Release factor-dependent ribosome rescue by BrfA in the Gram-positive bacterium *Bacillus subtilis*

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Rescue of the ribosomes from dead-end translation complexes, such as those on truncated (non-stop) mRNA, is essential for the cell. Whereas bacteria use trans-translation for ribosome rescue, some Gram-negative species possess alternative and release factor (RF)-dependent rescue factors, which enable an RF to catalyze stop-codon-independent polypeptide release. We now discover that the Gram-positive *Bacillus subtilis* has an evolutionarily distinct ribosome rescue factor named BrfA. Genetic analysis shows that *B. subtilis* requires the function of either trans-translation or BrfA for growth, even in the absence of proteotoxic stresses. Biochemical and cryo-electron microscopy (cryo-EM) characterization demonstrates that BrfA binds to non-stop stalled ribosomes, recruits homologous RF2, but not RF1, and induces its transition into an open active conformation. Although BrfA is distinct from *E. coli* ArfA, they use convergent strategies in terms of mode of action and expression regulation, indicating that many bacteria may have evolved as yet unidentified ribosome rescue systems.
Faithful translation requires accurate initiation, elongation, and termination. In translation termination, the stop codon situated in the A-site of the ribosome recruits a release factor (RF), which then hydrolyzes the peptidyl-tRNA ester bond to release the polypeptide product from the ribosome. In bacteria, RF1 recognizes UAA and UAG while RF2 recognizes UAA and UGA through their PxT and SPF codon recognition motifs, respectively6. These RFs contain the hydrolysis active site motif, GGQ, for catalysis. Polypeptide release is then followed by dissociation of the ribosome from mRNA into the small and large subunits by a process mediated by ribosome recycling factor and elongation factor G7,8. However, the termination/recycling event can be perturbed when mRNA has aberrant features, one of which is the absence of an in-frame stop codon. The mRNA lacking a stop codon, called a non-stop mRNA, causes stalling of the ribosome at the 3′ end because recruitment of RFs to the ribosome requires the interaction of a stop codon recognition motif of the RF with a cognate stop codon. Since the role of termination is not only to define the end of the protein but also to recycle the ribosome for the next round of translation initiation, a failure in termination lowers the cellular capacity of protein synthesis, unless dealt with by the cellular quality control mechanisms. Indeed, a loss of function in the quality control machinery leads to an accumulation of dead-cellular capacity of protein synthesis, unless dealt with by the end of the protein but also to recycle the ribosome for the next player in lethality4,5.

In translation, the ribosome reads the mRNA according to the mRNA-like coding function of tmRNA until the built-in stop codon is reached. The result is the formation of the ribosome-tmRNA complex which then accepts the non-stop peptide and is elongated further (tmRNA), which is encoded by ssrA9. tmRNA cooperates with SmpB, which mediates ribosomal accommodation of tmRNA at the ribosomal A-site10,11. The tmRNA is composed of trnaA- and mRNA-like domains. The former can be charged with alanine, which then accepts the non-stop peptide and is elongated further according to the mRNA-like coding function of tmRNA until the built-in stop codon is reached. The result is the formation of the non-stop polypeptide bearing an extra ssrA-encoded sequence (15 amino acids in B. subtilis) and dissociation of the ribosome from the non-stop mRNA. The SsrA tag sequence promotes proteolytic elimination of the non-stop polypeptide via targeting to cellular proteases. Trans-translation is essential for the growth of some bacteria9,12. The essentiality lies in the liberation of the ribosome from the non-stop mRNA, but not in proteolytic degradation of the translation products13,14. Bacterial species that can survive without trans-translation often possess one or more alternative ribosome rescue factor(s), such as ArfA, ArfB, or ArfT, which are involved in stop-codon-independent cleavage of the non-stop peptidyl-tRNA5. ArfA was identified in E. coli by a genetic screening for a mutation showing synthetic lethality with the loss of ssaA15. ArfB (YaeJ), identified as a multicopy suppressor of the ssaA/arfA double mutant16,17, contains its own GGQ catalytic motif, enabling it to act as an RF-independent ribosome rescue factor21. Although the physiological role of ArfB in E. coli is unknown, its homologs are widely distributed among both Gram-positive and -negative bacteria5,18,22 as well as eukaryotic mitochondria23. ArfT in Francisella tularensis, a member of γ-proteobacteria that lacks both ArfA and ArfB homologs5, is essential in the absence of tmRNA. Like ArfA, ArfT is an RF-dependent ribosome rescue factor, although ArfT can function with either RF1 or RF2. Phylogenetic distribution of ArfA is limited to a subset of β- and γ-proteobacteria whereas that of ArfT is limited to a subset of γ-proteobacteria. To date, RF-dependent ribosome rescue factors have only been reported in Gram-negative bacteria.

