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Release Factor Inhibiting Antimicrobial Peptides Improve Nonstandard Amino Acid Incorporation in Wild-type Bacterial Cells

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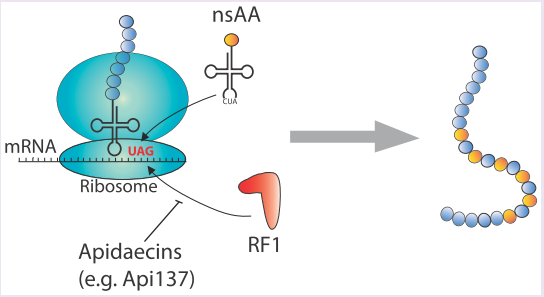
ABSTRACT: We report a tunable chemical genetics approach for enhancing genetic code expansion in different wild-type bacterial strains that employ apidaecin-like, antimicrobial peptides observed to temporarily sequester and thereby inhibit Release Factor 1 (RF1). In a concentration-dependent matter, these peptides granted a conditional lambda phage resistance to a recoded *Escherichia coli* strain with nonessential RF1 activity and promoted multisite nonstandard amino acid (nsAA) incorporation at in-frame amber stop codons *in vivo* and *in vitro*. When exogenously added, the peptides stimulated specific nsAA incorporation in a variety of sensitive, wild-type (RF1+) strains, including *Agrobacterium tumefaciens*, a species in which nsAA incorporation has not been previously reported. Improvement in nsAA incorporation was typically 2−15-fold in *E. coli* BL21, MG1655, and DH10B strains and *A. tumefaciens* with the >20-fold improvement observed in probiotic *E. coli* Nissle 1917. In-cell expression of these peptides promoted multisite nsAA incorporation in transcripts with up to 6 amber codons, with a >35-fold increase in BL21 showing moderate toxicity. Leveraging this RF1 sensitivity allowed multiplexed partial recoding of MG1655 and DH10B that rapidly resulted in resistant strains that showed an additional approximately twofold boost to nsAA incorporation independent of the peptide. Finally, in-cell expression of an apidaecin-like peptide library allowed the discovery of new peptide variants with reduced toxicity that still improved multisite nsAA incorporation >25-fold. In parallel to genetic reprogramming efforts, these new approaches can facilitate genetic code expansion technologies in a variety of wild-type bacterial strains.



INTRODUCTION

Proteins are translated by ribosomal decoding of mRNAs into polypeptides. This process continues until a stop codon is reached and a specific release factor (RF) is recruited to terminate translation. For each new residue, the correct amino acid is selected using specific tRNAs (tRNAs) as adaptors, which are selectively charged with one of the naturally occurring amino acids by aminoacyl tRNA synthetases (aaRS). The protein translation machinery of numerous bacterial and eukaryotic species has been successfully engineered to site-specifically encode a variety of different synthetic, nonstandard amino acids (nsAAs) in target proteins (Figure 1).[1−5] This technology enables the expansion of the protein chemistry space with a diverse set of new synthetic functionalities that include fluorescence, photo cross-linking, bioorthogonal tags, and post-translational modifications, among many others.[1−7]

Typically, genetic code expansion technologies rely on the use of engineered orthogonal aaRSs that do not charge native tRNAs or natural amino acids but instead charge a specific nsAA to an amber-suppressing cognate $tRNA_{CUA}$, which is not recognized by native aaRSs. As a result, the ribosome site-specifically incorporates this nsAA into target proteins with in-frame amber (UAG) stop codons (Figure 1a). In bacteria, this approach is used to encode nsAAs of different sizes and functionalities[2,4−6,8,9] without any significant incorporation to native/off-target amber stop codons.[2,10] Due to extensive optimizations over the last decades, encoding a single nsAA into a target protein via amber suppression can now provide expression yields close to native expression in wild-type (RF1+) *E. coli*.[5,11−13] However, efficiencies of incorporation begin to drop when a certain nsAA is encoded in a multisite manner in a single polypeptide. This is chiefly because amber codon-specific RF1 competes with the nsAA-charged $tRNA_{CUA}$, which can also lead to formation of undesired, truncated protein products.[14] Multisite incorporation of an nsAA can amplify the desired new chemical property in a target protein toward a variety of new applications, such as improving the biostability of protein therapeutic agents,[15] facilitating the

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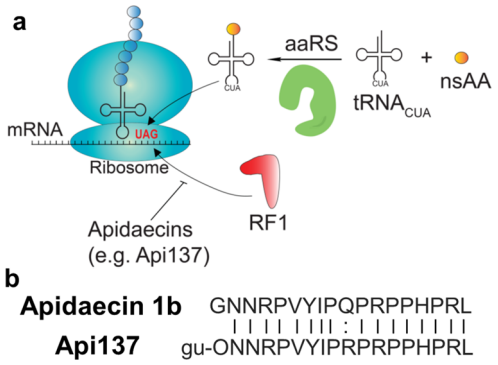

Figure 1. Genetic code expansion enables incorporation of nsAAs into proteins by providing orthogonal aminoacyl tRNA synthetase (aaRS)/tRNA$_{CUA}$ pairs to insert nsAAs at in-frame amber stop codons. (a) Amber codon suppression allows nsAA incorporation at UAG codons, but such incorporation competes with RF1 which terminates translation. Our strategy uses apidaecins to inhibit RF1. (b) Amino acid alignment of a naturally occurring Apidaecin 1b (Api1b) and its potent synthetic analogue Api137 used in this work. (O: ornithine, gu: *N*,*N*,*N*′,*N*′-tetramethylguanidino).

development of new antibody−drug conjugates,[16,17] or modulating the brightness of a fluorescent nsAA-labeled protein. Therefore, efficient cellular production of proteins with multiple nsAAs is an outstanding challenge for realizing the potential of genetic code expansion.

