without nuclease inhibitors. To compare protective activities of the nuclease inhibitors more directly, we repeated the experiments using *in vitro* T7-based transcription assay with normalized protein concentrations of each cell lysate. This allowed a more confined reactions with fewer variables (e.g., promoter compatibility and transcriptional efficiency) to specifically assess the nuclease inhibitors against exonucleases in cell lysates. The T7-based assay reproduced the constitutive promoter-based results with similar trends of protective activities by the nuclease inhibitors, but with normalized transcriptional yields (Figure S4). Taken together, these results highlight the importance of characterizing different types of nuclease inhibitors to enhance linear DNA stability in the ever-growing suite of bacterial CFSs. We expect that future efforts that explore Chi sequences or dsDNA binding proteins from different bacterial species will help accelerate the development of useful cell-free synthetic biology applications.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.0c00277.

Materials and Methods: (1) cell-free lysate preparation, (2) cell-free transcription reactions, (3) production and purification of Ku in *E. coli*. Supplementary Tables: (S1) bacterial species used in this study and their growth

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ACS Synth. Biol. XXXX, XXX, XXX−XXX

conditions, (S2) exonuclease inhibitors used in this study, (S3) plasmids used in this study, (S4) DNA parts used in this study. Supplementary Figures: (S1) DNA binding activity of Ku from *Mycobacterium tuberculosis*, (S2) transcription from plasmid DNA and linear DNA in *E. coli* cell-free expression system, (S3) translation from linear DNA templates using the nuclease complex inhibitors in *E. coli* cell-free expression system, (S4) T7-based assessment of linear DNA stability in diverse cell-free systems (PDF)

Plasmid maps, raw data (ZIP)

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Author Contributions

S.S.Y., N.I.J., V.N., and H.H.W. developed the initial concept. S.S.Y. performed experiments and analyzed the results under the supervision of H.H.W.; S.S.Y. and H.H.W. wrote the mansucript with input from all authors.

Notes

The authors declare no competing financial interest.

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