Bacillus subtilis, a Gram-positive bacterium, can also survive without ssrA25, but no alternative factor for ribosome rescue has been reported in this organism, raising the question of whether the ribosome rescue function is non-essential or alternative factors have escaped identification in B. subtilis. In this study, we have addressed this question and identified BrfA (Bacillus ribosome rescue factor A; formerly YqkK) as a ribosome rescue factor. BrfA has no obvious sequence similarity to other Arf proteins. We show that BrfA is an RF2-dependent ribosome rescue factor, which induces hydrolysis of peptidyl-tRNA in non-stop translation complexes. BrfA is produced naturally from a non-stop mRNA and is thus negatively regulated by trans-translation, revealing a conceptually similar regulatory crosstalk as documented for E. coli ArfA. Lastly, using single-particle cryo-electron microscopy (cryo-EM), we reveal how BrfA recognizes the presence of truncated tmRNAs and recruits and stabilizes an open conformation of RF2 to rescue the stalled ribosomes. BrfA uses a mechanism that is similar but distinct from ArfA. Collectively, our findings lead us to suggest that Gram-positive and -negative bacteria have independently acquired their own unique RF-dependent ribosome rescue systems equipped with a convergent scheme of regulation.

Results

BrfA and trans-translation exhibit synthetic lethality. The dispensability of trans-translation in B. subtilis raises the possibility that it contains an alternative ribosome rescue factor. With the reasoning that the loss of such a factor would make the bacterial growth dependent on trans-translation proficiency, we searched for the chromosomal gene knockouts that cause synthetic lethality with the deficiency of trans-translation. We used a strain with chromosomal deletion of smpB, which encodes the trans-translation co-factor but having a plasmid carrying the wild-type smpB as well as lacZ genes (Fig. 1a). This rescue plasmid was a derivative of pLOSS26 driven by a temperature sensitive (Ts) replicon, such that it is lost frequently at high temperature. We prepared chromosomal DNA from a mixture of the B. subtilis strain, a collection of mutants individually disrupted for the 3968 non-essential B. subtilis genes by replacement with the erythromycin resistance marker (ery)27, and used it to transform (by homologous recombination) the strain for the screening described above. Transformant mixtures were then incubated at 50 °C to destabilize the rescue plasmid and plated on selective agar containing X-Gal (see Methods). Whereas clones that did not depend on SmpB had segregated out the plasmid and formed white colonies, those requiring SmpB survived only when they had retained the plasmid and formed blue colonies due to the plasmid-encoded β-galactosidase (Fig. 1a). Among ~74,000 transformants, we picked up 42 blue colonies and determined the
chromosomal locations of the *ery* inserts by DNA sequencing, followed by elimination of false-positive clones by retransformation experiments. These procedures left clones with an *ery* disruption of *yqkK* (renamed *brfA*, see below), which makes SmpB indispensable for survival.

We validated the growth requirement features of *B. subtilis* for BrfA and the trans-translation system by an independent approach. We used CRISPR interference (CRISPRi) to conditionally silence either *ssrA* or *smpB* (Fig. 1b). To do this, the catalytically inactive and xylose-inducible variant of Cas9 (dCas9) was integrated into the chromosomal *lacA* locus of the wild type (WT) and the Δ*brfA* strains. In addition, the constitutively expressed small guide RNA (sgRNA) that targets either *ssrA* or *smpB* was integrated into the *amyE* locus of the same strains. The bacterial strains grew normally when dCas9 was uninduced in the absence of xylose (Fig. 1b, left panels). Growth of the Δ*brfA*^+^ (WT) cells was not affected by 1% xylose, which induced dCas9 to silence *ssrA* or *smpB* (lanes 1, 4, 7, and 10, right panels).

**Fig. 1** Simultaneous loss of BrfA and trans-translation leads to synthetic growth defects. a A schematic representation of the synthetic lethal screening to identify genes whose absence causes synthetic lethal phenotype with the deficiency of trans-translation. b Xylose-inducible CRISPRi was targeted to *ssrA* (lines 1–3, 7–9) or *smpB* (lines 4–6, 10–12) in the *B. subtilis* strains indicated at the left by the genotypes of the *brfA* gene (Δ*brfA*/Δ*brfA*^+^ signifies the presence of *brfA*^+^ in an ectopic locus). Cultures prepared in the absence of xylose were serially diluted (from 10^{-2} to 10^{-5}) and spotted onto LB agar plates with or without 1% xylose, as indicated at the top, for incubation at 30 °C (upper) or 37 °C (lower) for 17 h.
BrfA expression is regulated by trans-translation. The brfA gene contains 71 sense codons followed by a stop codon. We note that it contains a typical rho-independent transcription terminator sequence within the coding region, raising an intriguing possibility that BrfA is translated naturally from a non-stop mRNA lacking the 3′ region including the stop codon (Fig. 2a). If this is the case, then synthesis should initially consist of approximately 62 amino acids in the form of peptidyl-tRNA. However, translation of such mRNAs is likely dealt with by the trans-translation mechanism of ribosome rescue, which would add the SsrA tag sequence of 15 amino acids to the BrfA non-stop product. It follows then that the BrfA product would be rapidly degraded by cellular proteases, such that the protein level should be strongly down-regulated in trans-translation proficient cells.

