SUPPLEMENTAL RESULTS

Phenotypic profiling of the *prfC* null strain

In an effort to identify novel properties of the *prfC* null strain in an unbiased manner, we utilized the phenotypic microarray service provided by Biolog (Hayward, CA) to identify chemical-sensitivities (PM11-20) or differences in metabolite-utilization (PM1-10). The *prfC* null strain (JW5873) exhibited no substantial growth defects in the metabolite-utilization tests (for the assayed carbon, nitrogen, phosphorous and sulfur sources) but did exhibit systematic growth defects in chemical-sensitivity tests, relative to its wild-type parent (BW25113) (Figure S2A, B). In particular, in the absence of RF3, we observed sensitivities to a variety of chemical agents affecting membranes, cell wall, respiration, ion channels and DNA/RNA synthesis, as well as to chelating, oxidizing, reducing, ionphoric and toxic agents, summarized in Figure S2A. We validated the Biolog data for a representative set of agents extending across the range of the identified processes; these are marked by asterisks in Figure S2A with the actual growth profiles shown in Figure S2B. The breadth of processes that appear to be affected in the *prfC* null strain is consistent with this gene playing a rather general role in stress response in the cell, rather than a particular role in a single process.

Given the rather generic stress response phenotype that emerged from the phenotypic profiling, we next asked whether *prfC* expression is induced under conditions of starvation or stress. Interestingly, the expression level of *prfC* steadily decreases as cells proceed from exponential into stationary growth, to the point where almost no expression is detected when OD_{600} reaches 2. Furthermore, expression of *prfC* was notably decreased in minimal media when compared with the levels in rich media (LB or YPD) at an equivalent density of growth (OD_{600} of 0.6) (Figure S2C). *prfC* expression was modestly induced in the presence of a representative set of toxic compounds identified in the phenotypic microarray analysis as affecting growth in the deletion strain (Figure S2C). The most significant responses were to nitrofurazone and low pH where the relative amount of the *prfC* transcript was seen to increase by approximately twofold (Figure S2C). We note that an analysis of the region upstream of

the *prfC* revealed no obvious sequence features that hint at stress-regulated gene expression (Figure S2C).

An additional concern in evaluating the generic-stress response derived from the phenotypic profiling was that *prfC* is located downstream of *osmY*, a known stress response gene (Yim and Villarejo, 1992) (Figure S2C). As a result, we wondered if the stress profile that we had documented simply reflected polar effects on *osmY* resulting from the disruption of *prfC*. Using northern analysis, we showed that expression of *osmY* was not affected in the *prfC* null strain (relative to the wild-type strain) either in log or stationary phase in rich media, or in log phase in minimal media.

The effects of streptomycin on the growth rate of Δ*prfC* strains cannot be explained by increased rates of peptidyl-tRNA dissociation

In addition to miscoding, streptomycin has been reported to increase the rate of the dissociation of peptidyl-tRNA from actively-translating ribosomes (using a multi-step steady state assay) (Karimi and Ehrenberg, 1996). Since RF3 has been shown to stimulate the same reaction (Heurgue-Hamard et al., 1998), these growth phenotypes in streptomycin do not distinguish between a role for RF3 in post PT QC or in peptidyl-tRNA drop-off. We provide several genetic arguments and experiments to allow us to distinguish between these possible explanations. First, if RF3 is principally involved in peptidyl-tRNA dissociation, then deletion of RF3 should result in reduced drop-off rates and as such should alleviate the growth defects observed in the presence of streptomycin; we observe the opposite (Figure 2A). Second, consistent with earlier data (Cochella et al., 2007; Gromadski and Rodnina, 2004) and inconsistent with reports of increased peptidyl-tRNA dissociation (Karimi and Ehrenberg, 1996), we see no stimulation by streptomycin of peptidyl-tRNA dissociation from dipeptidyl-tRNA programmed ribosome complexes (Figure S1A). Third, while RF3 has been shown to genetically interact with PTH (an interaction that led to the identification of its role in peptidyl-tRNA dissociation) (Heurgue-Hamard et al., 1998), no such interaction between streptomycin and PTH was observed (Figure S1B). These observations suggest that the

genetic interaction that we document for RF3 with streptomycin report on compromised fidelity for the *ΔprfC* strain and is likely to be independent of a role for RF3 in peptidyl-tRNA dissociation.

Distinguishing between a role for RF3 in premature termination and peptidyl-tRNA dissociation

While our data were consistent with post PT QC being responsible for production of the truncated protein product, there are other potential mechanisms that might lead to premature termination events during elongation. In particular, RF3, in cooperation with RRF and EFG, has been implicated in promoting peptidyl-tRNA drop-off. We note, however, that the stimulation of peptidyl-tRNA dissociation by RF3/RRF/EFG has been shown to be limited to ribosomal nascent chains early on during translation (Heurgue-Hamard et al., 2000), where the length of the nascent peptides has not exceeded 7 amino acids (considerably shorter than the ~ 90 amino acid length of the prematurely terminated product seen in our assays). We also note that in other studies, RF3 did not appear to contribute to ribosomal recycling from non-stop, sense codon stalled complexes (Janssen and Hayes, 2009).

