Collateral Toxicity Limits the Evolution of Bacterial Release Factor 2 toward Total Omnipotence

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Abstract

When new genes evolve through modification of existing genes, there are often tradeoffs between the new and original functions, making gene duplication and amplification necessary to buffer deleterious effects on the original function. We have used experimental evolution of a bacterial strain lacking peptide release factor 1 (RF1) in order to study how peptide release factor 2 (RF2) evolves to compensate the loss of RF1. As expected, amplification of the RF2-encoding gene *prfB* to high copy number was a rapid initial response, followed by the appearance of mutations in RF2 and other components of the translation machinery. Characterization of the evolved RF2 variants by their effects on bacterial growth rate, reporter gene expression, and in vitro translation termination reveals a complex picture of reduced discrimination between the cognate and near-cognate stop codons and highlights a functional tradeoff that we term "collateral toxicity." We suggest that this type of tradeoff may be a more serious obstacle in new gene evolution than the more commonly discussed evolutionary tradeoffs between "old" and "new" functions of a gene, as it cannot be overcome by gene copy number changes. Further, we suggest a model for how RF2 autoregulation responds to alterations in the demand not only for RF2 activity but also for RF1 activity.

Key words: **experimental evolution, peptide release factor, tradeoff, new gene evolution.**

Article

Introduction

The appearance of new functions is a very fundamental biological phenomenon. New genes can evolve by modification of existing genes through mutations that introduce new functions or enhance existing promiscuous activities. Usually, such mutations have negative effects on the original function of the gene. This causes a tradeoff between the new function and the original function of a gene, which controls if and how the gene will evolve (Tokuriki et al. 2008, 2012).

Gene copy number variation enables two ways of escaping from the evolutionary tradeoff between two conflicting selection pressures (Francino 2005; Bergthorsson et al. 2007; Deng et al. 2010; Näsvall et al. 2012): 1) The extra gene copies can allow for higher expression, which provides more of both the original and the evolving new function, and 2) it allows the original function to be maintained in one gene copy whereas "redundant" gene copies are free to evolve. In order to study the process of new gene evolution, we generated a *Salmonella enterica* mutant lacking peptide release factor 1 (RF1), with the aim to follow how peptide release factor 2 (RF2) evolves to complement the loss of RF1.

Translation of a growing polypeptide chain on the ribosome is terminated by class 1 peptide release factors, which recognize stop codons in the ribosomal A-site and trigger hydrolysis of the peptidyl-tRNA, thereby releasing the nascent peptide chain. Eukaryotes and archaea have only one class 1 release factor (eRF1 and aRF1, respectively) that recognize all three stop codons, whereas most bacteria have two release factors (RF1 and RF2) that recognize two stop codons each. RF1 (encoded by the *prfA* gene) recognizes UAA and UAG, and RF2 (encoded by the *prfB* gene) recognizes UAA and UGA.

The two bacterial class 1 release factors, possessing high resemblance in sequence and structure (Petry et al. 2005), appear to have originated from a common ancestral release factor, whereas the archaeal/eukaryotic counterparts (aRF1 and eRF1, respectively) most likely have a distinct origin. The aRF1/eRF1 uses a different mechanism for stop codon recognition and shares very little similarity with RF1 and RF2 (Vestergaard et al. 2001; Brown et al. 2015).

Another difference between the bacterial and eukaryotic termination is that the eRF1 is brought to the ribosome as a ternary complex with a Guanosine triphosphate (GTP) bound class 2 release factor (eRF3). Upon stop codon recognition by eRF1, eRF3 hydrolyzes GTP and dissociates, allowing eRF1 to accommodate into the A-site and trigger peptidyl-tRNA hydrolysis. The eRF3 thus uses a kinetic proofreading

mechanism analogous to EF-Tu in aminoacyl-tRNA selection during translation elongation (Salas-Marco and Bedwell 2004).

In contrast, bacterial RF1 and RF2 enter the ribosome by themselves, and termination is not subject to any proofreading, thereby relying on a high specificity of stop codon recognition by the two. Additionally, many bacteria have a class 2 release factor, RF3 (not related to eRF3), that accelerates dissociation of the class 1 factors after peptidyl-tRNA hydrolysis (Freistroffer et al. 1997).

It was previously assumed based on experiments on *Escherichia coli* K12 that both RF1 and RF2 are essential (Gerdes et al. 2003). However, more recent studies have reported that RF1 is not essential for *E. coli*, provided there is a fully functional RF2 (Johnson et al. 2012) (*E. coli* K12 strains have a defective RF2[A246T] [Uno et al. 1996]). Furthermore, replacement of the UAG stop codon in six of the seven UAG-ending essential genes makes it possible to delete the *E. coli* K12 gene encoding RF1 (*prfA*), even in the presence of the defective RF2 (Mukai et al. 2010).

The first organism with a recoded genome (C321.ΔA [Lajoie et al. 2013]) was produced by replacing all 321 UAG stop codons in the *E. coli* K12 genome by UAA stop codons, followed by the removal of the *prfA* (RF1) gene. This organism had reduced fitness, which was hypothetically caused by a combination of an increased demand for RF2 activity and off-target mutations that were accidentally introduced during the extensive engineering of its genome in the absence of functional methyl-directed mismatch repair.

Later, Church and coworkers have allowed C321.ΔA to evolve to compensate for these detrimental fitness effects (Wannier et al. 2018). They found that backmutations that corrected the preexisting mutation in *E. coli* K12 RF2[A246T] provided the single largest fitness improvement. Furthermore, modest fitness gains were witnessed with a mutation that improved termination activity on UAA (RF2 [E170K]). Beside these mutations in RF2, mutations in RF3, that potentially accelerated the release of RF2 from ribosomes after termination, were also found.

In order to study the potential of RF2 to evolve for replacing RF1, and the potential evolutionary constraints that may have prevented bacteria from evolving a single omnipotent class 1 release factor, we have generated *S. enterica* strains lacking RF1 (Δ*prfA*) but with all UAG stop codons intact.

The *prfA* gene could only be deleted in cells containing a mutation (E167K) or a preexisting duplication of the *prfB* gene, showing that RF1 is essential in *S. enterica* unless RF2 is overexpressed or contains the E167K mutation known to cause termination on UAG (Ito et al. 1998).

Strains with a duplication of *prfB* and a deletion of *prfA* are viable but have extremely reduced growth rates, which are quickly compensated by amplification of the *prfB* (RF2) gene to high copy number during the course of evolution. We have allowed multiple lineages of Δ*prfA* to evolve for up to 1,000 generations to compensate for the loss of RF1 and found that adaptive evolution of *S. enterica* Δ*prfA* largely mirrors that of *E. coli* C321.ΔA by mutations in RF2 and RF3.

Some of the mutations we identify are in identical positions as the ones found by the Church group (Wannier et al. 2018) and others (Ejby et al. 2007; Johnson et al. 2011; O'Connor and Gregory 2011) to compensate for RF1 deficiency and defective RF2. Our data suggest that the RF2 mutations allow RF2 to functionally replace RF1 by improving termination on UAG by effects on two levels of release factor function: 1) by increasing the rate of peptide release on the near-cognate codons and/or 2) by increasing the occupancy of RF2 in the release complex (referred hereafter as RC).

In addition, we found that the RF2 mutations that show improved termination efficiency on UAG stop codons also demonstrate a concomitant and detrimental increase in termination on UGG sense codons (coding for tryptophan). These results uncover the existence of an evolutionary trade-off between efficient and accurate stop codon recognition and faithful avoidance of premature termination on sense codons.