To test this possibility, we examined the impact of trans-translation on the accumulation of a series of BrfA-derived proteins. We constructed a translational gene fusion consisting of the coding sequences for green fluorescence protein (GFP), BrfA (full length), and FLAG that are connected in-frame in this order from the N-terminus to the C-terminus. We also constructed transcriptional fusions, in which independently translatable lacZ follows gfp-brfA-flag to verify the occurrence of transcription termination in brfA (Fig. 2b). We expressed GFP-BrfA-FLAG in the wild type and the ΔsmpB cells and detected the products with anti-GFP and anti-FLAG immunoblotting. Strikingly, the wild-type cells did not produce any anti-GFP detectable protein from the gfp-brfA(WT)-flag construct (Fig. 2c, lane 1, upper panel). By contrast, the ΔsmpB cells produced a product that formed an intense band near the 37 kDa marker (Fig. 2c, lane 2, upper panel). This species of protein did not react with anti-FLAG (lane 2, lower panel), indicating that it lacked the C-terminal region including the stop codon (Fig. 2c, lane 2, upper panel). This species of protein did not react with anti-FLAG (lane 2, lower panel), indicating that it lacked the C-terminal region including the stop codon (Fig. 2c, lane 2, upper panel). These results are consistent with the notion that the mRNA ends within brfA due to internal termination. The translation products are SsrA-tagged and degraded rapidly in the wild-type strain, while they accumulate in the trans-translation-deficient ΔsmpB cells.

To examine the above scenario further, we disrupted the transcription terminator either by internal deletion or by synonymous substitutions (Fig. 2a, b). In the former, we deleted the codons 63–71 of brfA in the context of GFP-BrfA-FLAG to eliminate the 3′ T stretch of the transcriptional terminator (GFP-BrfA62-FLAG). In the latter, we introduced synonymous mutations to disrupt the secondary structure required for transcription termination (GFP-BrfA(no_term)-FLAG) (Fig. 2a, b). Both of these terminator-less mutant forms of GFP-BrfA-FLAG accumulated equally in the wild type and ΔsmpB strains and are reactive with both anti-GFP and anti-FLAG (Fig. 2c, lanes 3–6).

We then confirmed the occurrence of internal transcription termination by examining the expression of lacZ attached at a 3′ region of the transcription unit. If the terminator within brfA indeed functions, the downstream lacZ would not be expressed. As expected, cells harboring the construct with the wild-type brfA sequence exhibited very low levels of β-galactosidase activity (Fig. 2d, columns 1 and 2), as compared with those having the terminator mutations, which exhibited high levels of β-galactosidase activity (Fig. 2d, columns 3–6). These data establish that the internal transcriptional termination indeed occurs for brfA, indicating that it is a major element that triggers the BrfA down-regulation by the SsrA tag-dependent degradation.

These results show that B. subtilis is equipped with at least two layers of ribosome rescue mechanisms, trans-translation and BrfA-dependent peptidyl-tRNA hydrolysis (see below). The trans-translation-dependent down-regulation indicates that BrfA is the secondary ribosome rescue factor that is only produced upon dysfunction of trans-translation, the primary ribosome rescue mechanism. Thus, the internal transcription terminator in BrfA provides the means for this bacterium to accomplish the compensatory and vectorial regulation for the maintenance of ribosome rescue capability. In this context, BrfA bears a striking similarity to the E. coli alternative rescue factor, ArfA, which is also synthesized from a non-stop mRNA17,18.

BrfA recruits RF2 to hydrolyze non-stop peptidyl-tRNAs. We characterized BrfA biochemically by examining whether it could induce polypeptide release, as expected for an alternative ribosome rescue factor. To do this, we purified BrfA in the form of BrfA62-His6, which lacks the C-terminal 9 amino acid residues encoded by the brfA gene but not by the brfA mRNA (see above); a hexahistidine (His6) tag was attached to the C-terminus to aid purification. We considered that the absence of the C-terminal 9 amino acids was physiological. Also, the absence of the 3′ coding region of brfA should disrupt the internal transcription terminator signal and enable the attachment of the His6 tag. Since BrfA lacks a GGQ motif critical for peptidyl-tRNA hydrolysis, we hypothesized that BrfA requires an RF to hydrolyze peptidyl-tRNA. Therefore, we also purified B. subtilis RF1 and RF2. For in vitro translation with defined translation components, we used the Bs hybrid PURE system29, a modified version of the PURE coupled translation–translation system30, in which the original E. coli ribosomes were replaced with B. subtilis ribosomes. We omitted RFs in the Bs hybrid PURE system unless otherwise stated.

We used DNA fragments encoding GFP but without an in-frame stop codon (GFP-ns) to direct in vitro transcription and translation with Bs hybrid PURE system and separated the translation products by neutral pH SDS-PAGE, which preserved the peptidyl-tRNA ester bond31. The major product, migrating between the 42 and the 55 kDa markers, represents the peptidyl-tRNA (GFP-tRNA; Fig. 3a, lane 1), as treatment of the sample with RNase A before electrophoresis down-shifted this band to the position near the 28 kDa marker, indicative of tRNA removal (GFP; lane 2). Thus, the non-stop template indeed produced a translation-terminated state of ribosome-nascent chain complex, which was unaffected when the reaction mixture included B. subtilis RF1 or RF2 (lanes 3–6), as expected from the absence of a stop codon in the template. We then addressed the effects of BrfA. Neither BrfA by itself nor its combination with B. subtilis RF1 affected the production of GFP-tRNA (lanes 7–10). By contrast, the addition of both BrfA and B. subtilis RF2 to the Bs hybrid PURE system resulted in the production of the hydrolyzed GFP band with a concomitant decrease in the level of GFP-tRNA (lanes 11 and 12). Thus, BrfA and RF2 cooperatively catalyze polypeptide release from the non-stop stalled ribosome.