Despite these data suggesting that peptidyl-tRNA dissociation was an unlikely mechanism to explain the appearance of the truncated products, we evaluated this potential contribution in our in vitro reconstituted system. We incubated purified mismatched dipeptidyl-tRNA containing ribosome complexes with a single mismatch in the P site with RF2 and RF3 in the absence or presence of RRF and EFG. Biochemical analysis of reaction products with PTH allowed us to determine whether the peptidyl-tRNAs were simply dissociated, or whether an actual release reaction took place. These data allow us to evaluate the potential relative contributions of these two distinct processes to the dissolution of the stalled complex. We note that these singly mismatched complexes should be optimal substrates for the dissociation process (Heurgue-Hamard et al., 2000) and suboptimal substrates for post PT QC (Zaher and Green, 2009). What we observed was that peptidyl-tRNA dissociation accounted for

about 30% of the ribosome complex dissolution with 70% accounted for by premature termination (i.e. post PT QC) (Figure S3D, E). Next we assessed the relative contribution of these pathways with more typical mismatched ribosomal complexes carrying considerably longer peptide products (i.e. from authentic elongation reactions). Here, we took advantage of the fact that the peptidyl-tRNA dissociation mechanism is coupled to the action of PTH in the cell. We prepared extracts from a temperature-sensitive PTH *E. coli* mutant (AA7852) (Atherly and Menninger, 1972) and carried out in vitro translation reactions with the reporter constructs (Rep-DimN, Rep-DimK and Rep-DimX) to determine whether peptidyl-tRNAs accumulate in the Rep-DimN reaction when PTH activity is inhibited. First we showed that while treating the extract from the PTH-wild-type strain at 45°C for 5 minutes had no effect on the activity of PTH, the same treatment greatly diminished the activity of the PTH in the temperature sensitive strain extract (Figure S3F). However, the same heat-treatment had no discernible effect on the accumulation of prematurely terminated product from the Rep-DimN reporter in the absence of asparagine and no accumulation of peptidyl-tRNA species was detected (Figure S3G). These findings suggest that dissociation of peptidyl-tRNA cannot account for the accumulation of prematurely terminated products seen upon asparagine starvation in our assays (Figures 3B and 3C).

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Streptomycin does not affect the dissociation of peptidyl-tRNA in vitro and in vivo, Related to Figure 2 (**A**) The rate of peptidyl-tRNA dissociation from an f-Met-Lys-$tRNA^{Lys}$ dipeptidyl ribosomal nascent chain (RNC) was monitored in the absence and presence of streptomycin by adding PTH to hydrolyze free peptidyl-tRNA as a function of time. A bar-graph shows the observed rates of peptidyl-tRNA drop-off. Error-bars represent the standard error obtained from the non-linear-regression fit to the time-course. (**B**) Streptomycin does not affect the growth of a thermosensitive PTH mutant strain. A spotting assay of a dilution series showing that both the WT (CP79) and *pth-1* (AA7852) strains are capable of tolerating streptomycin at low concentration equivalently regardless of temperature. In contrast, the *ΔprfC* strain (JW5873) is incapable of growth at the same concentration of streptomycin.

Figure S2. The deletion of RF3 leads to broad chemical-sensitivities, Related to Figure 2 (**A**) Results of the phenotypic microarray assays conducted by BioLog. List of toxic agents - and the corresponding impacted processes - that affect growth of the Δ*prfC* strain (JW5873) relative to its parent (BW25113). Compounds marked by asterisks were chosen at random and their effect on growth was studied in more detail (panel **B**). Black asterisks represent compounds that reproduced the BioLog data in our hands, while the red one represents the compound that did not reproduce the Biolog data and appears to be a false-positive. (**B**) Growth curves of the WT (BW25113) and Δ*prfC* (JK463) strains in the presence of the indicated compounds at the indicated concentrations. The doubling times for each condition shown below the curves was determined by fitting the data to an exponential growth equation: $OD_{600} = OD_{(600)t_0} \times e^{kt}$, and using the obtained growth rate *k* to obtain the doubling time T_d, which is expressed as $T_d = ln(2)/k$. Except for EDTA, all tested chemicals slowed the growth (increased the doubling time) of the Δ*prfC* strain relative to the WT. (**C**) Expression of *prfC* under conditions of stress. Schematic of the gene organization around *prfC* is shown at top. Northern blot showing the expression of *prfC* under different phases of growth and media (left panels) and in

the presence of stress-inducing agents (right panels). *prfC* expression is low as cells enter stationary phase, and is maximal in rich media. The expression level and pattern of downstream stress-induced *osmY* appear not to be affected by *prfC* deletion (left). *prfC* is modestly induced under numerous chemical-induced stress conditions (right), in particular in the presence of nitrofurazone and at pH 5.0.