Results

Mutation or Overproduction of RF2 by Gene Amplification Enables Loss of RF1 in *S. enterica*

We attempted to replace *prfA* in two different backgrounds: a strain with the wild-type *prfB* and a strain carrying the E167K allele of *prfB*. Our attempts to delete RF1 in the wild-type RF2 (RF2[wt]) background failed, whereas in the RF2[E167K] background we obtained slow-growing Δ*prfA* transformants capable of growth on minimal medium but not on rich medium. These results indicated that RF2[E167K] is capable of partially substituting for RF1, whereas the wild-type is not.

In order to determine if overexpression of the wild-type *prfB* by gene amplification would enable loss of RF1, we constructed strains with a duplication of the *prfB* region held by an ampicillin resistance cassette and further increased the copy number of the duplicated area by selecting in a high concentration of ampicillin before attempting to delete *prfA*. In a strain with a single copy of the wild-type *prfB*, no colonies appeared despite up to 64 days of incubation. In the presence of multiple (two or more) copies of *prfB*, even the RF2[wt] was enough to support viability in the absence of RF1 (table 1). Additionally, preexisting higher-order gene amplifications in the recipient culture resulted in faster-growing transductants.

Thus, RF1 is essential unless RF2 is already overexpressed (by gene duplication or amplification), or if it contains the E167K mutation. In addition to being very slow growing, the resulting mutants were difficult to maintain stably in rich medium, which is consistent with our inability to generate *prfA* deletion mutants by selection on rich medium.

Compensatory Evolution of an *S. enterica* Strain Lacking RF1

Previously, Church and coworkers have evolved *E. coli* strains lacking RF1 but where every occurrence of UAG stop codons had been replaced by UAA stop codons (Lajoie et al. 2013). They found that mutations in RF2 and RF3 that reverted or compensated for the RF2 mutation (A246T) present in *E. coli*

Table 1. *prfB* (RF2) Mutation or Gene Duplication/Amplification Enables Deletion of *prfA* (RF1).

Culture[a]	[amp, mg l^{-1}][b]	*prfB* Copy Number[c]	# Recombinants	Time (days)[d]
***prfB*[wt]**	**0**	**1×**	**0**	**64**
***prfB*[wt]**	**0**	**1×**	**0**	**64**
***prfB*[wt]**	**0**	**1×**	**0**	**64**
dup(*prfB*[wt])	**0**	**2×**	**>323[e]**	**11**
dup(*prfB*[wt])	**0**	**2×**	**>473[e]**	**11**
dup(*prfB*[wt])	**0**	**2×**	**>248[e]**	**11**
dup(*prfB*[wt])	**500**	**13.2× (85%)**	**706**	**5**
dup(*prfB*[wt])	**500**	**13× (52%)**	**510**	**5**
dup(*prfB*[wt])	**500**	**14.3× (25%)**	**284**	**5**
***prfB*[E167K]**	**0**	**1×**	**725**	**3**
***prfB*[E167K]**	**0**	**1×**	**789**	**3**
***prfB*[E167K]**	**0**	**1×**	**723**	**3**
dup(*prfB*[E167K])	**0**	**2×**	**507**	**3**
dup(*prfB*[E167K])	**0**	**2×**	**561**	**3**
dup(*prfB*[E167K])	**0**	**2×**	**576**	**3**
dup(*prfB*[E167K])	**500**	**12.6× (85%)**	**583**	**2**
dup(*prfB*[E167K])	**500**	**12.9× (35%)**	**496**	**2**
dup(*prfB*[E167K])	**500**	**17× (18%)**	**362**	**2**

[a]All strains contained a deletion of the native *hisA* gene and a *syfp2* gene near *prfB*. Each strain was grown as independent triplicate cultures and was tranduced with the same amount of the same transducing P22 lysate containing the *prfAΔ903::j1his* allele as described in Materials and Methods.
[b]The duplication was constructed with a bla^{TEM-1} cassette (conferring ampicillin resistance) at the duplication junction. Ampicillin (500 mg l^{-1}) was added to preselect a population containing a higher copy number of the duplicated area.
[c]An *syfp2* gene (contained within the duplicated area) was used to estimate the copy number of *prfB* in the recipient cultures prior to the transductions. In the ampicillin selected cultures, two subpopulations expressing either 2× YFP or the indicated amount were evident. The numbers within parentheses indicate the fraction of each population that had >2 copies.
[d]Colonies were counted after the indicated number of days, when most colonies were larger than ~1 mm in diameter. The transductions with the wild-type recipient cultures were incubated for 64 days without the appearance of any colonies.
[e]A few (4–40) colonies started to become visible after ~6 days. Colonies ranging in size from barely visible to >1 mm in diameter were counted on day 11. As some colonies were probably still not visible, the numbers probably underestimate the true number of transductants.

K12 and for the increased demand for RF2 activity for termination on UAA codons when RF1 is missing.

We wanted to see if, and by which routes, loss of RF1 could be compensated when all natural UAG stop codons were still present in the genome. In addition to the increased demand for RF2 activity, selection in this situation would favor RF2 mutants capable of termination on UAG or perhaps mutations that change the stop codon in particularly problematic UAG-ending genes.

In order to select mutations that compensated for the loss of RF1, we evolved strains containing a deletion of the *prfA* gene and a duplication of either the wild-type or E167K mutant *prfB* gene, by passaging 16 cultures each for 500 generations (RF2[wt] ancestor) or 1,000 generations (RF2[E167K] ancestor).

The populations evolved from the RF2[wt] ancestor remained difficult to maintain in complex medium even after 500 generations and were thus evolved only in minimal M9 + glucose medium. The populations evolving from the RF2[E167K] ancestor were able to grow in complex medium after the first passage in minimal medium and were thus allowed to continue evolving in both media.

Whole-genome sequencing (WGS) of the 32 ancestral clones revealed that although most of the E167K lineages had only two copies at the start of the evolution experiment, all the RF2[wt] lineages already had higher-order gene amplification with between 17 and 44 copies (table 2). As the increase in copy number from 2 to 44 happened during our very minimal posttransduction cleanup procedure (see Materials and Methods), there must have been a large selective advantage from having multiple copies of *prfB*.

Fitness changes were estimated by measuring the growth rates of the ancestral and the evolved populations (fig. 1). The ancestral lineages had very low growth rates in M9 minimal medium, between 20% and 50% of the wild-type growth rate for the RF2[wt] lineages and 50% and 65% for the RF2[E167K] lineages (fig. 1*a* and *b*). None of the ancestral lineages showed any growth in rich lysogeny broth (LB) medium. The evolved lineages had greatly improved growth rates compared with their ancestors, with an average 2.5-fold (RF2[wt] lineages, fig. 1*a*) and 1.5-fold (RF2[E167K] lineages, fig. 1*b*) improvement in M9 minimal medium. The RF2[E167K] lineages evolved in both media reached closer to wild-type growth rates (fig. 1*b* and *c*) compared with the evolved RF2[wt] lineages (fig. 1*a*), reflecting the higher fitness of their starting point and the longer time they were allowed to evolve.

WGS of the evolved end-point populations revealed copy number changes and mutations affecting RF2 and RF3 and recurring mutations affecting other components of the translation machinery (table 2 and supplementary table S1, Supplementary Material online). Interestingly, mutations in RF2 or RF3 were almost mutually exclusive, and only one lineage acquired mutations in both factors. The lineages starting from the E167K ancestor only rarely acquired additional RF2 mutations and no RF3 mutations were found, indicating the E167K mutation in combination with *prfB* gene amplification compensates relatively well for loss of RF1 (table 2). As our focus for this study was new gene evolution, we chose to

Table 2. Evolution of RF2 and RF3 after Loss of RF1.