Distinct requirements of RF2 for rescue and termination. Given that BrfA and RF2 cooperatively induce hydrolysis of non-stop peptidyl-tRNA, the role of RF2 would be to execute the catalysis. Consistent with this expectation, a catalytically inactive RF2 variant, RF2(GAQ), whose GGQ active site had been mutated to GAQ, no longer stimulated hydrolysis of GFP-tRNA.
Fig. 2 The cellular abundance of BrfA is negatively regulated by trans-translation. **a** A schematic representation of the brfA open reading frame, showing the intrinsic transcriptional terminator sequence within the coding region (indicated by “terminator” at the top and the wild type (WT) enlarged view below). Shown also are the corresponding sequences of the gfp-brfA(2–62)-flag and gfp-brfA(no_term)-flag constructs (see **b**), in which nucleotide substitutions and the FLAG tag are indicated by reverse and underline, respectively. **b** Schematic representations of the gfp-brfA-flag constructs with wild-type terminator sequence (gfp-brfA(2–71)-flag) and its derivatives with defective terminator signals. These gfp-brfA derivatives were placed under the constitutive mifM promoter. The lacZ gene is also placed downstream of the gfp-brfA derivatives. “SD” and asterisks indicate Shine-Dalgarno sequence and synonymous mutations, respectively. **c** Cellular accumulation of the products of the wild-type construct (lanes 1, 2) as well as the brfA(2–62) (lanes 3, 4) and the brfA(no_term) (lanes 5, 6) constructs. They were expressed in the smpB+ (odd numbers) or the ΔsmpB (even numbers) strains and analyzed by anti-GFP (upper) or anti-FLAG (lower) immunoblotting. **d** β-Galactosidase activities (mean ± s.d., n = 3) of the cells harboring lacZ at the downstream of wild type (columns 1, 2), the brfA(2–62) (columns 3, 4), or the brfA(no_term) (columns 5, 6) derivatives of the gfp-brfA in the presence (odd numbers) or absence (even numbers) of smpB. “s.d.” indicates standard deviation. Source data are provided as a Source Data file.
even in the presence of BrfA (Fig. 3b, lanes 5 and 6). RF2 possesses the conserved SPF motif as an essential element for the stop codon recognition in termination.32 We addressed whether this motif is required for the ribosome rescue function of RF2 by mutating it to SPT, which abolishes the termination activity.32 In the in vitro peptidyl-tRNA hydrolysis assay, the RF2(SPT) was as active as the wild-type RF2 (lanes 3, 4, 9, and 10), RF2(GAQ) (lanes 5 and 6), and RF1 in the presence of combinations of BrfA, wild-type RF2 (lanes 3, 4, 9, and 10), RF2(GAQ) (lanes 5 and 6), and RF1 (lanes 11 and 12), as indicated. The translation products were analyzed by anti-GFP immunoblotting as described above.8 Interactions compatibility of the RF-dependent rescue factor functions. In vitro translation of gfp-ns was carried out using Bs hybrid PURE system (lanes 1–8) or Ec PURE system (lanes 9–16) in the presence of combinations of purified BrfA (lanes 1–4, 9–12), E. coli ArfA (lanes 5–8, 13–16), RFs (RF1 plus RF2) purified from B. subtilis (lanes 1, 2, 5, 6, 9, 10, 13, and 14), and RFs purified from E. coli (lanes 3, 4, 7, 8, 11, 12, 15, and 16). The translation products were analyzed by anti-GFP immunoblotting as described above. Source data are provided as a Source Data file.

Ribosome stalling can also be induced by specific amino acid sequences of nascent polypeptides for regulatory purposes. Such regulatory nascent polypeptides include E. coli SecM, B. subtilis MifM, and Vibrio alginolyticus VemP.33,34 Ribosome stalling in these cases needs to be adequately regulated, such that it is subject to conditional and specific mechanisms of cancellation.35,36 Unregulated rescue could be counterproductive in these cases. Our in vivo and in vitro analyses show that the B. subtilis MifM stalling is refractory to both the trans-translation and the BrfA mechanisms of ribosome rescue (Supplementary Fig. 1). Thus, we conclude that BrfA is an RF-dependent ribosome rescue factor in...
**B. subtilis** that rescues stalling on non-stop mRNAs, but not regulatory stalling peptides, leading us to suggest the renaming of Tykk to BrfA (**Bacillus ribosome rescue factor A**). To our knowledge, RF-dependent ribosome rescue factors have not been reported previously in Gram-positive bacteria.

**Low interspecies compatibility of ribosome rescue systems.** BrfA homologs are conserved among a subset of **Bacillaceae** family members, mainly among those belonging to the **Bacillus** genus. Although we do not rule out the possibility that BrfA and ArfA share the same evolutionary origin, their distinct phylogenetic distributions and the unique sequence features (Supplementary Fig. 2) seem to favor the notion that BrfA and ArfA have evolved independently of each other and independently from ArfT and ArfB. The narrow phylogenetic distributions of the RF-dependent rescue factors also implies that they emerged relatively late in evolution. Translation components are largely conserved across the species, but they have undergone some micro diversifications. If the alternative rescue factors evolved more recently than the translation factors, their interactions with the translation components, such as the ribosome and an RF, might be species-specific. Indeed, **Francisella tularensis** ArfT can work with **F. tularensis** RF1 or RF2, but not **E. coli** RFs. Also, **E. coli** ArfA fails to recruit **Thermus thermophilus** RF2 (refs. 37, 38). We tested the compatibility of **B. subtilis** BrfA and **E. coli** ArfA with heterologous RFs in vitro. While BrfA efficiently hydrolyzed GTP-tRNA in the Bs PURE system supplemented with RF1 and RF2 of **B. subtilis** (Fig. 3c, lanes 1 and 2), it did not work with the **E. coli** RFs (lanes 3 and 4). Interestingly, BrfA was functional when the substrate was translated by the **E. coli** ribosome, provided that the **B. subtilis** RFs were available (lanes 9–10). These results suggest that BrfA interaction with RF2 is species-specific, but its interaction with the ribosome is rather promiscuous.

