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Technical Note

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

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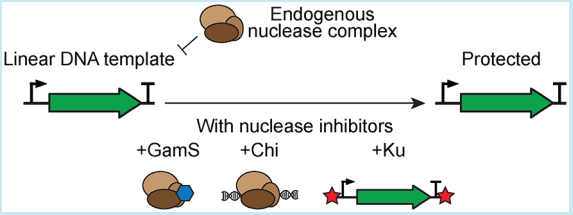
ABSTRACT: Recent advances in cell-free systems have opened up new capabilities in synthetic biology from rapid prototyping of genetic circuits and metabolic pathways to portable diagnostics and biomanufacturing. A current bottleneck in cell-free systems, especially those employing non-*E. coli* bacterial 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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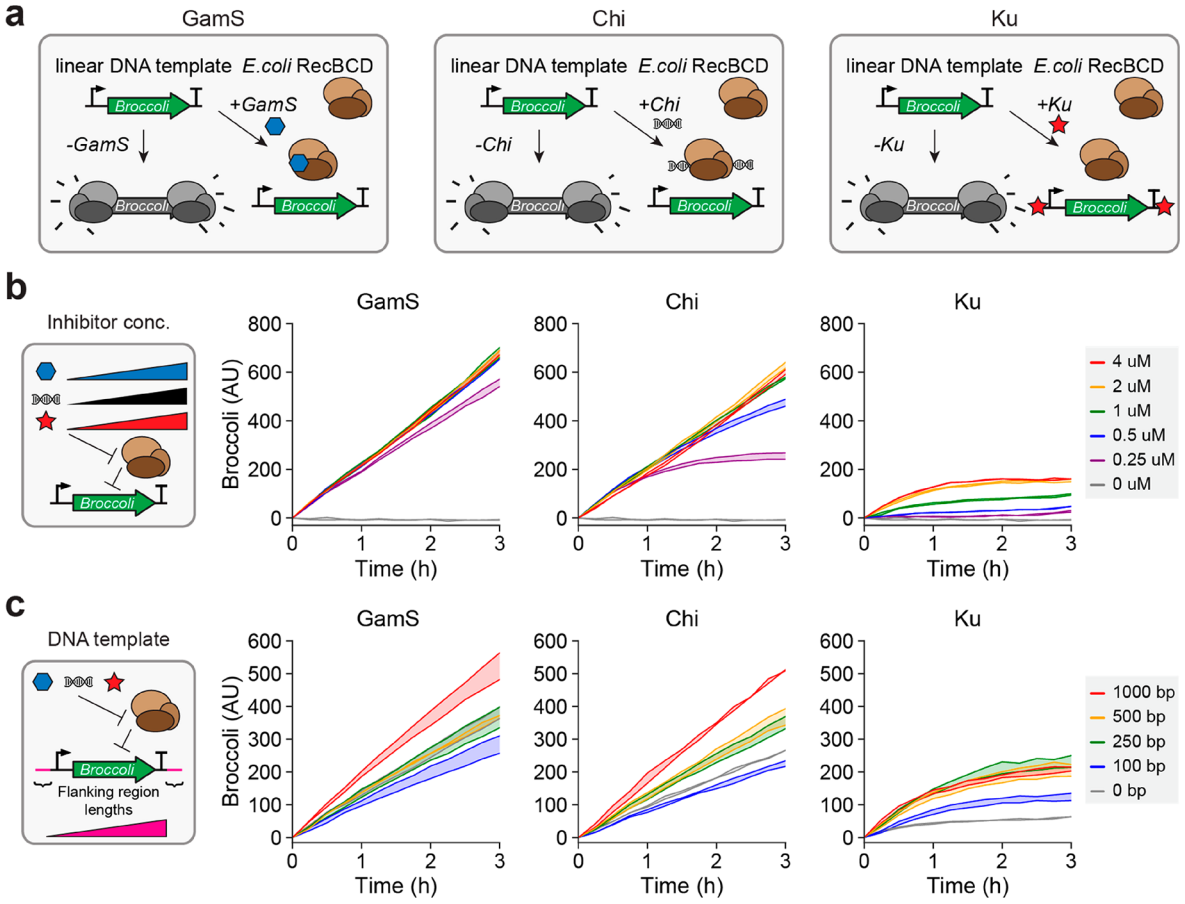