This limitation can be addressed in strains in which RF1 can be deleted.[18−22] For example, whole-genome recoding of all 321 native amber stop codons (TAG) to ochre (TAA) in *E. coli* MG1655[19] allowed for the unconditional deletion of RF1 resulting in the strain C321.ΔA (Addgene #48998 referred here as C321.ΔRF1) and enabling efficient and accurate multisite nsAA incorporation.[23] However, such recoding approaches are resource-intensive and require the availability of powerful genetic tools and an advanced understanding of genome architecture and function and have been accomplished in only a few *E. coli* strains. Instead of genetically knocking out RF1, we sought to develop a modular, chemical genetics approach relying on a RF1-inhibiting small molecule. Recently, apidaecins, a broad class of proline-rich antimicrobial peptides first isolated from honeybees[24−26] (Figure 1), were shown to have RF1- and RF2-inhibiting activity.[27−29] Inspired by this newly elucidated mechanism of action, here we show that apidaecin-like peptides serve as an effective agent to improve site-specific, multisite nsAA incorporation in a variety of wild-type bacterial species. This approach allows for rapid, transient, and tunable RF1 inhibition in a variety of strains where the alternative option of recoding may be technically unfeasible.

RESULTS AND DISCUSSION

Apidaecins Improve nsAA Incorporation in a Cell-Free Translation System. In wild-type bacteria, premature translation termination by RF1 limits amber suppression and incorporation of nsAAs into target proteins.[14] Recent work with bacterial cell-free translation systems tied the antimicrobial activity of apidaecins to their ability to trap and deplete release factors RF1 and RF2 in *E. coli* (Figure 1).[27−29] Given

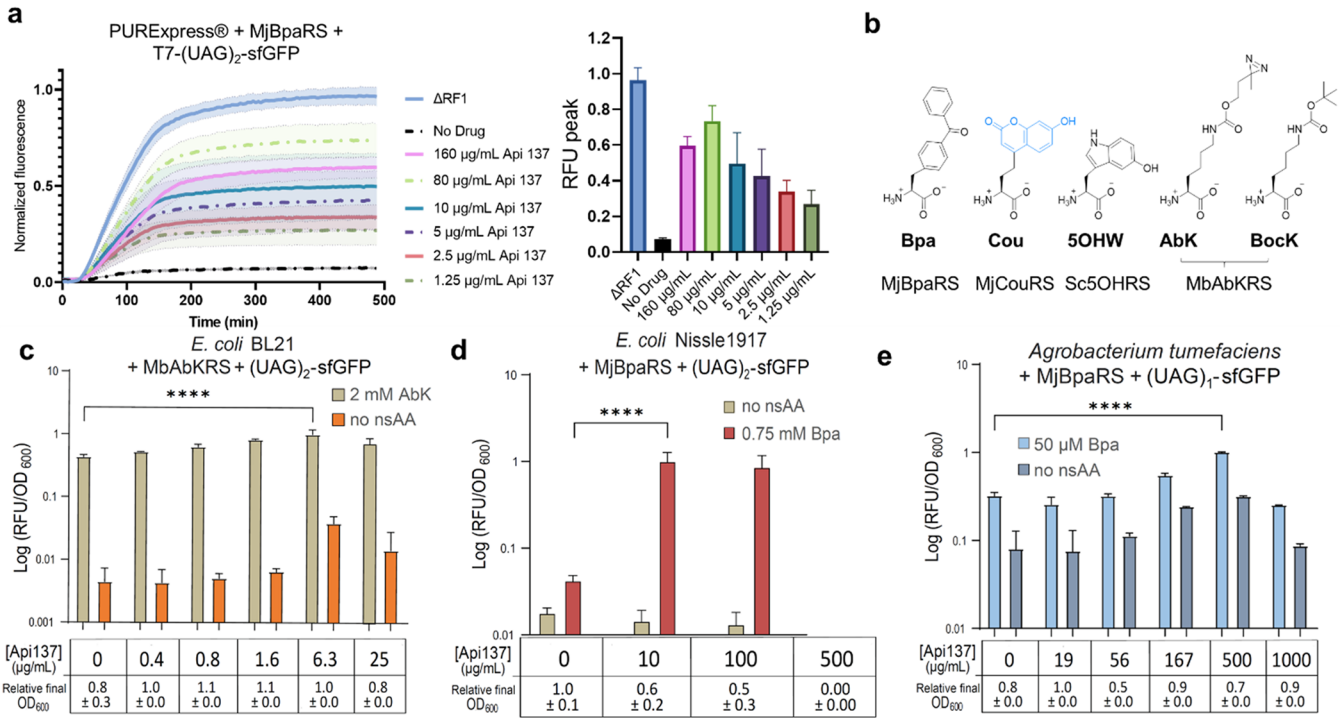

Figure 2. Apidaecins improve nsAA-dependent reporter expression *in vitro* and in different bacteria. (a) In a dose dependent manner, Api137 improved nsAA-dependent (UAG)$_2$-sfGFP expression in an RF1+ cell-free translation system, PURExpress. The ΔRF1 condition represents the same conditions applied in a specialized PURExpress Δ*RF123* kit lacking RF1. (b) Three distinct orthogonal aaRS systems and their cognate nsAAs used in this work. **Cou** is a fluorescent nsAA. (c−e) In a dose dependent manner and with modest effect in final cell density, Api137 improved nsAA-dependent reporter expression indicative of increased (c) **AbK** incorporation in *E. coli* BL21, (d) **Bpa** incorporation in *E. coli* Nissle 1917, and (e) in *Agrobacterium tumefaciens* C58 cells. At concentrations higher than 100 μg mL^{-1}, Api137 inhibited the growth in *E. coli* Nissle 1917. ****, $P < 0.0001$