By contrast, we could show that **E. coli** ArfA requires both the RF2 as well as the ribosomes to be derived from **E. coli**. Specifically, ArfA did not work if combined with the **E. coli** RF2 when the substrate was translated by the **B. subtilis** ribosomes (Fig. 3c, lanes 7 and 8), nor did it work with **B. subtilis** RF2 (lanes 5 and 6), even if the substrate was translated by **E. coli** ribosomes (lanes 13 and 14). The **E. coli** ArfA-mediated hydrolysis of GTP-tRNA was only observed when the ribosomes and RFs were both derived from **E. coli** (lanes 15 and 16). Thus, **E. coli** ArfA is incompatible with the **B. subtilis** ribosomes and RF2, analogously to what was previously observed between **E. coli** ArfA and **T. thermophilus** RF2 (refs. 37, 38). The high specificity of molecular interactions involving the RF-dependent rescue factors is in contrast to the broader interactions in the tmRNA- and ArfB-based rescue pathways (see Discussion).

**Cryo-EM structure of a BrfA-RF2 non-stop ribosome complex.** Since **B. subtilis** BrfA and RF2 can rescue **E. coli** ribosomes stalled on truncated non-stop mRNAs (Fig. 3c, Supplementary Fig. 3), we formed BrfA-RF2 non-stop 70S ribosome (ns70S) complexes by incubating **B. subtilis** BrfA and RF2 with **E. coli** ns70S complexes used previously for ArfA (39). By substituting the wild-type **B. subtilis** RF2 with a catalytically inactive GGP mutant (40, 41), peptidyl-tRNA hydrolysis and therefore recycling of the ns70S complex was prevented (Supplementary Fig. 3). Cryo-EM analysis of the BrfA62His-RF2-GGP-ns70S complex (herein referred as BrfA-RF2-ns70S) and extensive in silico sorting of this dataset yielded a major subpopulation of ribosomal particles (>80%) that contained stoichiometric occupancy of BrfA, RF2 and P-site tRNA (Supplementary Fig. 4). Refinement of this subpopulation led to a final cryo-EM reconstruction of the BrfA-RF2-ns70S (Fig. 4a), with an average resolution of 3.06 Å (Supplementary Fig. 4, Supplementary Table 1). The cryo-EM density for BrfA was well-resolved with local resolution ranging between 3.0–3.6 Å (Fig. 4b), enabling residues 2–55 of BrfA to be modeled de novo (Fig. 4c, d). BrfA contains an N-terminal α-helix α1 (residues 4–17) followed by a short α-helical turn (α2, residues 21–25) and β-strand (β1, residues 35–38) as well as a short C-terminal α-helix (α3, 40–47) followed by a positively charged region (residues 48–55) (Fig. 4d).

**Interaction of BrfA with the non-stop 70S ribosome.** The binding site of BrfA is located predominantly on the 30S subunit in the vicinity of the decoding center, where it spans from the top of helix 44 (h44) of the 16S tRNA past the ribosomal protein uS12 and reaches into the mRNA channel formed by the head and body of the 30S (Fig. 4a). The overall binding site of BrfA on the ribosome (Fig. 4a) is similar, but distinct, to that observed previously for **E. coli** ArfA (37–39, 42–44). The N-terminal helix α1 of BrfA resides within the intersubunit space, where highly conserved charged residues (Supplementary Fig. 2) establish interactions with the major groove of h44 and the minor groove of H71 of the 23S tRNA (Fig. 4e). In addition, Arg25 within helix α2 of BrfA, which is conserved in all BrfA sequences (Supplementary Fig. 2), stacks upon U1915 and flips C1914 out of H69, where it stacks upon His133 of RF2 (Fig. 4f). This contrasts with the canonical conformation of C1914 within H69 that is observed during translation termination (Fig. 4g) as well as ArfA-mediated ribosome rescue. Indeed, these N-terminal α-helices have no counterpart in ArfA, instead the N-terminus of ArfA is unstructured and folds back to interact with uS12 (refs. 37–39, 42–44) (Supplementary Fig. 5a–c).

The C-terminal region of BrfA extends from the decoding center into the mRNA channel and would be incompatible with the presence of a full-length mRNA (Fig. 4h), but compatible with a truncated non-stop mRNA (Fig. 4i). BrfA exhibits a modest overlap with the second (+2) and third (+3) nucleotide of the A-site codon, but extensive steric clashes would be expected for the subsequent positions (+4 onwards) (Fig. 4i), similar to that observed previously for ArfA (37–39, 42–44) (Supplementary Fig. 5d–i). Thus, BrfA may also recycle ribosomes stalled on non-stop mRNAs with 1–3 nucleotides extending into the A-site, as shown experimentally for ArfA (45–47). The positively charged C-terminus of BrfA can form multiple hydrogen bond interactions with 16S rRNA nucleotides that comprise the mRNA channel (Fig. 4j). While the interaction network is generally distinct from that observed for ArfA, we note that the mode of contact between the side chains of Lys49 and His50 of BrfA with U534 of the 16S tRNA appears to be shared by ArfA (37–39, 42–44) (Supplementary Fig. 5j–l).