Population[a]	0 Gen.[b] Copy Number[c]	1,000 Gen.[b] Copy Number[c]	RF2 Mutations[d]
RF2[E167K]_M9_L1	2.0	10.9	E167K -
RF2[E167K]_M9_L2	2.1	14.7	E167K -
RF2[E167K]_M9_L3	2.0	9.6	E167K -
RF2[E167K]_M9_L4	1.9	13.8	E167K -
RF2[E167K]_M9_L5	1.9	13.1	E167K E170K[e] D329G[e]
RF2[E167K]_M9_L6	1.9	13.0	E167K -
RF2[E167K]_M9_L7	1.9	9.8	E167K -
RF2[E167K]_M9_L8	2.2	15.3	E167K -
RF2[E167K]_M9_L9	1.9	13.2	E167K -
RF2[E167K]_M9_L10	2.0	12.2	E167K -
RF2[E167K]_M9_L11	2.0	11.7	E167K -
RF2[E167K]_M9_L12	1.9	13.8	E167K -
RF2[E167K]_M9_L13	4.8	11.4	E167K -
RF2[E167K]_M9_L14	1.9	13.6	E167K -
RF2[E167K]_M9_L15	1.9	8.2	E167K -
RF2[E167K]_M9_L16	1.8	10.5	E167K -
RF2[E167K]_LB_L1	2.0	7.6	E167K -
RF2[E167K]_LB_L2	2.1	8.9	E167K -
RF2[E167K]_LB_L3	2.0	2.7	E167K D329A
RF2[E167K]_LB_L4	1.9	2.5	E167K D329G G23Fs (38%), E167K D329G G23Fs K177R (19%), E167K E170K (6%), E167K E172G (21%), E167K (16%)[f]
RF2[E167K]_LB_L5	1.9	4.5	E167K D329G
RF2[E167K]_LB_L6	1.9	8.1	E167K -
RF2[E167K]_LB_L7	1.9	4.1	E167K -
RF2[E167K]_LB_L8	2.2	4.2	E167K -
RF2[E167K]_LB_L9	1.9	9.8	E167K -
RF2[E167K]_LB_L10	2.0	7.3	E167K -
RF2[E167K]_LB_L11	2.0	13.4	E167K -
RF2[E167K]_LB_L12	1.9	6.7	E167K D329A, E167K D329G[g]
RF2[E167K]_LB_L13	4.8	8.3	E167K D329A, E167K D329G[g]
RF2[E167K]_LB_L14	1.9	9.8	E167K -
RF2[E167K]_LB_L15	1.9	13.4	E167K -
RF2[E167K]_LB_L16	1.8	11.6	E167K -
RF2[wt]_M9_L1	22.1	4.8	A293E
RF2[wt]_M9_L2	44.1	6.8	E172K K294N
RF2[wt]_M9_L3	23.0	29.0	—
RF2[wt]_M9_L4	19.5	6.5	E172K
RF2[wt]_M9_L5	27.8	16.6	—
RF2[wt]_M9_L6	29.1	11.6	E167K
RF2[wt]_M9_L7	27.4	22.1	—
RF2[wt]_M9_L8	34.6	9.5	—
RF2[wt]_M9_L9	32.4	11.8	E172K
RF2[wt]_M9_L10	25.4	29.1	—
RF2[wt]_M9_L11	31.4	8.3	D26fs("FsFix") D131Y
RF2[wt]_M9_L12	16.9	12.6	E167K E172K
RF2[wt]_M9_L13	22.4	11.8	E172K
RF2[wt]_M9_L14	26.4	6.0	E172K
RF2[wt]_M9_L15	32.4	11.2	A293E
RF2[wt]_M9_L16	25.2	15.6	—

[a]The populations are listed according to their ancestral RF2 variant (E167K or wt) and which medium they were evolved in (M9 or LB), followed by a number (1–16).
[b]Samples for WGS were withdrawn from the populations after 0, 500, or 1,000 generations in the indicated medium (M9+glucose or LB). The "0" generation cultures were grown from a single colony in M9 + glucose and were used to inoculate the first cycle of the respective evolution experiments. The E167K_LB lineages were started by subculturing from the corresponding E167K_M9 lineages after the first passage (ten generations).
[c]The copy number of *prfB* was estimated from WGS data as the average read depth in a 7,005-bp region within the duplicated region relative to the average read depth in a 7005-bp region 10 kb away from the duplicated region (set to 1.0).
[d]See supplementary table S1, Supplementary Material online, for a comprehensive summary of all detected mutations.
[e]These mutations were present at low frequency (2–2.5%).
[f]The most likely alleles were deduced from the percentage of the reads supporting each mutation and by searching for reads that contained more than one mutation.
[g]These populations shared several identical mutations in *prfB* and other genes, likely due to cross-contamination.

characterize only the evolved RF2 variants. For a brief discussion of potential mechanisms for other compensating mutations, see supplementary Discussion, Supplementary Material online.

Reconstructing Strains with the Evolved RF2 Variants

For characterizing the evolved RF2 variants, we generated strains where the evolved *prfB* alleles or the corresponding single amino acid substitutions were put under the control of

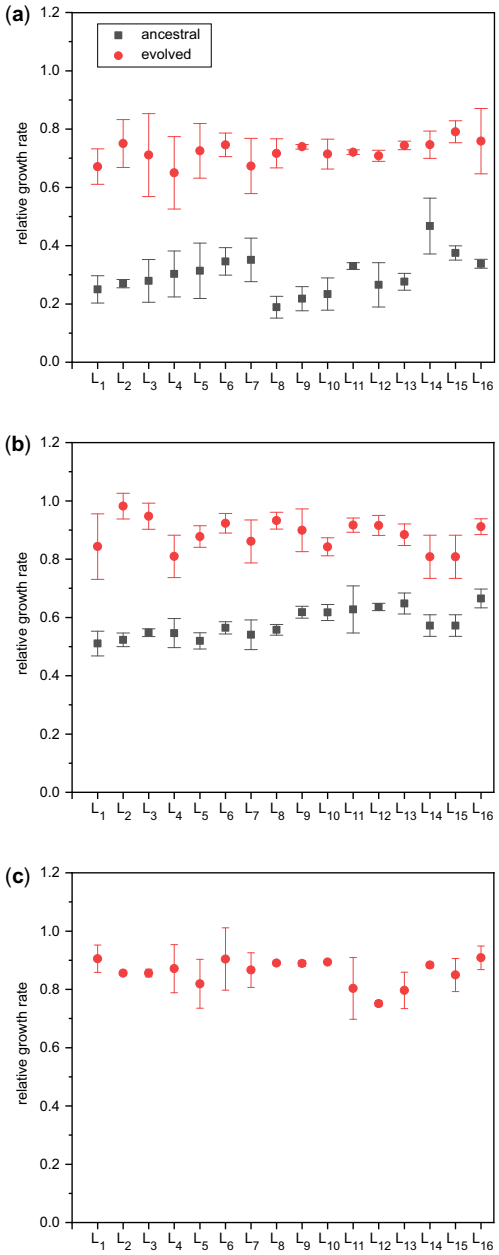

FIG. 1. Growth rate recovery of evolved Δ*prfA* strains. (*a*) Growth rates of the 16 lineages evolved from the RF2[wt] ancestor in M9 + glucose. Black, samples taken from cultures frozen after the first cycle (~10 generations); red, samples taken from cultures frozen after cycle 50 (~500 generations). (*b*) Growth rates of the 16 lineages evolved from the RF2[E167K] ancestor in M9 + glucose. Black, samples taken from cultures frozen after the first cycle (~10 generations); red, samples taken from cultures frozen after cycle 100 (~1000 generations). The error bars show the standard deviations of three replicates. (*c*) Growth rates of the 16 lineages evolved from the RF2[E167K] ancestor in LB. The samples were taken from cultures frozen after cycle 100 (~1000 generations). The ancestral lineages could not grow in this medium. All growth rates are expressed relative to a wild-type *Salmonella enterica* strain (set to 1.0) grown at the same time, and the error bars show standard deviations of at least three replicates.