Figure 1. Nuclease complex inhibitors enhance the stability and activity of linear DNA in *E. coli* cell-free expression system. (a) Nuclease inhibiting mechanisms of GamS, Chi-sites, and Ku. (b) Various concentrations of the inhibitors and (c) lengths of flanking region were examined. A linear DNA template (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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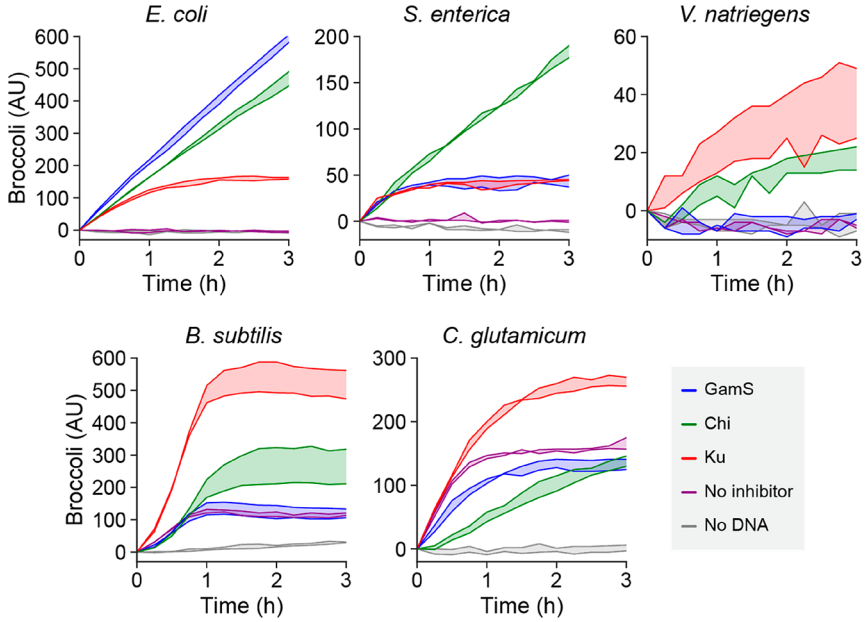

Figure 2. Transcription from linear DNA templates in diverse bacterial cell-free systems. A linear DNA template (12 nM) encoding RNA fluorescence aptamer Broccoli under the control of a strong broad-host-range constitutive promoter (P_{Gen_18145}) was used to quantify the level of protection from exonuclease 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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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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Supplementary Information

Protecting linear DNA templates in cell-free expression systems from diverse bacteria

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This PDF file includes:

Materials and Methods

Supplementary Tables S1 to S4

Supplementary Figures S1 to S4

MATERIALS AND METHODS

Cell-free lysate preparation

Cell-free lysates were prepared based on our previous work in Yim et al., *Molecular Systems Biology* **15**, e8875, (2019). Strain information and growth conditions used for each species can be found in **Supplementary Table 1**. Briefly, single colonies of each species were inoculated into 4 mL liquid media and grown overnight. 3.3 mL from the overnight culture was transferred to two flasks containing 330 mL growth medium in 1-L flasks and grown to mid-exponential growth phase. Flasks were rapidly chilled on ice and cells were washed three times with 50 mL S30A buffer. The mass of the final pellet was measured and 0.8 mL S30A buffer was added per gram of pellet mass. The resuspensions were sonicated on ice using a Qsonica125 sonicator. Lysates were centrifuged at 12,000 x g for 10 min at 4 ºC. Supernatant was transferred to 2-mL microtubes and run-off reactions were performed by incubating clarified lysates at growth temperature. Samples were centrifuged at 12,000 x g for 10 min at 4 ºC, and supernatant was dialyzed in S30B buffer in dialysis cassette (Slide-A-Lyzer 10k MWCO, Thermo Scientific) for 2-3 h at 4 ºC. Samples were centrifuged at 12,000 x g for 10 min at 4 ºC, then supernatant was aliquoted and stored at -80 ºC.