**BrfA stabilizes an open conformation of RF2 on the ribosome.** The structure of the BrfA-RF2-ns70S reveals that BrfA recruits RF2 by establishing an extensive interaction surface, specifically encompassing the central portion (residues 30–40) of BrfA and domain 2 (d2) of RF2 (Fig. 5a). Similar to ArfA (37–39, 42–44) (Supplementary Fig. 6a–c), BrfA also donates the small β-strand (β1) to augment the β-sheet of the superdomain d2/d4 of RF2 (Fig. 5a). The overall position of RF2 in BrfA-RF2-ns70S is similar, but slightly shifted, compared to that observed during canonical translation termination (40, 48) (Fig. 5b, Supplementary Fig. 6d–f). The shift is larger and more global than reported previously for ArfA (37–39, 42–44) (Supplementary Fig. 6a–c), which may arise in part due to the differences between **B. subtilis** and **E. coli** RF2s. The shift affects the loop between the β4 and β5 strands of d2 bearing the SPF (**B. subtilis** 202-Ser-Pro-Phe-204) motif, which is involved in the specificity of recognition of the first and second positions of UGA/UAA stop codons (32, 40, 48).
Importantly, the structure illustrates that BrfA, like ArfA, does not interact with the SPF motif and therefore does not directly mimic the presence of a stop codon (Fig. 5c, Supplementary Fig. 6g–i), which is consistent with our observation that mutations in the SPF motif that impair RF2 termination activity do not affect BrfA-RF2-mediated ribosome rescue (Fig. 3b).

During canonical termination, recognition of the stop codon by RF2 (and RF1) is proposed to stabilize a distinct conformation of the switch loop that directs domain 3 into the PTC (49). The switch loop conformation is stabilized by stacking interactions between Trp318 (B. subtilis numbering) of RF2 and A1492 (h44) as well as between A1493 (h44) and A1913 (38).

![Fig. 4 Cryo-EM structure of BrfA-RF2-ns70S complex.](image)

**Fig. 4** Cryo-EM structure of BrfA-RF2-ns70S complex. a Different views of the cryo-EM map of the BrfA-RF2-ns70S complex with isolated densities highlighting the 30S (yellow: hd, head; bd, body) and 50S (gray: CP, central protuberance) subunits, P-site tRNA (green), RF2 (orange), and BrfA (blue). b,c Isolated electron density for BrfA (b) colored according to local resolution and (c) shown as mesh (gray) with fitted molecular model for BrfA. d Model for BrfA with features highlighted corresponding to the schematic of BrfA protein, including α-helical and β-strand regions. e The N-terminus of BrfA (blue) interacts both h44 of the 16S rRNA (yellow) and H69 and H71 of the 23S rRNA (gray). f The conserved R25 of BrfA (blue) stacks upon U1915 and causes C1914 to flip out and stack upon H133 of RF2. g Same view as f but showing the RF2stop (lime) and the conformation of H69 for a canonical termination complex (PDB ID 4V5E). h Transverse section of the 30S subunit (yellow) to reveal the mRNA channel showing a superimposition of full-length mRNA (FL-mRNA, cyan) with truncated non-stop mRNA (TR-mRNA, teal), P-site tRNA (green), and surface representations of BrfA (blue). i Superimposition of FL-mRNA (cyan) with TR-mRNA (teal), P-site tRNA (green), and transparent surface representation of BrfA (blue). The first (+1), second (+2), and third (+3) nucleotides of the A-site codon of the FL-mRNA are indicated. j Interaction of the C-terminus of BrfA (blue) with the 16S rRNA showing potential hydrogen bonds with yellow dashed lines.
(Fig. 5d). In the BrfA-RF2-ns70S, the presence of BrfA precludes a direct interaction between Trp318 and A1492 (Fig. 5e). Rather, BrfA appears to stabilize a similar conformation of A1492 through stacking interactions with His34 and Arg36 (Fig. 5e), whereas a completely distinct conformation of A1492 (and A1493) is adopted in the presence of ArfA (Supplementary Fig. 7a–c). Additional stacking interactions are also observed between Phe31 of BrfA and Trp318 within the switch region of RF2 (Fig. 5e), which we suggest facilitates the transition from the closed to the open form of RF2 (Fig. 5f, Supplementary Fig. 7d–f) and thereby enables placement of the GGQ motif within domain 3 (d3) of RF2 at the PTC of the ribosome.

**Discussion**

We have shown that Gram-positive bacteria, such as *Bacillus*, possess an RF-dependent ribosome rescue pathway, which had previously been known to occur only in Gram-negative bacteria (Fig. 6a). In this pathway in *B. subtilis*, BrfA plays a critical role in the hydrolytic release of the incomplete polypeptide from the non-stop stalled ribosomes. It does so by recruiting RF2 in a stop-codon-independent manner to the otherwise dead-end translation complex, as shown by our biochemical experiments using purified components. Our cryo-EM structure also reveals that BrfA recognizes the empty mRNA channel of a non-stop ribosome complex to recruit and stabilize the active (open) conformation of RF2 on the ribosome (Fig. 6b), in a similar but distinct manner to ArfA37–39,42–44 (Fig. 6c).