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the limited abundance of RF1 relative to RF2 and ribosomes in the bacterial translation machinery,[30] we hypothesized that apidaecins could promote nsAA incorporation in response to amber suppression in a dose-dependent manner. As a first test of this idea, we expressed a super folder GFP (sfGFP) reporter DNA template carrying an in-frame amber codon (T7-$(UAG)_1$-sfGFP) in a modified real-time, cell-free translation monitoring system based on PURExpress[31,32] in the presence of an orthogonal aaRS/$tRNA_{CUA}$ pair (MjBpaRS and $tRNA^{Tyr}_{CUA}$) and its cognate nsAA, **Bpa.** We found we could obtain sfGFP protein at levels comparable to a control template without an amber codon (T7-$(UAG)_0$-sfGFP) when the system lacks RF1 (using the specialized PURExpress Δ*RF123* kit) (SI Figure 1a). However, the addition of RF1 reduced the signal ∼18-fold (SI Figure 1a). When RF1 was present, both naturally occurring Api1b and its synthetic analogue Api137 (Figure 1b) promoted nsAA-dependent increase of T7-$(UAG)_1$-sfGFP signal (SI Figure 1b). sfGFP yields were concentration dependent with Api137 having a significantly greater effect than the same concentrations of Api1b (SI Figure 1b). Moreover, 80 μg mL^{-1} Api137 promoted a ∼10-fold increase of the nsAA-dependent expression of a sfGFP reporter carrying two in-frame amber codons (T7-$(UAG)_2$-sfGFP) (Figure 2a). Higher concentrations of Api137 (>80 μg mL^{-1}) appeared to inhibit the translation of both the reporter and the control templates (Figure 2a, SI Figure 1a). These results are consistent with Api137 dependent depletion of ribosomes in conjunction with RF1 and RF2 at high concentrations.[27] These results also suggest that apidaecin dosage could be modulated to favor RF1 depletion in cell lysates or other in vitro translation systems from other RF1+ organisms.[33,34]

Apidaecins Preferably Inhibit RF1 in bacteria. A variety of Gram-negative bacterial species, including *E. coli* and *Agrobacterium tumefaciens*, are known to be sensitive to apidaecins.[24,30] In liquid medium, both Api1b and Api137 inhibited the total cell mass and the growth rate in a concentration dependent manner of common wild-type *E. coli* strains MG1655, BL21, DH10B, and 1917 (a standard probiotic strain), and *A. tumefaciens* C58 (SI Figure 2). As a general trend, Api137 was a significantly more potent inhibitor than Api1b. In addition, the growth of the engineered *E. coli* strains in which all UAG codons were replaced by UAAs (C321.RF1 and C321.ΔRF1, derived from MG1655,[19] where RF1 was retained as wild-type in C321.RF1 but deleted in C321.ΔRF1) was minimally inhibited even at the highest Api137 concentrations tested (2560 μg mL^{-1}, SI Figure 2). On solid media, 125 μg mL^{-1} Api137 inhibited colony formations of wild-type *E. coli* strains, MG1655, BW25113, BL21, and DH10B. However, C321.ΔRF1 was resistant to Api137 even at the highest concentration tested, 750 μg mL^{-1} (SI Figure 3). These results suggest that apidaecins preferably inhibit RF1 in *E. coli* and do not cause significant cell toxicity when RF1 function is redundant.

C321.ΔRF1 is also resistant to different *E. coli*-specific bacteriophages, such as λ phage, that require RF1 activity to accurately express lytic genes ending with the UAG codon (SI Figure 4a).[19,35] In contrast, the recoded *E. coli* that still contains RF1 (C321.RF1) is sensitive to phages (SI Figure 4b).[19,35] Upon the induction of the λ phage lytic cycle, Api137 did not affect the growth of the C321.ΔRF1 with genomically integrated λ, C321.ΔRF1 (λcl857); however, Api137 rescued the growth of otherwise isogenic C321.RF1 (λcl857) (SI Figure 4a and b). In this strain, Api137 inhibited the λ phage lytic cycle and conferred a "conditional" phage resistance in a dose-dependent manner (SI Figure 4b); a phenotype that could be exploited as a conditional biocontainment system in genetically modified organisms.[36]

Apidaecins Improve nsAA-Dependent sfGFP Expression in Different Bacteria. We next tested if we could utilize preferential RF1-inhibiting activity of apidaecins to promote nsAA incorporation in response to amber stop codon(s) in live bacteria. We focused on three previously engineered primary classes of orthogonal aaRS and their cognate $tRNA_{CUA}$ pairs. These include a *Methanocaldococcus jannaschii* (Mj) tyrosyl-RS, Mj**Bpa**RS,[37] previously evolved to charge an orthogonal $tRNA^{Tyr}_{CUA}$ with **Bpa**, a photo-cross-linker nsAA. We also utilized a variant of *Saccharomyces cerevisiae* (Sc) tryptophanyl-RS, Sc**5OHW**RS, that charges an orthogonal $tRNA^{Trp}_{CUA}$ with **5OHW**,[6,9] a serotonin precursor. We also adopted a variant of *Methanosarcina barkeri* (Mb) pyrrolysyl-RS, Mb**AbK**RS, that can charge an orthogonal $tRNA^{Pyl}_{CUA}$ with both **AbK**, a photo-cross-linker nsAA and **BocK**, a chemically protected lysine derivative that is more readily available than **AbK**.[8] Finally, to directly measure the nsAA-dependent fluorescence signal increase, we also included Mj**Cou**RS[2] evolved to charge an orthogonal $tRNA^{Tyr}_{CUA}$ with a fluorescent nsAA, **Cou** (Figure 2b). To probe site-specific nsAA incorporation in live cells of various strains, we expressed these aaRS/$tRNA_{CUA}$ pairs constitutively on one plasmid and in parallel an inducible sfGFP reporter on another and used a range of sfGFP constructs containing different numbers of in-frame UAG codons. We quantified nsAA incorporation using established bulk culture plate-reader assays and by normalizing sfGFP signal to final optical density[6] or via more sensitive and higher information single-cell microscopy.[38,39]