Figure S3. Post PT QC appears to be dependent on RF2 and does not involve peptidyl-tRNA dissociation, Related to Figure 3 (**A**) An autoradiograph of an electrophoretic TLC showing that the asparagine AAU codon is efficiently misread by lysine in the S30 extract in the absence of asparagine. The indicated f-Met-Lys (MK) or f-Met-Asn (MN) programmed initiation complexes (ICs) were incubated in the S30 extract in the absence of asparagine and the products of peptidyl transfer resolved on the electrophoretic TLC. As expected, the MK IC produced one product band that migrates to the same spot as a f-Met-Lys dipeptide. In contrast, the MN IC produced two product bands, a minor one corresponding to f-Met-Asn dipeptide and a major one corresponding to f-Met-Lys dipeptide. (**B**) Depletion of RF2 from the S30 extract results in inhibition of premature termination. The left panel shows a western blot of the extract showing that immuno-depletion of RF2 is efficient while RF1 levels are unaffected. The left panels show SDS-PAGE analysis of the in vitro translation assays with the indicated reporters and conditions. The starvation (Rep-DimN) construct in the normal extract and in the absence of asparagine prematurely terminates to produce a monomer-size product, whose ratio to the dimer is 2.7. In the RF2-depleted extract, this ratio decreases to 1.7, suggesting that RF2 plays a role in terminating miscoded products. Consistent with the activity of RF2 being compromised, we also observe readthrough of the UGA stop-codon found in the Rep-DimX construct. In the presence of paromomycin, which is a competitive inhibitor of class 1 RFs, the efficiency of premature termination is significantly reduced. (**C**) 2D-PAGE analysis of the reporter products produced in the asparagine auxotroph JK463 (Figure 3C) used to examine the nature of the prematurely terminated products. Products produced from the Rep-DimN and Rep-DimX constructs

(and as indicated in the presence or absence of asparagine) were resolved using 2D-PAGE and co-resolved on the same gel. The latter gel was used to examine the relative mobility of the prematurely-terminated products relative to product from a normal stop-codon (Rep-DimX). These data confirm that a lysine residue (or at a minimum a positively charged amino acid) was added before termination took place. (**D** and **E**) Dissociation of peptidyl-tRNA from a short mismatched complex on sense-codons as initiated by RF3, RRF and EFG competes poorly against peptide-release as initiated by RF2 and RF3. (**D**) Matched MKF and mismatched MNF complexes were incubated with the indicated reagents/factors for 10 minutes. At the end of the reaction, PTH was added to half the reaction to determine the extent of peptidyl-tRNA dissociation from the ribosome complexes. Phe TC indicates addition of Phe-tRNA^{Phe} ternary complex, which is the cognate aa-tRNA for the A-site codon. Elongation upon its addition was used to confirm the functional integrity of the complexes. Comparison of the – PTH lane (used to follow peptide release) to the + PTH lane (used to follow the combined effects of peptide-release and peptidyl-tRNA dissociation) was used to directly asses the competition of peptide release with peptidyl-tRNA dissociation. For example, in the presence of EDTA or RF3, RRF and EFG, release of dipeptide is only observed in the presence of PTH suggesting that only peptidyl-tRNA is being released from the ribosome. On the other hand, for the mismatched complex in the presence of RF2 and RF3, release of dipeptide is observed in the absence of PTH, and the addition of PTH increases the signal by an amount corresponding to the amount of peptidyl-tRNA that was dissociated before the termination reaction took place (see the – lanes). The addition of RRF and EFG to the reaction decreased the efficiency of peptide-release relative to peptidyl-tRNA dissociation by ~30 %, as shown in the analysis in **E**. (**E**) Yield of f-Met-Lys dipeptide released from the dipeptidyl tRNA on (-PTH) and off (+PTH) the ribosomes. The plot represents the average from two independent experiments with the error bars representing the standard deviation. (**F**) An S30 extract prepared from the *pth-1* AA7852 strain (PTH ts) has profoundly impaired peptidyl hydrolase activity relative to an extract from the CP79 parent (PTH-WT). The indicated dipeptidyl- (MK) or tripeptidyl- (MKK)

tRNAs were incubated with extracts from the two strains that were either untreated or heat-shocked at 45°C for 5 minutes. The time courses show that hydrolysis of the peptidyl-tRNA is significantly slowed with the extracts prepared from the PTH-ts strain at 37°C relative to the PTH-WT strain, and nearly completely inhibited with the 45°C-treated extract. (**G**) Autoradiographs of Tris-Tricine gels showing that premature termination is not affected by inactivation of PTH, and that no accumulation of peptidyl-tRNA is observed upon starvation. The denoted constructs were incubated with the indicated extracts from the designated strains in the absence of asparagine. The Rep-DimN, as seen earlier, produces a half-length monomer product, whose ratio to the full-length product does not change upon the inactivation of PTH. The tryptophanase upstream open reading frame (TnaC) (Gong and Yanofsky, 2002) construct was used as a control for peptidyl-tRNA accumulation upon addition of tryptophan and confirmed our ability to visualize peptidyl-tRNA species in our gel system. Disappearance of the peptidyl-tRNA band upon KOH-treatment was used to confirm its identity as a peptidyl-tRNA species.