In vivo, the *brfA* deletion mutation exhibits a synthetic lethal phenotype when combined with CRISPRi-mediated knock-down or deletion of either SsrA or SmpB. Thus, BrfA is essential for growth in the absence of *trans*-translation activity. These findings answer the long-standing question of why the *trans*-translation system is not essential in *B. subtilis*, which lacks ArfA, ArfB and ArfT. Instead, BrfA is the alternative ribosome rescue factor in this organism and some other *Bacillus* species. The growth requirement of *B. subtilis* for BrfA and *trans*-translation is observed under normal growth conditions without the imposition of proteotoxic stresses. Thus, clearing of constitutively produced aberrant states of translation might be critical for cell survival, and BrfA and *trans*-translation components are the major players in this basic quality control. In this regard, the recently reported RqCH pathway, which adds a protein-degrading polyalanine sequence to the arrested polypeptide on the split 50S subunit of the ribosome50, appears to play a minor role in ribosome rescue under normal growth conditions, since RqCH is dispensable for *trans*-translation deficient strains to grow in the absence of stress. By contrast, RqCH appears to be required under more harsh conditions, such as high temperature and the presence of translation-disturbing drugs50.

During BrfA-mediated ribosome rescue, peptidyl-tRNA hydrolysis depends on an intact GGQ motif of RF2, indicating...
BrfA appears to facilitate ribosome rescue using a different mechanism than reported previously for ArfA. For example, during stop codon decoding, the switch loop Trp stacks directly upon A1492 of the 16S rRNA (Fig. 5d, Supplementary Fig. 7a), whereas during BrfA-mediated recycling, a series of stacking interactions appear to indirectly relay this information—specifically, Phe31 of BrfA stacks upon the switch loop Trp318 of RF2, while His34 (and Arg36) of BrfA stacks upon A1492 (Fig. 5e, Supplementary 7b). By contrast, during ArfA-mediated rescue, the equivalent Trp residue inserts into a hydrophobic pocket created by ArfA and A1492 adopts a completely unrelated conformation (Supplementary Fig. 7c).

Perhaps the most dramatic difference between BrfA and ArfA is found in the N-terminal region, where BrfA contains two short α-helices that establish multiple interactions with H69 and H71 of the 23S rRNA, whereas the N-terminus of ArfA is unstructured and does not establish any interactions with the 50S subunit (Supplementary Fig. 5a–c). Despite this lack of sequence and structural homology, there are some common features between the two rescue systems: Firstly, both ArfA and BrfA (as well as SmpB and ArfB) appear to utilize positively charged C-terminal extensions to interact with the negatively charged 16S rRNA comprising the mRNA channel. Although the details of the interactions are unrelated, we identified a Lys-His (K49-H50 in B. subtilis) motif that is conserved between BrfA and ArfA, which is used to establish contacts to the backbone of nucleotide U534 of the 16S rRNA (Supplementary Fig. 5j–l). Second, both ArfA and BrfA contain short β-strands that augment the β-sheet in domain 2/4 of RF2, which we presume is important to recruit RF2 to the ribosome (Supplementary Fig. 5a–c).

Here we show that BrfA works with RF2, but not RF1, reminiscent of the partner selectivity described previously for ArfA. Analysis of the contacts between BrfA and RF2 within the BrfA-RF2-ns70S complex, and comparison with the sequence alignments between B. subtilis RF2 and RF1, indicated that there are two main regions in the RFs that are likely to be responsible for the selectivity of BrfA (Supplementary Fig. 8). These encompass residues within the β-sheet of domain 2/4 of RF2 that are in proximity of Phe31 of BrfA. For example, in RF2 residues 197 and 216 are generally Val and Phe, respectively, whereas in RF1 the equivalent residues are replaced with the smaller Ala and Thr residues, respectively (Supplementary Fig. 8a). More dramatic, however, is the lack of sequence conservation between the switch loops of B. subtilis RF1 and RF2. In fact, the switch loop of RF1 is one residue longer than in RF2 (Supplementary Fig. 8b, d). We also note that the RF-dependent rescue factors show low interspecies compatibility (Fig. 3c). The incompatibility of BrfA to work with E. coli RF2 is not surprising given the relatively low sequence conservation observed within the switch region (Supplementary Fig. 8d). Moreover, sequence differences are also observed with α-helix 7 between B. subtilis and E. coli RF2 that could contribute to the interactions with BrfA (Supplementary Fig. 8c). Collectively, these differences suggest that even if BrfA could recruit B. subtilis RF1 or E. coli RF2 to the ns70S, the sequence differences in the switch loop are unlikely to stabilize the open conformation of RF2 on the ribosome. The lack of interspecies compatibility of BrfA (and ArfA) contrasts with other, more ubiquitous ribosome rescue factors. For example, B. subtilis tmRNA is functional in E. coli, albeit with lower efficiency, and the human mitochondrial ArfB homolog, ICT1, is functional in C. crescentus and vice versa. This low interspecies compatibility of the RF-dependent rescue factors reinforces the notion that these rescue systems emerged late in evolution.