We first tested the effect of Api1b on nsAA incorporation in C321.ΔRF1 and C321.RF1, already shown to be resistant to apidaecins. Unsurprisingly, the presence of RF1 in C321.RF1 caused a ∼80% reduction in nsAA-dependent sfGFP signal in comparison to C321.ΔRF1 (SI Figure 4c). Exogenously added Api1b partially rescued nsAA-dependent sfGFP signal in C321.RF1 but not in C321.ΔRF1 (SI Figure 4c and d). This effect was dose-dependent and improved the reporter expression 1.4−2-fold in C321.RF1 coexpressing MbAbKRS, Sc5OHWRS, or MjBpaRS systems (SI Figure 4c and d). These results suggest that apidaecins preferably inhibit RF1 in live cells and therefore stimulate nsAA incorporation. As apidaecins also inhibit RF2[27] but are not toxic to C321.ΔRF1, these peptides may also facilitate nsAA incorporation into the UAA/UGA codons in parallel to the UAG codon in this strain.[40]

We hypothesized that by tuning the apidaecin dosage and exposure, we could promote nsAA incorporation also in sensitive bacterial strains that have not been recoded and retain their native UAG codons and RF1. When added in the late-exponential phase, Api137 promoted **AbK**−dependent sfGFP signal increase ∼2-fold, indicative of increased nsAA incorporation, in *E. coli* BL21 in a dose-dependent manner with minimal inhibitory effect on the final cell density (Figure 2c). Consistent with its higher inhibitory potential,[26] Api137 improved nsAA-dependent signal more than Api1b (Figure 2c and SI Figure 5a). These results motivated us to test the effects of apidaecins in other sensitive strains where nsAA technology has not been demonstrated. In probiotic *E. coli* Nissle 1917 with plasmids expressing inducible $(UAG)_2$-sfGFP and constitutive MjBpaRS/$tRNA^{Tyr}_{CUA}$, Api137 improved **Bpa**-

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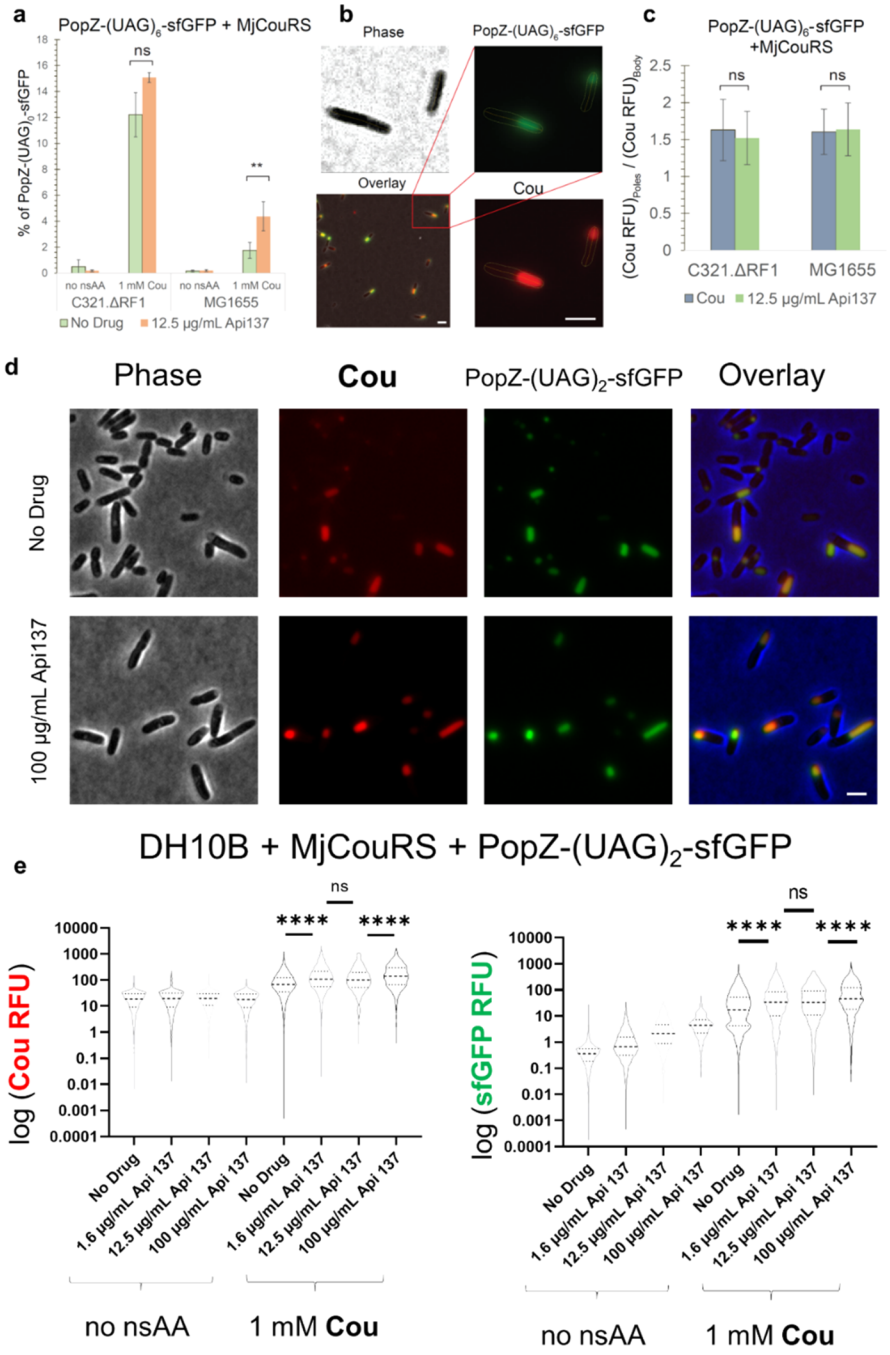