Cell-free transcription reactions

Cell-free transcription reactions were performed based on our previous work in Yim et al., *Molecular Systems Biology* **15**, e8875, (2019) with minor modifications to use linear DNA template. Briefly, Cell lysates were combined with amino acids, PEG, energy buffer, Mg-glutamate and K-glutamate at concentrations that were previously optimized (Yim et al., *Molecular Systems Biology* **15**, e8875, (2019)) in a skirted white 96-well PCR plate (Bio-rad). A Broccoli expression cassette from TOPO-F30-Broccoli plasmid was used as a DNA template either as a plasmid or as a linear PCR product, and nuclease-free water was used as a negative control. 1 µL of exonuclease inhibitors (GamS, Chi, and Ku) were added at a final concentration of 2 µM, unless otherwise stated. Sequence information of the exonuclease inhibitors can be found in **Supplementary Table 2**. DNA template (1 µL) and 10 mM DFHBI-1T (0.5 µL, Tocris Bioscience) was added to each well immediately before time course measurements. Fluorescence was tracked for 3 h using a Synergy H1 plate reader (BioTek) at 30 ºC using excitation and emission wavelengths of 482 and 505 nm respectively.

Production and purification of Ku in *E. coli*

ku gene from *Mycobacterium tuberculosis* was synthesized (IDT) and cloned into pET28c with riboJ (BBa_K1679038) and RBS (BBa_B0034) to yield pET28c-Ku. The pET28c-Ku plasmid was introduced to *E. coli* BL21(DE3). Ku was produced in the strain by 0.2 mM IPTG (isopropyl-b-D-thiogalactopyranoside) induction at 18 °C for 16 h. Soluble lysate of the

culture was prepared by sonication on ice using a Qsonica125 sonicator with 3.2-mm probe at 40% amplitude for 50 rounds of 30 seconds, with 30 second breaks and clarification by centrifuging at 12,000 x g for 10 min at 4 ºC. Recombinant Ku protein in the soluble lysate was purified using Nickel-NTA-agarose. Ku was recovered in the 300 mM imidazole eluates. Eluted Ku protein was dialyzed in PBS for 2-3 h at 4 ºC, and stored at -20 °C.

Supplementary Table S1. Bacterial species used in this study and their growth conditions

Species	Strain (Source)	Medium	Temp.	Aeration
E. coli	BL21	2xYT+P	37 ℃	Shaking (220 rpm)
S. enterica	Serovar Typhi Ty2	2xYT+P	37 ℃	Shaking (220 rpm)
V. natriegens	ATCC 14048	BHI+v2 salt	37 ℃	Shaking (220 rpm)
B. subtilis	BD3182 (168 derivative)	2xYT+P	30 ℃	Shaking (220 rpm)
C. glutamicum	ATCC 13032	BHI	30 ℃	Shaking (220 rpm)