Figure S4. Deletion of RF3 results in wide-spread differential protein expression that is most prominent in an error-prone background, Related to Figure 4 (**A**) 2D-PAGE analysis of total newly synthesized proteins (using [^{35}S]-methionine pulse-labeling) in the denoted strains. Differences in the banding pattern are marked by arrows, while Kan-label indicates the NPT II/Neo protein produced from the *Kan* cassette that was incorporated with the deletion of *prfC*. Most notable were differences observed in the *rpsD* strain and in particular in the acidic-small-length region of the gel (marked by []), where a collection of products was observed in the presence of RF3 and is absent in the Δ*prfC* background. (**B**) Line-analysis (using ImageQuant software) of the denoted region of the gel showing differential patterns for the WT and *rpsD* strains (marked by asterisks), but not for the *rpsL* strain.

Figure S5. RF3 deletion appears to have little impact on transcriptional rate as assessed by a run-on-type assay, Related to Figure 4 (**A**) The radio-labeled samples, generated by carrying out run-on transcription in the presence of [α-^{32}P]-UTP, were resolved on a formaldehyde denaturing agarose gel and stained with ethidium bromide to assess the steady-state levels of total RNA. (**B**) An autoradiograph of the gel in **A** used to look at the total newly-synthesized transcripts. Relative global transcription was determined by first dividing the signal from [α-^{32}P]-UMP incorporation to that from the ethidium bromide and normalizing the resulting ratios to the uninduced sample. (**C**) Slot-blot probing showing that the relative amount of nascent *Rep-DimK* transcript to the 5S transcript does not vary significantly between the Δ*prfC* strains and their respective parents.

Figure S6. Effects of RF3 deletion on polysome profiles in WT and hyperaccurate strains and under conditions of starvation, Related to Figure 5 (**A**) Polysome profile of the Xac (WT) strain and its HZ002 (Δ*prfC*) derivative showing a modest increase in the polysome levels as a result of the deletion. (**B**) Polysome profile of the hyperaccurate US157 (*rpsL*) strain and its HZ003 (Δ*prfC*) derivative showing no discernible change in the polysome profile as a result of the deletion. (**C**) Polysome profiles of the asparagine auxotrophic JK463 strain and its Δ*prfC* derivative showing significant increases in the polysome levels in minimal media and under amino acid starvation as a result of the deletion. Fractional area was determined by cutting the appropriate peaks from the traces and weighing them.

Figure S7. Mutation of the programmed frameshifting sequence reduces the levels of frameshifting from the Rep-Dimfs construct, Related to Figure 6 (**A**) Schematic of the reporters used to assess the levels of frameshifting with the relevant elements labeled. Rep-Dimfs is the construct described in the body of the text with an intact frameshifting site, while the variant Rep-Dimfsm was constructed by altering the Shine-Dalgarno sequence of the *prfB*-programmed frameshifting site. Note that the myc-tag is in the -1 frame and as a result is only expressed when a +1 frameshift occurs. (**B**) Western-blot

analysis using antibodies against the N-terminal His-tag and the out of frame C-terminal myc-tag. While for the Rep-Dimfs noticeable frameshifting is observed when *prfC* is deleted (see Figure 6), for the Rep-Dimfsm the level of frameshifting is significantly reduced in all tested strains. These observations suggest that a majority of frameshifting observed with the Rep-Dimfs construct stems from the designed frameshifting sequence (and occurs on the designated AGG arginine codon).

Table S1 Strains used in this study

Strain	Genotype	Notes	Reference
BW25113	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ*$^-$, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*	Parent for JW5873	(Datsenko and Wanner, 2000)
JW5873	BW25113*, ΔprfC770::kan*	Δ*prfC*	(Baba et al., 2006)
JK463	F+, *asnA, asnB, lacI*^{q} *, relA, spot, thi*	Asn auxotroph	(Precup and Parker, 1987)
HZ001	JK463, *ΔprfC770::kan*	JK463 Δ*prfC*	This work
Xac	*ara Δ[lacproAB], gyrA, rpoB, argE[amber]*	Parent for US157 and UD131	(Andersson et al., 1982) (
HZ002	Xac, *ΔprfC770::kan*	Xac Δ*prfC*	This work
US157	Xac, *rpsL141, zcg-174::Tn10*	Hyperaccurate	
HZ003	US157, *ΔprfC770::kan*	US157 Δ*prfC*	This work
UD131	Xac, *rpsD12*	Error-prone	
HZ004	UD131, *ΔprfC770::kan*	UD131 Δ*prfC*	This work
CP79	F-, *thr-1*, *leuB6*(Am), *fhuA2*, *glnV44*(AS)?, *gal-3*, *his-65*, *relA2*, *malT1*(λ^R), *xyl-7*, *mtlA2*, *argH46*, *thi-1*	Parent for AA7852	(Fill, 1969)
AA7852	F-, *thr-1*, *leuB6*(Am), *fhuA2*, *glnV44*(AS), *gal-3*, *pth-1*, *his-65*, *rfbC1*, *relX201*, *malT1*(λ^R), *xyl-7*, *mtlA2*, *argH46*, *thi-1*	Ts due to *pth* mutation	(Atherly and Menninger, 1972)
N3431	Hfr(PO1), *lacZ43*(Fs), *λ*$^-$, *rne-3071*(ts), *relA1*, *spoT1*, *thi-1*	Ts due to *rne* mutation	(Apirion, 1978)
HZ004	N3431, *ΔprfC770::kan*	N3431 Δ*prfC*	This work