Figure 3. Apidaecins improve specific multisite incorporation of a fluorescent nsAA. (a) Exogenously added Api137 doubles multi-**Cou** incorporation to a PopZ-$(UAG)_6$-sfGFP reporter in MG1655 cells but not in C321.ΔRF1. (b) Signals from PopZ-$(UAG)_6$-sfGFP fusion (false colored in green) and the **Cou** (false colored in red) colocalized at the poles of the MG1655 *E. coli* cells imaged in phase, DAPI, and EGFP channels. (c) Exogenously added Api137 does not change the ratio of **Cou** signal at the poles to the rest of the cells in either MG1655 or C321.ΔRF1. (d) Micrographs showing subcellular signals from **Cou** (false colored in red) and PopZ-$(UAG)_2$-sfGFP fusions (false colored in green) colocalized at the poles of the DH10B *E. coli* cells imaged in phase, DAPI, and EGFP channels and overlaid on phase (false colored in blue). (e) Violin plots of single cell quantification by light microscopy showed that Api137 improved both Cou and sfGFP signals ~2-fold. The scale bars are 2 μm. ****, $P < 0.0001$; **, $P = 0.0059$.

dependent sfGFP expression dramatically (>23-fold, Figure 2d), potentially due to inherently higher activity of RF1 in this strain.

Similarly, double transformation of the Gram-negative bacterium, *A. tumefaciens*, with plasmids expressing codon optimized, inducible $(UAG)_1$-sfGFP and constitutive MjBpaRS/$tRNA^{Tyr}_{CUA}$ showed a **Bpa**-dependent increased sfGFP expression suggestive of nsAA incorporation (SI Figure 5b). Tandem mass spectrometry (MS/MS) analysis confirmed the incorporation of **Bpa** at the expected UAG codon (~63% of the total ions) on top of a high confidence evidence for glutamate incorporation (~26% of the total ions) among lower confidence evidence for tyrosine (~9% of the total ions), lysine, and methionine (SI Figure 5c and d). These results demonstrated the expansion of the nsAA technology to *A. tumefaciens*, to our knowledge for the first time. In this strain

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too, Api137 improved **Bpa**-dependent sfGFP expression in a dose-dependent manner (up to ∼3.1−fold) with moderate toxicity (Figure 2e). These results suggest that apidaecins could facilitate functional nsAA experiments, such as site-specific probing of protein−protein interactions by photo-cross-linker nsAAs in previously uncharacterized bacterial strains.

As a general trend, the presence of apidecins increased background sfGFP signal also in the absence of added nsAAs (Figure 2c and e and SI Figure 5a). This is likely a consequence of RF1 inhibition, as this phenomenon is widely reported in ΔRF1 strains.[6,41] Although the exact reasons for this background signal in ΔRF1 strains are still unclear, the contributing factors are linked to a combination of promiscuity of engineered AARSs for natural amino acids, increased near cognate suppression and codon skipping in the absence of cognate nsAAs in the medium.[6,10,41]

A New Autoinducible Plasmid System to Encode nsAAs. In RF1+ cells, nsAA-dependent protein expression efficiency is known to decay significantly if multiple nsAAs are encoded in a polypeptide.[14] We wanted to examine if apidaecins could address this problem, but to accurately observe expected low levels of protein expression, we switched to a new system that features lower background noise and higher experimental reproducibility. We first cloned our aaRS/$tRNA_{CUA}$ pairs into a pDule plasmid (p15A origin, TcR, aaRS, and tRNA genes constitutively expressed). In addition, into a pBAD plasmid, we cloned sfGFP reporters with 2, 6, or 8 UAG in-frame codons (in addition to a 0 UAG control) as a C-terminal fusion of the arabinose-inducible *Caulobacter* PopZ that forms polar, subcellular foci in *E. coli*.[42] This new plasmid system allows late-exponential autoinduction of reporters in glycerol minimal media (GMML) supplemented with glucose and arabinose.[43,44] The autoinduction in GMML increased the reproducibility of nsAA incorporation experiments and reduced the background sfGFP signal in the absence of added nsAAs, a nonspecific signal that is known to be exacerbated by the excess natural amino acids in rich media.[6] For example, BL21 cells expressing Sc5OHWRS/$tRNA^{Trp}_{CUA}$ in GMML had less "no nsAA" signal than when the same experiment was performed in rich medium (SI Figure 6a−b). In addition, apidaecins improved **5OHW**-dependent sfGFP signal only ∼1.3-fold in LB and up to ∼5-fold in GMML (SI Figure 6a and b). One possible explanation for the improved expression observed in this system is that growth in minimal media can increase uptake of peptides like apidaecins and/or of nsAAs.[6,45] Added to late-exponential cells, apidaecins improved nsAA-dependent reporter expression in both BL21 and DH10B indicative of increased nsAA incorporation: In BL21 cells with the PopZ-$(UAG)_2$-sfGFP reporter and expressing MbAbKRS, Sc5OHWRS, or MjBpaRS systems, Api137 or Api1b improved nsAA-dependent sfGFP increase 4−14-fold, depending on the AARS/nsAA used (SI Figure 6b−d). In DH10B cells this improvement varied between 2- to 16-fold (SI Figure 6e−g).