Supplementary Table S2. Exonuclease inhibitors used in this study

Inhibitor	Sequence	Note
GamS	(AA) MNAYYIQDRLEAQSWARHYQQLAREEKEAELADDMEK GLPQHLFESLCIDHLQRHGASKKSITRAFDDDVEFQERM AEHIRYMVETIAHHQVDIDSEV	Purchased from Arbor Biosciences
Chi	(DNA) TCACTTCACTGCTGGTGGCCACTGCTGGTGGCCACT GCTGGTGGCCACTGCTGGTGGCCACTGCTGGTGGCC ACTGCTGGTGGCCA	Annealed sense and antisense oligonucleotides
Ku	(AA) MHHHHHHRAIWTGSIAFGLVNVPVKVYSATADHDIRFH QVHAKDNGRIRYKRVCEACGEVVDYRDLARAYESGDG QMVAITDDDIASLPEERSREIEVLEFVPAADVDPMMFDR SYFLEPDSKSSKSYVLLAKTLAETDRMAIVHFTLRNKTRL AALRVKDFGKREVMMVHTLLWPDEIRDPDFPVLDQKVEI KPAELKMAGQVVDSMADDFNPDRYHDTYQEQLQELIDT KLEGGQAFTAEDQPRLLDEPEDVSDLLAKLEASVKARS KANSNVPTPP	Purified using N-terminal 6xHis-tag

Annotated plasmid maps are provided as Supplementary Data

Supplementary Table S3. Plasmids used in this study

Plasmid	Origin	Antibiotic	Description
pTOPO-F30-Broccoli	pUC	Carbenicillin	P_{Gen_18145}-F30-Broccoli-T_{B0015}*
pTXTL-P70a-deGFP	pBR322	Carbenicillin	P_{70a}-deGFP-T_{T500}**
pET28c-F30-Broccoli	pBR322	Kanamycin	P_{T7}-F30-Broccoli-T_{T7}***
pET38c-Ku	pBR322	Kanamycine	P_{T7}-Ku-T_{T7}

Annotated plasmid maps are provided as Supplementary Data

*[Filonov et al., 2014. *J Am Chem Soc* **136**, 16299-308]

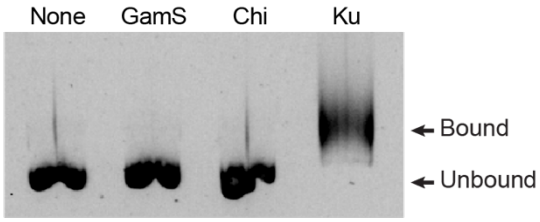
[Sun et al., 2013. *J Vis Exp* **79, e50762]

***[Filonov et al., 2015. *Chem Biol* **22,** 649-660]

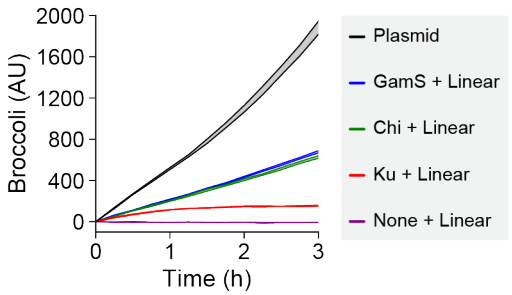
Supplementary Table S4. DNA parts used in this study

DNA parts	Sequence	Note
P_{Gen_18145}	ATTGGGGGCGACGGTCAATTAAGTCCAGTC ATAGTGGGACTGGTGTCCTAATAATTTAAGC ATTCTTTTCTAGAAATACTTTGTCTTTCCTTG ACTTTCATCAGGGTGGTTGTTATATTAGTAA T	Promoter
P_{70a}	TGAGCTAACACCGTGCGTGTTGACAATTTTA CCTCTGGCGGTGATAATGGTTGCA	Promoter
P_{T7}	TAATACGACTCACTATAGGG	Promoter
T_{B0015}	CCAGGCATCAAATAAAACGAAAGGCTCAGT CGAAAGACTGGGCCTTTCGTTTTATCTGTTG TTTGTCGGTGAACGCTCTCTACTAGAGTCA CACTGGCTCACCTTCGGGTGGGCCTTTCTG CGTTTATA	Terminator
T_{T500}	CAAAGCCCGCCGAAAGGCGGGCTTTTCTGT	Terminator
T_{T7}	CTAGCATAACCCCTTGGGGCCTCTAAACGG GTCTTGAGGGGTTTTTTG	Terminator