Table S2 Plasmids used in this study

Plasmid	Description
pBAD*prfC*	The *prfC* gene cloned into the pBAD/HisA vector using the NcoI and EcoRI cut sites. The gene is under control the *ara*BAD promoter and is induced by arabinose.
pASK*prfC*	The *prfC* gene cloned into the pASKIBA5C vector using the BsaI cut sites. The gene is under control of the *tet* promoter/operator *ara*BAD promoter and is induced by anhydrotetracycline.
pACYC*rep-DimK*	The dimer reporter (described in the text) was cloned into the pACYC184 vector using the EcoRI and NcoI sites. The reporter contains a lysine (K) residue between the two monomers that make up the full-length protein. The protein contains an N-terminal HA tag and a C-terminal myc tag.
pACYC*rep-DimN*	Similar to pACYC*rep-DimK*, but contains an asparagine (N) residue between the two monomers.
pACYC*rep-DimX*	Similar to pACYC*rep-DimK*, but contains a UGA stop-codon (X) residue between the two monomers.
pPROEX*rep-DimN*	The reporter dimer was cloned in to the pPROEX Htb vector, whose TEV cleavage site was altered to contain aspartate instead of asparagine, using the NcoI and BamHI cut sites. The reporter also contains an I88M mutation.
pPROEX*rep-DimX*	Similar to pPROEX*rep-DimN*, but contains a stop codon between the two monomers.
pASK*rep-DimK*	The rep-DimK gene cloned into the pASKIBA5C vector using the BsaI cut sites.
pPROEX*rep-Dimfs*	Similar to pPROEX*rep-DimN*, but a sequence derived from *prfB* frameshifting site was introduced between the two monomers altering the frame by -1. An in-frame stop-codon was moved 2 amino acids downstream from where the frameshifting occurs.

DETAILED EXPERIMENTAL PROCEDURES

Growth conditions for the bacterial strains,

Unless otherwise stated, strains were grown in Luria Broth (LB) and where applicable in the presence of the desired antibiotic (Carbenicillin 100 μg/mL, Kanamycin 50 μg/mL, Tetracycline 10 μg/mL and Chloramphenicol 33 μg/mL). For the starvation experiments, cells were grown in M9 minimal media.

Plasmids, factors and antibodies

Plasmids used in this study are listed in Table S2. Briefly, *prfC* was cloned into the pBAD/His vector (Invitrogen) using the NcoI and EcoRI cut sites, which disposes of the His-tag. The gene was also cloned into the vector pASK-IBA5C (IBA BioTAGnology) using the BsaI cut sites. The reporter-dimer (Rep-Dim) series of reporters were made by first generating the monomers from annealing and subsequent extension of three DNA oligonucleotides (Zaher and Unrau, 2005). The oligonucleotides had the following sequences:

Oligo1

AGATCTCCCAGAATTCATTAATACGACTCACTATAGGGGAACTTTAAGAAGGAGATATACATAT
GGCATACCCATACGACGTCCCAGACTACGCTATGTCTAAGATTAAAGGTGACGTTAAGTGGTTT
GACGAGTCCAAAGGATTCG,

Oligo2

GGCCTTTGGCACCGTCAGTGATTTCGAACTCTACGCGCTGACCTTCAGCAAGAGTTTTAAAACC
GTCAGTCTGGATTGCAGAGAAGTGTACGAACACGTCTTTGCTGCCGTCTTCCGGAGTAATGAAA
CCGAATCCTTTGGACTCG

Oligo3

TGACGGTGCCAAAGGCCCTTCTGCTGCAAATGTAATCGCTGAACAAAAACTTATTTCTGAAGAA
GATCTGTAAGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTT
TGCCATGGAAGTAGCTTA

The monomers were then amplified in two different reactions with two different primer pairs. The 3′-end of the monomer from the first reaction overlapped with the 5′-end of the monomer from the second reaction. The sequence overlap was then used to anneal

the monomer for strand extension to take place and ultimately generate the desired dimers.