the L-arabinose inducible P_{araBAD} promoter (replacing the rest of the *araBAD* operon) (supplementary fig. S1, Supplementary Material online). The wild-type *prfB* gene is autoregulated by RF2 through a mechanism that involves a competition between translation termination and a programmed +1 frameshift at an internal UGA stop codon at the 25th position (supplementary fig. S2, Supplementary Material online).

To be able to test the activities of RF2 at different expression levels, we constructed RF2 variants with a 1-nt deletion of the U in the internal UGA stop codon. Variants containing this mutation translate the entire RF2 from the same reading frame and are referred in text and figures as "FsFix" (supplementary fig. S2, Supplementary Material online). Some mutations that only appeared in double mutants in the evolved lineages (E170K, K177R, K294N, D329A, and D329G) were

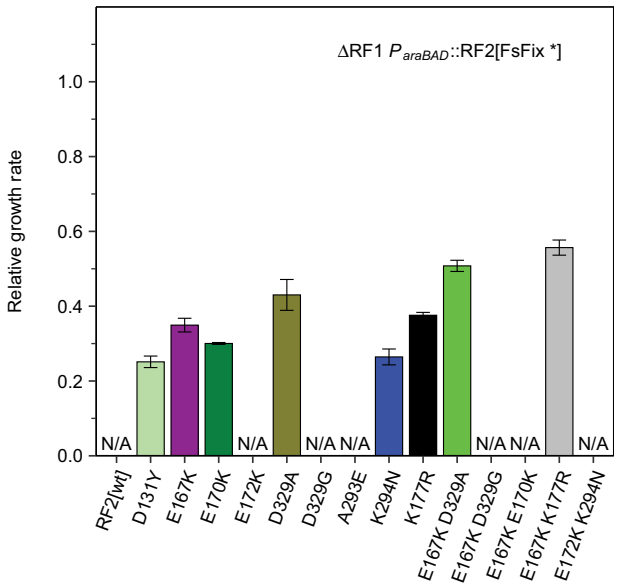

Fig. 2. Evolved RF2 variants compensate for the loss of RF1. Strains expressing the indicated RF2 variants from the chromosomal P_{araBAD} promoter were deleted for *prfA* (RF1), and the growth rates of viable recombinants in M9 + glycerol supplemented with 0.05% L-arabinose were determined. All growth rates are expressed relative to a wild-type *Salmonella enterica* strain (set to 1.0) grown at the same time, and the error bars show standard deviations of at least three replicates. N/A, not available (the corresponding strains could not be constructed).

reconstructed as single mutants in order to test their effects in isolation. Additionally, in order to examine the biochemical basis for the altered termination properties of a subset of evolved RF2 variants, we generated the corresponding mutations in *E. coli* RF2 and measured termination kinetics in sensitive assays based on a highly optimized *E. coli* in vitro translation system (Bouakaz et al. 2006).

When constructing strains carrying the "FsFix" variants of *prfB* under the control of the P_{araBAD} promoter (supplementary fig. S1, Supplementary Material online), we noticed that expression of all the evolved RF2 variants in the presence of the wild-type RF1 and RF2 caused considerable toxicity, causing a decrease in growth rate (supplementary fig. S3, Supplementary Material online).

The Evolved RF2 Variants Compensate the Loss of RF1 by Termination on UAG

To test how well the evolved RF2 variants could replace RF1, we attempted deletion of the *prfA* gene in strains expressing evolved RF2 "FsFix" variants from the P_{araBAD} promoter. In some of the strains (including the strain expressing RF2[wt]), we were unable to delete the *prfA* gene, despite several attempts. For RF2[wt], the reason might be that the expression level was not good enough for terminating on UAG stop codons, whereas for others the toxicity of expression may have prevented the isolation of viable recombinants.

For the variants where we were able to delete the *prfA* gene, we measured the relative growth rates (fig. 2) and developed a reporter gene assay where the *dTomato* (red fluorescent protein [RFP]) gene was terminated with a single UAG and no other stop codons before the terminator (*dTomato*-UAG-nonstop, supplementary fig. S4*c*, Supplementary Material online). Poor termination at UAG causes readthrough of the *dTomato*-UAG-nonstop reporter and ribosomes reaching the end of the mRNA, leading to tmRNA-dependent trans-translation which ultimately leads to the destruction of both the mRNA and the protein (Keiler et al. 1996; Venkataraman et al. 2014). Improved termination at UAG codons was thus expected to increase the expression of *dTomato*-UAG-nonstop (supplementary fig. S4*c*, Supplementary Material online) by stabilizing both the mRNA and the protein. As determined by this in vivo reporter assay, all of the reconstructed variants of RF2 terminated on UAG stop codons and, as expected, the double mutants showed significantly higher degree of UAG termination (fig. 3*a*).

As the reporter gene assay was not expected to be quantitative, we complemented it with an in vitro stopped-flow-based fluorescent tripeptide release assay using the evolved RF2 variants with UAG-programmed RCs (fig. 3*b* and supplementary fig. S5, Supplementary Material online). As expected, RF2[wt] released the tripeptide on UAG with a very low maximal rate ($\sim$0.1 s^{-1}). Compared with this almost all evolved RF2 variants showed moderate to significant increase in the rate of peptide release on UAG (fig. 3*b*). The most dramatic increase was seen with the double mutants E167K E170K (about 70-fold) and E167K K177R (about 40-fold).

The rates of peptide release by these RF2 variants on near-cognate UAG are comparable with the activity of RF1 on its cognate UAG codon. The single mutants E167K, E172K, and K177R and the double mutant E167K D329A also showed 30- to 40-fold increase. The single mutants E170K and D329A and the double mutant E167K D329G showed relatively lower but still higher peptide release on UAG. Thus, the results from the in vitro biochemical assay match well with the in vivo reporter gene assays with UAG as a stop codon. These assays confirm that RF2 evolves by increasing its low inherent rate of peptide release on UAG codons in order to compensate for the loss of RF1.

Weak Tradeoff between the Termination on Cognate and Near-Cognate Stop Codons

As evolution of a new function often comes at the cost of loss of the original function of an enzyme (Tokuriki et al. 2008, 2012), the gain in termination on the near-cognate UAG was expected to cause a decrease in termination efficiency on the cognate UAA and UGA codons.