Apidaecins Improve Specific nsAA Incorporation. To directly and quantitively link the nsAA-dependent sfGFP signal increase in the presence of apidaecins to increased nsAA incorporation, we utilized the MjCouRS/$tRNA^{Tyr}_{CUA}$ system encoding the fluorescent nsAA, **Cou**. In a bulk plate reader assay with DH10B, Api137 treatment improved both **Cou** and **Cou**−dependent PopZ-$(UAG)_2$-sfGFP signals (SI Figure 7a). These results directly supported that apidaecins increase nsAA-dependent sfGFP signal because they promote nsAA incorporation. For a given aaRS/$tRNA_{CUA}$ system, C321.ΔRF1 cells represent the current limit of high nsAA incorporation efficiencies.[14,19] To estimate the extent to which apidaecins can promote multisite **Cou** incorporation, we next compared the case with C321.ΔRF1 to its RF1+ and UAG+ parent, MG1655. Similar to previous estimations,[14] in C321.ΔRF1, **Cou**-dependent PopZ-$(UAG)_6$-sfGFP expression was around 12% of PopZ-$(UAG)_0$-sfGFP and was minimally affected by Api137 (Figure 3a). Under the same conditions, in MG1655, Api137 improved the expression levels of the PopZ-$(UAG)_6$-sfGFP from ∼1.8 to ∼4.4% of the PopZ-$(UAG)_0$-sfGFP, a ∼2.4-fold increase (Figure 3a). These results suggest that apidaecins can remarkably promote multisite nsAA incorporation in unrecoded strains retaining RF1.

To test if apidaecins cause significant nonspecific signal, we devised a quantitative, single cell fluorescence microscopy approach that is sensitive enough to resolve subcellular nsAA incorporation. As opposed to diffuse localization of typical GFP constructs,[46] PopZ fusion recruits the nsAA incorporated sfGFP specifically to cell poles, as demonstrated here by the colocalization of spectrally distinct signals from fluorescent nsAA, **Cou**, and sfGFP in *E. coli* MG1655 expressing PopZ-$(UAG)_6$-sfGFP and MjCouRS/$tRNA^{Tyr}_{CUA}$ (Figure 3b). Two lines of evidence distinctly supported that Api137 did not cause significant nonspecific signal. First, Api137 did not change the **Cou** signal ratio of the "fluorescent poles" to the rest of the cell body in neither C321.ΔRF1 nor MG1655 cells expressing PopZ-$(UAG)_6$-sfGFP (Figure 3c). Because C321.ΔRF1 represents the case without any UAG containing native genes, the lack of change in this ratio also in the UAG+ parent suggests that apidaecins do not cause significant nonspecific **Cou** incorporation into genomic amber codons in wild-type *E. coli*.[3] This is consistent with previous observations about the stop codon context effect preventing nsAA incorporation into native amber stop codons.[10]

Second, single cell quantification by light microscopy in DH10B cells expressing the MjCouRS/$tRNA^{Tyr}_{CUA}$ and PopZ-$(UAG)_2$-sfGFP or PopZ-$(UAG)_6$-sfGFP, showed that Api137 treatment improved both **Cou** and sfGFP signals comparably (∼2-fold) without affecting the colocalization of **Cou** or sfGFP signals (Figure 3d, e and SI Figure 7b, c). These results suggest that apidaecins do not cause significant undesired amber suppression in a target protein, e.g. by promoting nonspecific incorporation of natural amino acids into PopZ-$(UAG)_2$-sfGFP reporter. Altogether, these results indicate that apidaecins improve multisite nsAA incorporation and cause minimal nonspecific incorporation events.

In-Cell Expression of Api1b Dramatically Improves nsAA Incorporation. Encouraged by the moderate toxicity of exogenously added apidaecins on late-exponential cells, we hypothesized that cells would tolerate autoinduction of a gene for Api1b (Table 1) under arabinose control. To test this, we cloned *api1b* into the plasmid that constitutively expresses the MbAbKRS/$tRNA^{Pyl}_{CUA}$ system. Optimization of the Api1b expression under different ribosome binding sequences (RBS) led to two such sequences, P_{araB}-RBS1-*api1b* and P_{araB}-RBS2-*api1b*, which improved **BocK** incorporation in BL21 cells up to ∼37-fold compared to no peptide condition despite a significant decrease of the final cell density (SI Figure 8a). Growth curves confirmed that the late-exponential autoinduction of these peptides mainly reduced the final cell mass (SI Figure 8b). Nevertheless, in-cell autoinduction of

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Table 1. Amino Acid Residues of the *api1b* Gene, the Apidaecin-like Peptide Library, and the Enrinched Variants Pursued Further in This Work[a]

position	*api1b*	library	*apiB5*	*apiB8*	*apiB10*	*apiC3*
1	M	fixed	M	M	M	M
2	G	A, E, G, K, R, T	**K**	**E**	**A**	**A**
3	N	fixed	N	N	N	N
4	N	fixed	N	N	N	N
5	R	A, G, R, T	**A**	**A**	T	A
6	P	fixed	P	P	P	P
7	V	A, I, T, V	**I**	**I**	V	V
8	Y	fixed	Y	Y	Y	Y
9	I	I, V	V	V	V	V
10	P	P, S	P	**S**	**S**	P
11	Q	A, E, G, K, P, Q, R, T	Q	**G**	Q	**K**
12	P	fixed	P	P	P	P
13	R	fixed	R	R	R	R
14	P	fixed	P	P	P	P
15	P	fixed	P	P	P	P
16	H	fixed	H	H	H	H
17	P	fixed	P	P	P	P
18	R	K, R	**K**	R	**K**	R
19	L	I, L	L	**I**	**I**	**I**
STOP	UAG	UAG, UAA, UGA, UGG (W)	**UAA**	**UGA**	**UAA**	**UGG**

[a]Divergence from the original Api1b sequence is highlighted. Each construct starts with an additional initiating N-terminal formyl-methionine that is not present in natural apidaecins. The library also contains a degenerate termination codon (TRR) sampling each of the three stop codons and Trp.