Translation factors were over-expressed and purified from *E. coli* using protocols described earlier (Zaher and Green, 2010). Monocolonal anti-His HRP conjugated, anti-HA HRP conjugated and anti-myc antibodies (all from mouse) were purchased from Qiagen, Santa Cruz Biotechnology and Sigma, respectively. The secondary antibodies of goat anti-rabbit IgG HRP conjugated and rabbit anti-mouse IgG HRP conjugated were purchased from Pierce and Promega. Purified His-tagged RF1 and RF2 were injected into rabbits (2 for each protein) to produce polyclonal sera (Covance). Total IgG was first purified from the sera using protein-A sepharose (GE Lifesciences) affinity-chromatography. The antibodies were then affinity-purified over the corresponding immunizing antigen (RF1 or RF2) coupled to activated sepharose 4B (GE Lifesciences).

SDS-PAGE analysis of cell-free in vitro translation reactions

Reactions were stopped by adding 5 fold excess of cold acetone to precipitate total proteins. The sample was then centrifuged at 16,000 $\times$ g for 5 minutes, followed by air-drying the pellet. The pellet was resuspended in SDS loading buffer in the presence of DTT or where applicable Tris-Tricine loading buffer. Radio-labeled proteins were resolved using 4-12% Tris-Bis SDS PAGE (Bio-rad) or where indicated using 12% Tris-Tricine PAGE and visualized using a Typhoon 9410 phosphorimager (GE Lifesciences). Analysis was carried out using the ImageQuant software package (GE lifesciences).

Analysis of reporter products under starvation conditions

pACYC*Rep-DimK*, pACYC*Rep-DimN* and pACYC*Rep-DimX* plasmids were transformed into the JK463 strain and its Δ*prfC* derivatives HZ001 and HZ001(pBAD*prfC*). Single colonies were inoculated into 5 mL of LB and incubated at 37°C overnight in the presence of the appropriate antibiotics. 2 mL of the overnight cultures were then used to inoculate 100 mL of fresh LB cultures but now in the presence of 0.01% arabinose to induce RF3 from the pBAD*prfC* plasmid. The cells were incubated at 37°C while shaking

until their OD_{600nm} reached 0.6; at this point the culture was centrifuged at 5,000 $\times$ g for 5 minutes and the cell-pellet washed 3 times with 50 mL of M9 minimal media. Each cell pellet was resuspended in 100 mL of M9 media and split into two 50 mL cultures, and asparagine was added to one of the cultures at a concentration of 100 μg/mL. The cultures were incubated for an additional 30 minutes before 200 μCi of [^{35}S]-methioinine was added. After a further incubation period of 30 minutes, a chase of unlabeled methionine was added to a final concentration of 1 mM. The cells were then harvested by centrifugation and lysed using a combination of lysozyme treatment (0.1 mg/mL) and freeze-thaw cycles in liquid nitrogen. Lysis was completed by the addition of deoxycholate to a final concentration of 0.3 %. The lysates were then clarified by centrifugation at 16,000 $\times$ g for 10 minutes. The reporters were purified using the Anti-HA Immunoprecipitation Kit from Sigma. The proteins were eluted from the matrix by adding Tris-Tricine loading dye and boiling for 5 minutes. The reporters were then resolved on 12% Tris-Tricine PAGE gels, visualized and analyzed as described earlier.

For 2D-PAGE analysis of the purified reporters, the proteins were first precipitated from the loading dye by the addition of 5 fold excess of cold acetone. The protein-pellet was resuspended in water and re-precipitated as before; this procedure was repeated 3 times to get rid of any contaminating SDS. The rest of the procedure used to resolve the proteins on both dimensions is described in detail in the GE Lifesciences handbook of "2-D Electrophoresis Principles and Methods". For the first dimension of isoelectric focusing (IEF), we used the 11-cm Immobiline DryStrip pH 3-5.6 NL gel (GE Lifesciences); for the SDS PAGE second dimension, we used 15 % Tris-Glycine SDS PAGE gels.

Purification of constructs used for MALDI-TOF analysis

pPROEX*Rep-DimN* and pPROEX*Rep-DimX* plasmids were transformed into the JK463 strain and its *prfC* deletion derivative HZ001. 500 mL of LB culture of each plasmid/strain pair was started as described earlier and incubated until an OD_{600nm} of 0.6 was reached. The cells were centrifuged and washed with M9 media. Each cell pellet

was split into two equal 250 mL M9 minimal media cultures with one culture containing asparagine at a concentration of 100 μg/mL. After 30 minutes of incubation at 37°C, IPTG was added to a concentration of 1 mM to induce the expression of the reporters. Cells were harvested 2 hours after induction and lysed using a French Press. The reporters were purified over Ni-NTA resin and eluted with 300 mM imidazole. The proteins were then precipitated using 10 % TCA, air-dried and resuspended in TEV-cleavage buffer (50 mM Tris-HCl (pH 8.0), 200 mM NaCl, 0.5 mM EDTA and 1 mM DTT). Cleavage of the N-terminal His-tag was carried out by adding His-tagged TEV protease to a final concentration of 50 μg/mL and incubating the mixture at room temperature for 4 hours. The cleaved-protein was then passed over Ni-NTA to collect the flowthrough; this step gets rid of TEV, the His-tag and any contaminating proteins that bound Ni-NTA nonspecifically. Before mass-spec analysis, the reporter proteins were cleaned up using C18 zip tips (Millipore).