To test this, we measured the rate of peptide release on UAA- and UGA-programmed release complexes by single-turnover tripeptide release assay (fig. 4 and supplementary fig. S5, Supplementary Material online). All the tested variants were functional to release tripeptide on these cognate codons. On UAA, a general loss of 20% activity was noticed in all the evolved RF2s with maximal 2-fold drop with D329A, K177R, and E167K K177R (fig. 4*a*). On UGA, most of the RF2

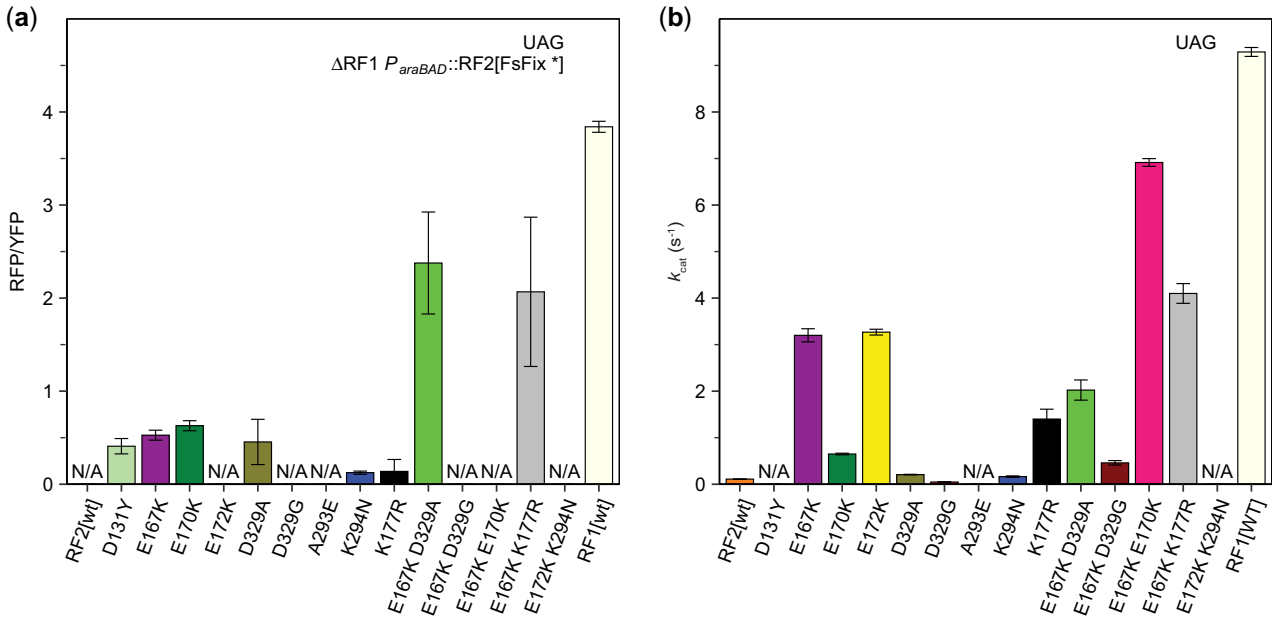

Fig. 3. Evolved RF2 replaces RF1 for UAG termination. (*a*) In vivo termination on UAG. The strains expressed RF2 variants as indicated from the chromosomal P_{araBAD} promoter (supplementary fig. S1, Supplementary Material online) were deleted for *prfA* (RF1) and contained *syfp2* (encoding a yellow fluorescent protein; YFP) (supplementary fig. S4*a* and *b*, Supplementary Material online) and dTomato-UAG-nonstop, which translates to a RFP only when translation is terminated on UAG at the end of the coding sequence (supplementary fig. S4*c*, Supplementary Material online). The data are reported as RFP/YFP ratios with standard deviations. The signal and standard deviation from a wild-type strain (expressing RF1 and RF2 from their native loci) are shown as horizontal dotted lines. (b) In vitro peptide release rates (k_{cat}, s^{-1}) on UAG-programmed release complexes. The error bars show standard errors. N/A, not available.

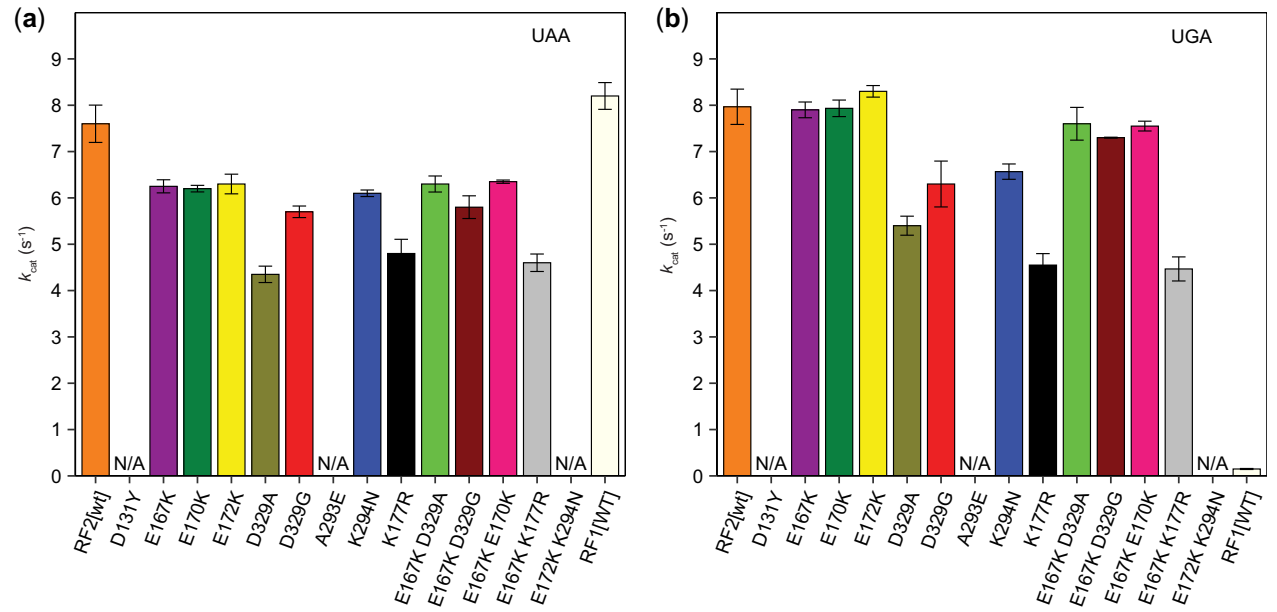

Fig. 4. Evolved RF2s are functional for its native function (termination on UAA and UGA). In vitro peptide release rates on (*a*) UAA-programmed and (*b*) UGA-programmed release complexes. The rates are expressed as k_{cat} (s^{-1}) with standard errors. N/A, not available.

variants showed wild-type like activity in tripeptide release, whereas the three variants mentioned above were defective by merely 2-fold (fig. 4*b*). Thus, even though some of the variants had lower rates of termination on cognate codons, this does not appear to have been a major limiting factor in our evolution experiment.