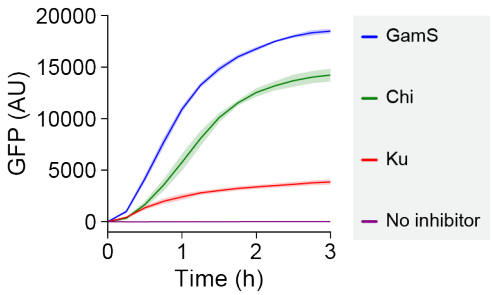
Annotated plasmid maps with these sequences are provided as Supplementary Data



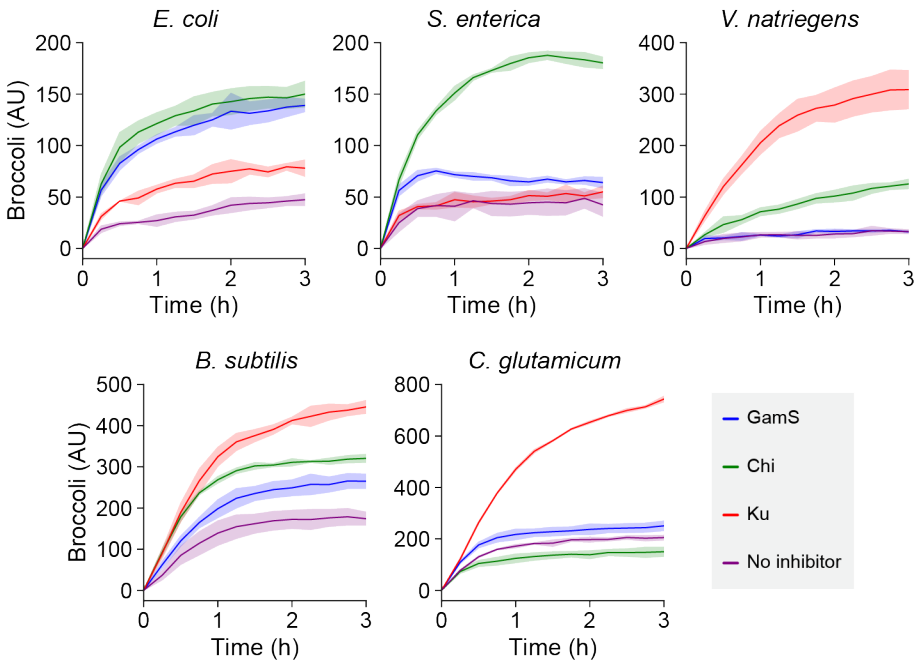
Supplementary Figure S1. DNA binding activity of Ku from *Mycobacterium tuberculosis*. A linear DNA (100 nM) was incubated with each of the nuclease complex inhibitors (1 μM) at 30 °C for 5 min, and interactions between them were analyzed by agarose gel electrophoretic mobility shift assay. The shifted band at the Ku lane indicates Ku from *M. tuberculosis* directly interacts with linear DNA templates.



Supplementary Figure S2. Transcription from plasmid DNA and linear DNA in *E. coli* cell-free expression system. 12 nM of plasmid or linear DNA templates 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.



Supplementary Figure S3. Translation from linear DNA templates using the nuclease complex inhibitors in *E. coli* cell-free expression system. A linear DNA template (12 nM) encoding GFP under the control of a strong sigma70-dependent constitutive promoter (P_{70a}) 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 three biological replicates, and shaded region represents standard deviation of the three biological replicates.



Supplementary Figure S4. T7-based assessment of linear DNA stability in diverse cell-free systems. *in vitro* T7-based transcription assay was performed using T7 RNAP (NEB M0251), 1x RNAPol Reaction Buffer, 1.25 mM NTPs, and 12 nM linear DNA template encoding RNA fluorescence aptamer Broccoli under the control of a T7 promoter. RNA fluorescence aptamer Broccoli 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. Cell lysates prepared from each bacterial species were added into the T7 reactions at a final concentration of 1 mg/mL. All measurements are based on three biological replicates, and shaded region represents standard deviation of the three biological replicates.