Western-blotting analysis of protein expression

pASK*Rep-DimK* plasmid was transformed into the Xac (WT), US157 (*rpsL*141) and UD131 (*rpsD*12) strains and their derivatives (Table S1). Cultures from each transformation (single colonies) were started as described earlier. The starter cultures were used to inoculate 5 mL of cultures, which were grown in the presence of arabinose (0.01%) until OD_{600nm} reached 0.6; at this point, the reporter was induced by the addition of anhydrotetracycline to a final concentration of 200 ng/mL. After 30 minutes of induction, the cells were split into two tubes (one used for Western analysis while the second was used for Northern analysis). Cells were pelleted and resuspended in 200 μL SDS loading buffer and the lysis completed by sonication and boiling. 20 μL of each sample (~30 μg of total protein) was loaded onto 4-12% Tris-Bis SDS PAGE gels. Proteins were then transferred onto PVDF membranes using a semi-dry apparatus. The efficiency of the transfer was determined by staining the membranes with Ponceau S. The membranes were then blocked using a 4% skim milk solution in PBST, before the addition of the appropriate antibody in PBST. The membranes were incubated with the

antibodies at 4°C overnight. For unconjugated primary antibodies, the membranes were washed extensively with PBST before the addition of secondary antibodies. Detection of the HRP-conjugate was accomplished using the SuperSignal ELISA Pico Chemiluminescent Substrate from Thermoscientific.

Northern-blotting analysis and determination of mRNA half-lives

Nucleic acids were isolated from the cell-pellet above using hot phenol extraction (Jahn et al., 2008), followed by chloroform extraction and ethanol precipitation. The pellet from the ethanol step was resuspended in water and the concentration of nucleic acids determined using the absorbance at 260 nm. 10 μg of total nucleic acid from each sample was resolved on a 1% formaldehyde denaturing agarose gel and then transferred onto Hybond XL Nylone membranes (GE Lifescieciences) using a vacuum manifold setup. The nucleic acids were cross-linked onto the membrane using a UV oven (Stratagene) followed by baking at 80°C for 20 minutes. The membranes were prehydbridized using the Rapid-hyb buffer (GE Lifesciences) at 68°C for at least 30 minutes in a hybridization oven. The radio-labeled DNA probe (described later) was then directly added to the Rapid-hyb buffer around the membrane, and incubated at 68°C overnight. Membranes were initially washed 3 × with a nonstringent buffer (2 × SSC buffer, 0.1% SDS), followed by washing 3 × with a stringent buffer (0.1 × SSC buffer, 0.1% SDS), both at 68°C. The membranes were sealed in heat-sealable bags, exposed to a phosphorimager screen and analyzed as described earlier using a Typhoon phosphorimager. The membranes were stripped using a 0.1% SDS solution and incubation at 95°C, before re-probing for a different transcript.

Radio-labeled DNA probes were made by generating 300-500 bp double-stranded DNA templates from plasmids or *E. coli* genomic DNA using a PCR-amplification reaction. The primers were then removed using a QIAquick PCR Purification Kit from Qiagen. The single-stranded labeled probe was then generated from this template by performing an amplification reaction with only the 3′-end primer in the presence of [^{32}P]-α-dTTP.

mRNA half-lives were determined by isolating nucleic acids (using a hot-phenol procedure) from cultures harvested at various time points after transcription was inhibited by the addition of Rifampicin (final concentration of 0.5 mg/mL). The half-lives were then measured by Northern-blotting as described earlier.

Polysome Analysis

Analysis of the polysome profiles in the different strains was carried out using published protocols (Ron et al., 1966) except that chloramphenicol was added to a final concentration of 100 μg/mL 5 minutes before initiation was halted by the addition of an equal volume of ice to the growing culture. The profiles were exported into a spreadsheet using DataThief III software, and the integration was done using GraphPad Prism5 software. For northern analysis, RNA was isolated from the polysome fractions using phenol/chloroform extraction followed by ethanol precipitation and analyzed as before. For western analysis, proteins were initially precipitated using 10% TCA and incubation at 4°C overnight. Protein pellets were resuspended in SDS loading buffer and analyzed as described earlier.

Peptidyl-tRNA dissociation

All assays involving purified in vitro translation components were conducted in polymix buffer (Jelenc and Kurland, 1979). Dipeptidyl-tRNA ribosomal complexes were generated using protocols described elsewhere (Zaher and Green, 2009). To measure the rate of peptidyl-tRNA dissociation, RNCs at ~200 nM were incubated with PTH at ~2 μM in the presence of varying concentrations of streptomycin. Aliquots were taken at different times and the reaction stopped by the addition of 2% formic acid. The dipeptide product was resolved from peptidyl-tRNA using an electrophoretic TLC setup (Youngman et al., 2004). Fractional radioactivity corresponding to the hydrolyzed dipeptide was quantified using the ImageQuant software package (GE Lifesciences). Rates were then determined by fitting the data to a single-exponential function using Prism software (GraphPad).