Some Evolved RF2 Variants Terminate Efficiently on UGG Codons

All of the evolved variants came with significant fitness costs when expressed without the native autoregulatory frameshift site ("FsFix" variants; supplementary fig. S3, Supplementary Material online). As RF2 normally discriminates against a G in

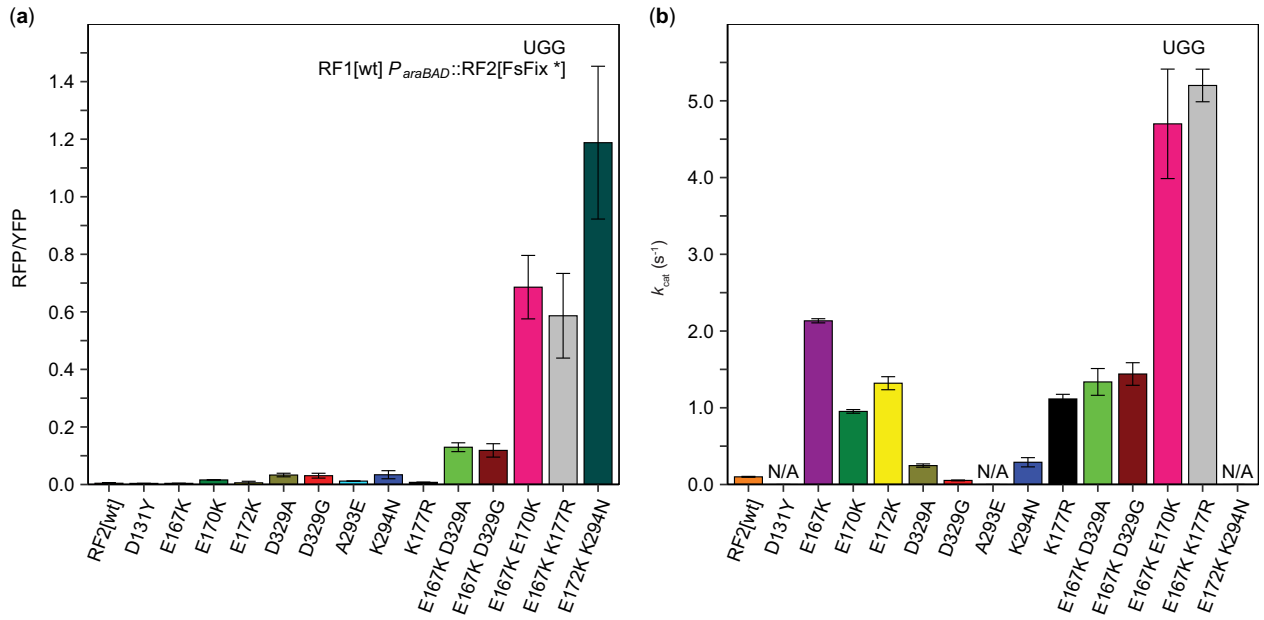

FIG. 5. Evolved RF2 variants terminate on UGG (Trp) codons. (*a*) In vivo termination on UGG codons. The strains expressed RF2 variants as indicated from the chromosomal P_{araBAD} promoter (supplementary fig. S1, Supplementary Material online) and contained *syfp2* (YFP) and dTomato-WWW-nonstop construct, which translates to RFP only when translation is terminated on the triple UGG codons at the end of the coding sequence (supplementary fig. S4*d*, Supplementary Material online). Expression of the indicated RF2 variants was induced with 0.05% L-arabinose. Without induction, no signal was detected. The data are reported as RFP/YFP ratios with standard deviations. (*b*) In vitro peptide release rates on UGG-programmed release complexes, expressed as k_{cat} (s^{-1}) with standard errors. N/A, not available.

the third position of the stop codon, while allowing both A and G in the second position, we hypothesized that mutations in RF2 that increased termination at UAG may also increase the rate of termination on UGG (tryptophan) codons.

To detect termination on UGG codons in vivo, we generated a *dTomato* reporter gene with three consecutive UGG codons instead of a stop codon and no stop codon before the transcriptional terminator (supplementary fig. S4, Supplementary Material online). Normal translation of this reporter construct results in ribosomes reaching the end of the mRNA, leading to tmRNA-mediated trans-translation and destruction of both the peptide and the mRNA, whereas an increased rate of mistermination on UGG codons would result in production of stable dTomato protein as well as stabilization of the mRNA. To be able to test all RF2 variants, the experiment was done with strains that had *prfA* (RF1) still in its native locus.

RF2[wt] and some of the single mutants showed no or barely detectable RFP fluorescence, whereas the D329A, D329G, and K294N single mutants and all the double mutants showed significant levels of RFP fluorescence (fig. 5*a*). The double mutants showed higher termination on UGG than the single mutants, as expected if increased UGG mistermination comes collaterally to improved UAG termination.

Complementary to this in vivo experiment, we also performed the stopped flow tripeptide release assay using UGG-programmed RC (fig. 5*b*). The estimated rate of tripeptide release on UGG by most of the evolved RF2 variants was similar to the rates obtained with UAG (fig. 3*b*). RF2[wt] released tripeptide on UGG with a rate of $\sim 0.1\,s^{-1}$. Compared with that, the double mutants E167K E170K and E167K K177R showed dramatic increase (~60- to 70-fold) in peptide release on UGG. All other RF2 variants except D329A and D329G showed moderate increase of 10- to 25-fold.

For the double mutants, the rates of peptide release on UGG correlated well with the signal from the reporter gene assay (fig. 5*a* and *b*), whereas the results differed for the single mutants. As the peptide release assay was done at saturating concentrations of RF2, the measured rates were insensitive to differences in the binding affinity. Additionally, the assay did not include any tryptophanyl-$tRNA^{Trp}$•EF-Tu•GTP ternary complex, which would be naturally present and compete for the UGG codon in the in vivo reporter gene assay. To test this, we conducted two different experiments.

First, we analyzed the binding of the RF2 variants to UGG-programmed RCs through sucrose cushion ultracentrifugation. Most of the RF2 mutants showed higher occupancy on the UGG RC than RF2[wt] (supplementary fig. S7, Supplementary Material online). In particular, two mutations that reverse or neutralize negative charge (E167K and D329G) showed strong binding to the UGG RC (supplementary fig. S7, Supplementary Material online).

In parallel, the ability of some of the RF2 variants to release the tripeptide on UGG in competition with trp-$tRNA^{trp}$-ternary complex was tested using the stopped-flow-based assay (supplementary fig. S8, Supplementary Material online). In the presence of trp-$tRNA^{trp}$-ternary complex, ~80% of peptide release by RF2[wt] was inhibited. For the E167K single mutant, the inhibition was 40%, whereas no inhibition of peptide release was observed with the double mutant

E167K E170K (supplementary fig. S8, Supplementary Material online). These experiments explain that the discrepancy between the in vivo and in vitro results appears due to mainly the difference in the composition of the assays.

Discussion

In *E. coli* K12, the *prfA* gene has previously been reported to be nonessential, provided one of the following: 1) a mutant RF2[E167K] from *S. enterica* is expressed from a plasmid (Ito et al. 1998) or 2) the A246T mutation present in the K12 variant of RF2 is reverted (Johnson et al. 2011, 2012) or 3) UAG stop codons in six out of seven essential genes that end with UAG are changed to UAA in combination with an amber (UAG) suppressor tRNA (Mukai et al. 2010).

Loss of RF1 leaves the UAG (amber) codon undefined, causing ribosomes to stall when the A-site presents a UAG codon. Such stalled ribosomes would be open to misreading errors by near-cognate tRNAs or to reading frame errors by "slippage" of the mRNA relative to the P-site peptidyl-tRNA, leading to extended proteins that may not always be functional. An additional expected "error" is termination by RF2 "misreading" at UAG, which would produce functional protein with wild-type sequence.

In an *E. coli* strain with fully functional RF2, deletion of *prfA* is reportedly not lethal despite a very low level of termination and high level of readthrough of UAG, which was ambiguously decoded as gln, tyr, and trp (Johnson et al. 2012). The growth phenotype of this *E. coli* strain was exacerbated by expression of an amber suppressor tRNA, indicating that translational readthrough of UAG codons was a major cause of the growth phenotype, and that "misreading" of UAG by RF2 was beneficial. In *S. enterica*, RF1 is essential unless RF2 is overexpressed, indicating that in *Salmonella* RF2 is less promiscuous than in *E. coli*. Alternatively, the effects of ribosome stalling and translational errors on UAG codons are more severe in *Salmonella* than in *E. coli*.