P_{araB}-*RBS2-api1b* in BL21 improved expression levels of the PopZ-$(UAG)_6$-sfGFP and PopZ-$(UAG)_8$-sfGFP from undetectable levels (<0.02% even with exogenously added Api137) to ∼6% and ∼2% of PopZ-$(UAG)_0$-sfGFP (Figure 4a). These results also suggest that the modest improvements by exogenously added Api1b might be due to its limited cellular uptake (SI Figure 5a). Consistently, exogenously added Api1b inhibited the growth of the *api1b* expressing cells more severely than the control cells (SI Figure 8b). The dramatic improvement of nsAA incorporation by *api1b* expression was strain-specific: in DH10B, in-cell autoinduction of *api1b* merely doubled the expression levels of the PopZ-$(UAG)_6$-sfGFP (from ∼0.05% to only ∼0.1% of PopZ-$(UAG)_0$-sfGFP, SI Figure 8c). Growth curves confirmed that in-cell autoinduction of *api1b* did not affect the growth in this strain, suggesting that DH10B is resistant to Api1b at these expression levels (SI Figure 8d).

Partial Recoding Promotes Apidaecin Tolerance and nsAA Incorporation. To improve apidaecin-dependent nsAA incorporation in other strains and reduce their toxicity, we pursued two distinct approaches: first, we explored if we could utilize RF1 inhibition by apidaecins as a selection for multiplexed recoding of essential UAG+ genes in different *E. coli* wild-type strains. *E. coli* DH10B and MG1655 are both known to have the same 13 essential genes ending with UAG (SI Table 1).[19,47] We were able to recover resistant transformants on Api137-containing selection plates by only performing 3 cycles of recombineering[48] with a mixture of 13 oligos which would recode the stop codons of these genes (UAG to UAA) (SI Figure 9a). Our attempts to delete RF1 in multiple isolates were unsuccessful, suggesting that RF1 was still essential in these strains. Nevertheless, growth curves with two selected isolates confirmed that each of the partially recoded isolates was significantly more resistant to Api137 than their parents (SI Figure 9b). Mismatch amplification mutation assay polymerase chain reaction[19] revealed that *atpE* and *coAD* for the DH10B and *atpE* and *lolA* for the MG1655 isolate were recoded. Moreover, these Api137 resistant, "partially recoded" isolates showed higher **BocK**-dependent $(UAG)_2$-sfGFP signals even in the absence of apidaecins, which was further improved in the presence of Api137 in DH10B (Figure 4b) and in MG1655 (SI Figure 9c). Using apidaecins as selection for rapid recoding can be broadly applicable to strains that are apidaecin sensitive, such as *A. tumefaciens*.

In-Cell Expression of New Apidaecin Variants Improves nsAA Incorporation. Second, in search of more potent novel apidaecin isoforms, we designed a focused peptide library based on natural and synthetic apidaecin-type, proline-rich antibacterial peptide sequences. This library covered isoforms like Api2, Api3, and other related antibacterial peptides first isolated from bumblebees (Bb+A), cicada killer wasps (CkA), or bald-faced hornets (Ho+).[25,26,49] In addition to the termination codon, we varied 8 positions with residues of known diversity, resulting in a library of ∼5.5 × 10^4 theoretical diversity (Table 1). We assembled this library (with at least 10× coverage) using the plasmid expressing P_{araB}-*RBS1-api1b* and the MbAbKRS/$tRNA^{Pyl}_{CUA}$ system as the template. We validated the diversity and quality of this library by randomly sequencing 50 library variants. Next, we grew the cells expressing the pooled library in GMML autoinduction media in the presence of **BocK** and sorted DH10B cells for $(UAG)_2$-sfGFP signal via fluorescence-activated cell sorting (FACS). We plated the ∼0.0005% (stringent sort) and ∼0.02% (loose sort) of most fluorescent cells on selective plates. Variants sequenced from these plates did not converge into common sequences (SI Figure 10a, b). Purification and retransformation of the 31 most enriched variants were further characterized in subsequent plate-reader assays. Of these, none of the 12 variants from the loose sort increased BocK+/no nsAA signal. However, 20 out of the 21 variants from the stringent sort significantly increased BocK+/no nsAA signal with varying levels of final cell densities (SI Figure 10c, d). Based on their reduced toxicity, but increased BocK+ signals, 4 optimal variants, *apiB5*, *apiB8*, apiB10, and *apiC3* were selected and further pursued (Table 1). Despite their sequence diversity (SI Figure 10e, f) each of these variants dramatically improved **BocK** incorporation in DH10B cells compared to *api1b* (SI Figure 8c and Figure 4c, d). In-cell autoinduction of these novel peptides improved expression levels of the PopZ-$(UAG)_2$-sfGFP and PopZ-$(UAG)_6$-sfGFP from ∼0.9 and ∼0.1% up to ∼38.9 and ∼9.1% of PopZ-$(UAG)_0$-sfGFP (Figure 4c, d). This represented a ∼43−91-fold increase of the BocK+ signal without the peptide and an increase up to ∼645-fold of the true nsAA signal over the background incorporation, i.e. no nsAA condition (Figure 4c, d). Growth curves suggested that the late-exponential autoinduction of these peptides did not slow the apparent growth rate but rather significantly reduced the final cell mass (SI Figure 10g). Given the conservation of RF1 across bacteria, this approach may be utilized to raise new apidaecin-like peptides in bacterial species that are naturally resistant to the natural apidaecins.