Assessing the effect of asparagine starvation on miscoding in S30 extracts

f[^{35}S]-Met-Lys (MK) and f[^{35}S]-Met-Asn (MN) initiation complexes (ICs) were prepared as described previously (Zaher and Green, 2010). In a typical 10 μL reaction, complexes (1 μM) were incubated with 4 μL S30 premix (Promega) and 3 μL of the S30 extract in the presence of 500 μM of each amino acid (minus asparagine) at 37°C for 5 minutes. The reaction was stopped by the addition of KOH to 200 mM, and product analysis was carried out using electrophoretic TLCs.

Immuno-depletion of RF2 from S30 extracts

RF2 was removed from the S30 extract using a protocol described earlier by Gong and Yanofsky (Gong and Yanofsky, 2002) but with our rabbit anti-RF2 antiserum (described in the experimental procedures section).

Competition assays

Matched and mismatched dipeptidyl-tRNA complexes were prepared as described earlier (Zaher and Green, 2009) and incubated (final concentration 0.5 μM) with 2 μM Phe-tRNA^{Phe} ternary complex at 37°C for 5 minutes. The yield of peptidyl transfer, assayed by the relative amount of f-Met-Lys-Phe tripeptide to the starting f-Met-Lys dipeptide, was then used to determine the integrity of the complexes. To determine the functional activity of PTH, EDTA was added to the complexes at 20 mM to dissociate the peptidyl-tRNA from the ribosomes before the addition of PTH to a final concentration of 2 μM. To determine the functional activity of RF3, RRF and EFG, the factors (final concentration 2 μM each) were incubated with the complexes (200 nM) at 37°C for 10 minutes before the reaction was split into two tubes and PTH added to one tube. The reaction was then stopped by the addition of 2% formic acid and the products analyzed by electrophoretic TLC. Similar procedures were used to determine the functional activity of RF2 and RF3. For the final competition experiment, all the factors were included in the reaction at 2 μM and analysis proceeded as described earlier.

Assaying the activity of PTH in the S30 extracts

To generate peptidyl-tRNAs, programmed dipeptidyl and tripeptidyl ribosomal complexes were broken apart through the addition of EDTA to a final concentration of 20 mM. 2 μL of untreated or 45°C-treated (for 5 minutes) S30 extract was then added to 18 μL of the 200 nM disassembled complexes (free peptidyl-tRNA). Aliquots were taken at various times by stopping the reaction with 2% formic acid. Analysis of the hydrolysis reaction was carried out by resolving the dipeptide and tripeptide hydrolysis products from the peptidyl-tRNA substrates using electrophoretic TLC.

Proteome-wide 2D-PAGE analysis

5 mL of culture was grown in LB until mid-logarithmic phase (OD600 ~ 0.6) and cells were pelleted and washed extensively with M9 minimal media. The cells were then resuspended in 5 mL of M9 minimal media and incubated for an additional 30 minutes. 50 μCi of [^{35}S]-methionine was added to the culture to label total proteins, followed by an additional incubation of 30 minutes before a chase of cold methionine (1 mM) was added. The cells were then immediately harvested by centrifugation, lysed by resuspending in SDS-loading buffer and boiled for 10 minutes. Cellular debris was removed by centrifugation. The SDS was then removed by multiple acetone precipitation steps (at least 3). 2D-PAGE analysis was carried out as detailed in the experimental procedures section.

Run-on transcription assay

5 mL of culture was grown to OD_{600} of 0.6. Rep-DimK was then induced by adding anhydrotetracycline to a final concentration of 200 ng/mL. After 30 minutes of induction, the cells were pelleted and washed once with the run-on buffer [20 mM Tris (pH 8.0), 10 mM $MgCl_2$, 120 mM KCl, 2 mM DTT]. The cells were then flash-frozen using liquid nitrogen. Transcription was initiated by resuspending the cells in 200 μL run-on buffer in the presence of ATP (1 mM), GTP (1 mM), CTP (1 mM), [α^{32}P]-UTP (0.34 μCi/μL, 111 nM)

and polymixin B sulfate (1 mg/mL). The mixture was incubated at 37°C for 10 minutes. The samples were then phenol/chloroform extracted and ethanol precipitated.

A slot blot apparatus was assembled with Hybond Neutral Nylon membranes (GE lifesciences). Rep-DimK, rpoB and 5S antisense single-stranded DNA oligos (30-50 nt long) were then cross-linked to the membranes as described previously (Nickenig and Murphy, 1994). Hybridization of the run-on products to the membranes and subsequent washings were carried out using protocols published earlier (Nickenig and Murphy, 1994).

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