On Possible Mechanisms for Gain of UAG Reading by Evolved RF2

Several of the mutations that appeared in our evolution experiment have been described previously (Ito et al. 1998; Nakamura et al. 2001; Uno et al. 2002; Ejby et al. 2007; O'Connor and Gregory 2011; Wannier et al. 2018), but any explanations for their effect on termination have been lacking or were based on poor or missing structural information. There are, however, several high-resolution structures of RF2 and RF1, both in isolation and in termination complexes with stop-codon-programmed ribosomes (Petry et al. 2005; Korostelev et al. 2008, 2010; Pierson et al. 2016; Fu et al. 2019; Svidritskiy et al. 2019).

In crystals grown in isolation, RF1 and RF2 adopt a compact "closed" conformation, where the active site (for peptidyl-tRNA hydrolysis) GGQ motif is only ~25 Å from the stop codon recognizing motif (PxT in RF1 and SPF in RF2), whereas in termination complexes both release factors accommodate into an extended "open" conformation and the GGQ motif reaches into the peptidyl transferase center ~75 Å away from the stop codon recognition loop.

The switch between the two conformations has been suggested to be part of an induced fit mechanism that explains the high degree of accuracy of translation termination; the RFs first bind (in the closed conformation) with low affinity to the ribosome, but after stop codon recognition they are allowed to adopt the high-affinity open conformation which moves the GGQ motif ~50 Å into the PTC where hydrolysis of the peptidyl-tRNA occurs (Rawat et al. 2003; Fu et al. 2019).

Out of the mutations found in our evolution experiment, only two mutations in position 329 (D329A and D329G) are close to any residue involved in stop codon recognition (fig. 4 and supplementary fig. S9, Supplementary Material online). The aspartic acid residue at position 329 lies just behind an arginine (R213) which has been suggested to be directly involved in RF2's mechanism of discrimination against G in the third position of the codon (Sund et al. 2010). An R213I mutant of RF2 has previously been shown to increase RF2 termination on UAG (Korkmaz and Sanyal 2017). We find it likely that the D329 mutations destabilize the R213 side chain, resulting in loss of discrimination against G in the third position.

Except for the D329 mutations, all other mutations are localized in the domain interface, which positions those close to each other in the closed conformation (supplementary fig. S9*a*, Supplementary Material online). The side chains of K177 and E172, and D131 and K294 are close enough (2.7 and 2.6 Å) to form strong salt-bridge interactions in the closed conformation, whereas in the open conformation the residues are separated by 7.0 and 32.1 Å, respectively (supplementary fig. S9*a* and *b*, Supplementary Material online). Mutations in any of these positions (K177R, E172K, D131Y, and K294N) would abolish interdomain interactions and destabilize the closed conformation. Similarly, in the closed conformation, the side chains of E167 and A293 are buried (supplementary fig. S9*c*, Supplementary Material online) but get fully exposed (supplementary fig. S9*a* and *b*, Supplementary Material online) in the open conformation.

Any larger or differently charged side chain in these positions (E167K and A293E) would thus likely destabilize the closed conformation. It has been demonstrated that the peptide release depends on proper accommodation of the release factors on the ribosome that requires closed to open transition (Indrisiunaite et al. 2015). Thus, by destabilizing the closed conformation, these mutations may allow the switch between the two conformations to occur even without proper stop codon recognition.

Evolutionary Constraints for Evolving RF2 into an Omnipotent Release Factor

An important source of new functions in biology is the modification of the existing functions through a process that often involves duplication of an ancestral gene and accumulation of mutations.

We have previously used a biosynthetic enzyme, HisA, from *S. enterica* to demonstrate that how a set of specialized paralogs (one with the ancestral function and one with a new

function) can evolve from a common ancestral gene in a relatively short timescale (Näsvall et al. 2012). In that experiment, selection for improving the new function and maintaining the ancestral function quickly selected for cells with high-order amplifications of the gene, and this was followed by a slower process of sequential accumulation of beneficial mutations that improved one or both of the functions and allowed the copy number to decrease, validating the Innovation–Amplification–Divergence model (Bergthorsson et al. 2007) for new gene evolution in a 3,000 generation evolution experiment. In this study, we set out to study the evolution of a new function in a different model system.

Our results again support the predictions of the Innovation–Amplification–Divergence model. A gene with a trace of a "new" function (in this case, the ability of RF2 of terminating on UAG) quickly became amplified when the "new" function became beneficial (in this case, by the loss of RF1), and then began accumulating mutations that improved this function. Additionally, we uncovered a constraint on the evolution of release factors, which we did not find in the HisA system; in the HisA model system, selection was mainly for improving the new function while maintaining the old function. Any tradeoff between the two functions was made practically irrelevant when the gene was amplified and each cell could have two alleles supporting one function each.

In the release factor system, we have uncovered another tradeoff which cannot be solved by gene amplification: The loss of apparent specificity required to increase the beneficial function of RF2 ("misreading" UAG codons) at the same time caused an increase in a deleterious activity of RF2 (misreading of near-cognate sense codons).

A commonly discussed constraint in evolution of new genes is the tradeoff between the old and new functions of an enzyme (Tokuriki et al. 2012), where the improvement of one beneficial activity leads to a loss in another beneficial activity. Here, we found another type of tradeoff, which potentially is an even more serious constraint on evolution, namely a collateral increase in a toxic activity that occurs as a result of the mutations that increase the beneficial new activity.

This "collateral toxicity" tradeoff does not necessarily make further evolution impossible, but it may result in evolutionary dead ends. Evolving proteins could become trapped on local peaks in the fitness landscape, where any further improvement in the beneficial function is balanced out by an increase in toxicity. It is likely the collateral toxicity can be overcome by additional mutations that allow better discrimination between nonsense and sense codons, eventually resulting in evolution of RF1-like specificity in a duplicate of RF2.

A Communicating Vessels Model for RF2 Autoregulation in Response to Total Termination Capacity

In a wide range of bacteria, expression of RF2 is negatively feedback regulated through competition between termination and ribosomal frameshifting at an internal UGA stop codon (supplementary fig. S2, Supplementary Material online; Craigen and Caskey 1986; Persson and Atkins 1998; Baranov et al. 2002).

RF1 is expressed from a tricistronic operon (*hemA–prfA–prmC*) and the only known regulation of RF1 expression is the growth-rate-dependent activity of one of the *hemA* promoters (Dahlgren and Rydén-Aulin 2004). No evidence for any feedback control has been reported, although the presence of a UAG stop codon at the end of the *hemA* gene initially led to the suggestion that RF1 may be under feedback regulation involving readthrough of this UAG stop codon (Elliott 1989).

So why, then, is RF2 autoregulated, whereas RF1 apparently is not? To answer this question, we suggest that the autoregulation of RF2 not only responds to changes in the demand for RF2 activity but also indirectly responds to changes in the demand for RF1 activity. As the two factors compete for the common UAA stop codon, any change in the amount of (or demand for) one factor will affect the demand for the other factor by altering the division of labor of the two factors on UAA stop codons (supplementary fig. S10, Supplementary Material online).

If autoregulation is on the more abundant factor RF2 it can be used to tune the total termination capacity to the demand, whereas if it was on the less abundant factor RF1 it would at best only be used for tuning RF1 concentration to the demand for termination on UAG. The relative ratios of UGA versus UAA in bacteria vary with GC content, whereas usage of UAG is low and relatively constant (Povolotskaya et al. 2012; Korkmaz et al. 2014). This results in a higher demand for RF2 than RF1 in all bacteria except those with very low GC content which may have a more similar demand.