The transient and tunable inhibition of RF1 activity by exploiting apidaecin-like peptides could be used to improve

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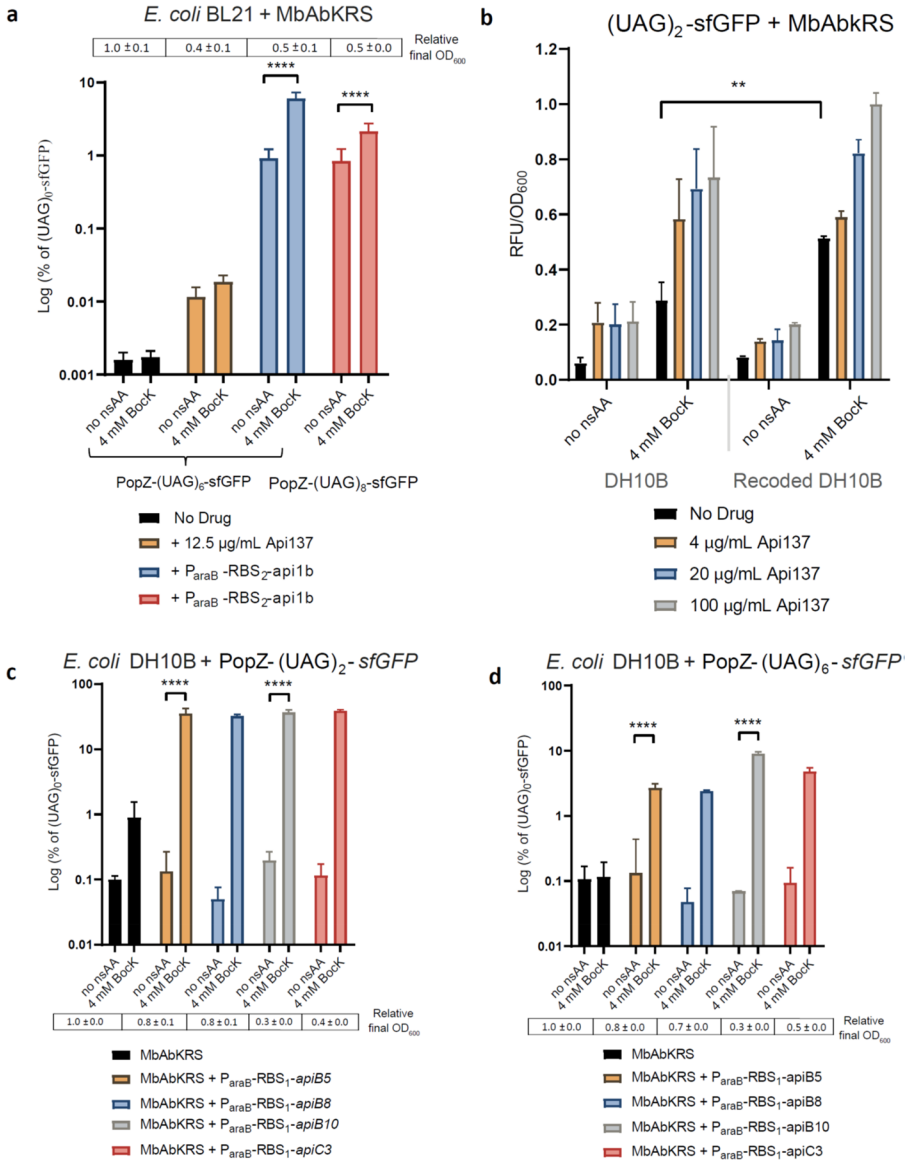

Figure 4. In-cell autoinduction of apidaecin-like antimicrobial peptides improves nsAA incorporation in different *E. coli* strains. (a) In-cell autoinduction of an *api1b* gene in BL21 dramatically improved multi-**BocK** incorporation to 6- and 8-UAG containing reporters despite significantly reducing the final cell density. (b) Recombineering with exogenously added apidaecins selected "partially recoded" and Api137 resistant DH10B cells that incorporated **BocK** more efficiently than the wild-type, even absent Api137. (c, d) In-cell autoinduction of enriched apidaecin-like variants in DH10B dramatically improved multi-**BocK** incorporation to (c) 2-UAG or (d) 6-UAG containing reporters while being nontoxic. ****, $P < 0.0001$; **, $P = 0.004$.

nsAA-based genetic code expansion approaches in various biotechnological applications and recoding projects in a variety of wild-type bacterial species. This class of peptides is a new tool to boost incorporation efficiencies of any nsAA in RF1+ contexts, including both cell-free translation systems and nonrecoded bacteria species. For example, by promoting multisite incorporation of small fluorescent nsAAs, apidaecins can rapidly amplify the brightness of a target protein and may find use in sensitive applications where large fluorescent protein fusions cannot be used.[50] Further characterization of new apidaecin-like peptides is ongoing.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.0c00055.

Additional methods for in vitro protein translation assays, lambda phage lysis assays, cloning and strain engineering, nsAA incorporation assays, MAGE recoding, and library generation by flow cytometry and cell sorting; nsAA incorporation in a cell-free translation system (SI Figure 1); apidaecins toxicity in RF1-dependent Gram-negative bacteria (SI Figure 2); inhibition of RF1-dependent *E. coli* strains by apidaecins (SI Figure 3); conditional phage resistance and improved nsAA incorporation by apidaecins in *E. coli* (SI Figure 4); improved nsAA incorporation in different bacteria by exogenously added apidaecins (SI Figure 5); reporter system comparison in *E. coli* (SI Figure 6); improved multisite nsAA incorporation by apidaecins (SI Figure 7); comparison of three ribosome binding sequences on cell growth (SI Figure 8); partial recoding

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and RF1 inhibition by apidaecins (SI Figure 9); evolution of new apidaecin-like peptides (SI Figure 10); sequences of key oligos (SI Table 1); sequences of new constructs (SI Table 2) (PDF)

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Author Contributions

⊥R.-M.M. and K.No. contributed equally. E.K., K.No., and R.M. were involved in plasmid and strain construction. E.K. performed nonstandard amino acid incorporation, microscopy and growth experiments with help from K.No., R.M, D.A.S. and K.Na. D.W. J.R. and E. K. purified proteins necessary for *in vitro* experiments and provided synthetic chemistry support. E.K., D.A.S. and G.M.C. wrote the manuscript with feedback from all other authors.

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Notes

The authors declare the following competing financial interest(s): G.M.C. has related financial interests in 64-x, EnEvolv, and GRO Biosciences. For a complete list of G.M.C.s financial interests, please visit arep.med.harvard.edu/gmc/tech.html.

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