Materials and Methods

Bacterial Strains and Growth Conditions

All strains (listed in supplementary table S2, Supplementary Material online) are derived from *S. enterica* subsp. *enterica* serovar Typhimurium strain LT2 (*S. enterica*). Generalized transductions using phage P22 HT 105/1 *int-201* (Schmieger 1972) were used for transferring chromosomal markers and plasmids between strains.

LB (10 g/l Tryptone [Sigma], 5 g/l yeast extract [Sigma], and 10 g/l NaCl) was used as liquid-rich medium, and LB agar plates (LA; LB with 15 g/l agar [Sigma]) were used as solid-rich medium. Salt-free LB (LB prepared without NaCl) was used for preparing cells for transformation by electroporation. SOC medium (Hanahan 1983) was used for recovery after transformations. Sucrose selection plates (salt-free LA, supplemented with 5% [w/v] sucrose [Sigma]) were used for selection against *sacB*-cassettes. Minimal medium was M9 (Miller 1992) with 0.2% glucose (M9 + glucose) or 0.2% glycerol (M9 + glycerol) as carbon source and was supplemented with 15 g/l agar to make solid medium.

Antibiotics (tetracyclin [tet; 7.5 mg/l, Sigma] or chloramphenicol [cam; 6.25 mg/l, Sigma]) were added to the media when needed for selection of transformants or transductants.

For detailed descriptions of bacterial strain constructions, see Supplementary Material online.

Evolution Experiments

In order to allow strains to undergo compensatory adaptive evolution after deletion of the *prfA* (RF1) gene, we conducted experimental evolution by serial passage in liquid media. Before the evolution experiment with the *prfB*[E167K] ancestor, the *prfA* gene was deleted by introducing the *prfA*Δ*903::cat* allele by P22 transduction using DA29516 (dup[*fldB xerD dsbC recJ prfB*(E167K) *lysS*-FRT-J23106-*SYFP2*]**bla*) as recipient.

Similarly, for the evolution experiment using the *prfB*(wt) ancestor, the *prfA* gene was deleted by introduction of the *prfA*Δ*903::j1his* allele into DA32747 (Δ*hisA* dup[*fldB xerD dsbC recJ prfB*(wt) *lysS*-FRT-J23106-*SYFP2*]**bla galE::cat-sacB-T0*). Transductants with the *prfB*[E167K] allele were purified from phage and verified to be phage free and phage sensitive before the start of the experiment.

As the transductants for the *prfB*(wt) lineages were extremely slow growing, we included a *galE* mutation (causing conditional resistance against phage P22 in the absence of galactose) to avoid P22 infection during the long incubation needed to form colonies. As M9 + glucose does not contain any galactose, we could minimize the posttransduction cleanup to include a single pure-streak step for isolating single clones. Thus, prior to the serial passage experiment, the *prfB*(wt) strains had undergone a minimal number of generations on plates, with only two single-cell bottlenecks and a total of <60 generations of growth.

Sixteen fresh clones from each transduction were used to inoculate starter cultures (in 2 ml M9 + glucose) for the evolution experiments. For the first passage, 50 μl starter culture was transferred into 5 ml M9 + glucose in 50-ml conical bottom tubes, 500 μl was pelleted and used to prepare DNA for WGS, and 1 ml was frozen in 10% dimethyl sulfoxide (DMSO). After the first passage, all populations were transferred into both M9 + glucose and LB; however, the *prfB*(wt) populations did not continue growing in LB and were maintained only in M9 + glucose for the remainder of the experiment.

Whole-Genome Sequencing

Genomic DNA from all populations was extracted before passage 1 ("0" generations) and after passage 50 (500 generations; *prfB*[wt] populations) or 100 (1,000 generations; *prfB*[E167K] populations) and sequenced using either Illumina HiSeq (by BGI, Beijing, China) or Illumina MiSeq (in-house). DNA was prepared using Genomic Tip-100 (Qiagen; for HiSeq) or MasterPure (Epicentre; for MiSeq), and libraries for MiSeq sequencing were made using Nextera DNA library prep and indexing kits (Illumina).

All analyses of Illumina sequence reads were done using CLC Genomics Workbench version 11 (Qiagen). Reads were trimmed based on quality to remove any ambiguities and poor-quality reads prior to mapping against a reference genome containing all the modifications present in the ancestral strains, but only one copy of the duplicated *prfB* region to simplify copy number estimation and single-nucleotide polymorphism calling. Single-nucleotide polymorphisms and indels were called using the low-frequency variant detector, and structural rearrangements (duplications and deletions) were found using the structural rearrangement tool in combination with visual scanning through the mapped read depth to find regions of different coverage flanked by partially mapped reads. Amplification copy numbers and frequencies of deletions in populations were estimated by comparing the mapped read depth in the amplified/deleted region with a region that was outside the affected area.

Growth Rate Determinations

For each strain to be assayed, cultures were started from three independent colonies and grown overnight at 37 °C. To measure the growth rates of evolved populations (fig. 1), three overnight cultures were started from each population by thawing the frozen cultures and inoculating samples (10 μl) into 1 ml medium. Each overnight culture was diluted 1:1,000, and 300 μl of each diluted culture was transferred to a 100-well honeycomb plate. Growth of the cultures was monitored in a BioScreen C plate reader with continuous shaking at medium speed at 37 °C. The optical density at 600 nm (OD_{600}) was measured every 4 min for 18 h (for *prfA*+ strains) or 48 h (for Δ*prfA* strains). The growth rates were calculated by fitting the OD_{600} data to the exponential growth equation $N_t = N_0 \times e^{kt}$, where k (min^{-1}) is the exponential growth rate and t is the time (min). Growth rates are reported as relative growth rates by dividing with the average growth rate of wild-type cultures from the same BioScreen run.

Reporter Gene Assays

Termination on UAG was detected in strains lacking RF1, whereas termination on UGG codons was detected in strains with native RF1 still present. All measurements were made in strains containing wild-type *prfB* in its native locus, and the RF2 variants to test expressed from the P_{araBAD} promoter. The strains additionally contained the *dTomato* (termination reporters) and *syfp2* (internal reference) constructs shown in supplementary figure S4, Supplementary Material online. For detection of termination, the strains were grown overnight in M9 + 0.2% glycerol supplemented with 0.05% arabinose in order to express the RF2 variants and then diluted 1:100 in phosphate buffered saline (PBS) for fluorescence measurements. Samples in PBS were incubated for 1 h in room temperature before the fluorescence intensities were measured by flow cytometry using a MACSQuant VYB (Miltenyi Biotech). To control for differences in expression caused by, for example, differences in growth rates, the termination efficiencies are reported as the ratio of red fluorescence (dTomato) to yellow fluorescence (sYFP2).

Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

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Author Contributions

J.N. conceived the study, constructed bacterial strains and reporter genes, performed evolution experiments, and wrote most of the manuscript. J.N. and H.A. planned experiments, analyzed whole genome sequencing data, and reconstructed evolved mutants. H.A. constructed bacterial strains and performed in vivo translation assays and growth rate analysis. S.P. and X.G. cloned and purified all mutant factors and performed and analyzed in vitro translation assays. S.S. planned experiments and analyzed in vitro translation data and wrote parts of the manuscript. All authors contributed in preparing the manuscript.

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