It is crucial for the ribosome rescue factors not to intervene during normal translation, nor when translation is arrested by nascent polypeptides for regulatory purposes. Our cryo-EM
structure of the BrfA-RF2-ns70 reveals that BrfA uses its C-terminal region to monitor the vacancy of the ribosomal mRNA channel, a hallmark of the ribosomes stalled on the 3′ end of mRNA, and thereby discern non-stop translation complexes from those of ongoing translation. Consistently, we have shown that neither BrfA, nor trans-translation, can resolve the MiIM-programmed elongation arrest complexes (Supplementary Fig. 1). In this case, the mRNA channel should remain occupied by the miIM mRNA. Also, the cryo-EM structures of the MiIM-stalled ribosomes reveal altered conformations of 23S rRNA residues near the peptidyltransferase center (PTC) such that they block accommodation of aminoacyl-tRNAs or mimics thereof into the A-site, explaining why MiIM stalling is refractory to the BrfA-RF2 and the SmpB-tRNA actions, too.

We have shown that brfA is transcribed as a non-stop mRNA because of the terminator sequence within the coding region. Thus, brfA itself is subject to a futile type of translation, producing a non-stop polypeptide that is extended by the SsaR tag sequence and rapidly eliminated by proteolysis. BrfA accumulates only when translation is impaired, and in the form of peptidyl-tRNA. However, once some free BrfA product is generated by spontaneous hydrolysis of the BrfA peptidyl-tRNA, or by the presence of residual BrfA in the cell, it actively liberates in trans the BrfA peptide in a self-perpetuating manner. We have shown clearly using biochemical assays that a liberated form of the non-stop BrfA peptide (residues 1–62) is active in mediating the RF2-dependent peptidyl-tRNA hydrolysis.

Because BrfA is not effectively produced in trans-translation proficient cells, but induced strikingly upon dysfunction of trans-translation, it is likely to represent a secondary, back-up rescue system that compensates for defects in trans-translation. This scenario reinforces the notion that the proteolytic function characterized associated with the SmpB-tRNA system is not essential for growth, as reported previously. Indeed, the GFP-ns non-stop product accumulates in the ssaR-deleted cell (Supplementary Fig. 1a), indicating that the products of the BrfA system are not necessarily toxic. Thus, the growth-essential roles of trans-translation and BrfA are in their ribosome recycling functions, rather than in the tagging-proteolysis that the BrfA system lacks. Cell viability would require a sufficient pool of the uncompromised ribosomes, which maintains the translation capacity of the cell. Whereas the two pathways share the essential function required for growth-supporting ribosome rescue, the proteolytic functions of the trans-translation and that of the RqH tail-adding system could become more important under more severe stress conditions.

The regulatory scheme of BrfA expression is strikingly similar to that elucidated for ArfA regulation in E. coli. The arfA mRNA is also subject to RNase III-dependent cleavage and/or transcription termination such that ArfA only accumulates when trans-translation is defective. Assuming that BrfA and ArfA are evolutionarily unrelated, it is noteworthy that they employ a similar scheme of regulation. Convergent acquisition of such regulatory mechanisms may be more common for factors that have evolved recently and which have functions related to the firmly established and relatively rigid constituent of the cell, such as the ribosome and translation factors.

In summary, our study reveals that bacteria, both Gram-negative and Gram-positive, have RF-dependent mechanisms of ribosome rescue that allow for the stop-codon-independent liberation of the polypeptide from the ribosome on the non-stop mRNA (Fig. 6a). However, the crucial adapter proteins, ArfA, ArfT, and BrfA, are unrelated in amino acid sequence (Supplementary Fig. 2). The modes of their interactions with the catalytic RF partner(s) are also divergent, indicative of the tailored nature of their evolution in different species. It remains possible that many more factors in this category exist in different organisms. As the present study suggests that sequence similarity alone cannot be used to identify additional factors that exist in the domains of life, we need better strategies to address this question. Further studies on the generality and diversity of independently evolved rescue factors and their manner of interaction with the ribosome and translation factor would provide invaluable insights into regulatory mechanisms of translation processes in the cell.

Methods

Bacterial strains and plasmids. B. subtilis and E. coli strains, plasmids, DNA oligonucleotides used in this study are listed in Supplementary Tables 2, 3, 4, and 5, respectively. The B. subtilis strains were derivatives of PY79 (wild-type; ref. 54) and constructed by transformation that involves homologous recombination with plasmids listed in Supplementary Table 6. These plasmids carried an engineered B. subtilis gene to be integrated, which was flanked by sequences from the integration target loci, and were constructed by standard cloning methods including PCR, PrimeSTAR mutagenesis (Takara), and Gibson assembly. The plasmid pCH74 was constructed by cloning a Spil–SfI fragment of pCH735 into psyqG21. Plasmids pCH735 and psyqG21 were constructed as described. Successful integration of a gene into the chromosome was accomplished by double crossing-over at the target loci. The resulting recombining clones were checked for their antibiotic-resistance markers, including the absence of those originally present on the plasmid backbone, and inactivation of the amyE, lacA, or thrC target locus. The marker-less deletion mutants of smpB, yesZ, and lacA were constructed by excising the drug resistance gene cassette by the Cre-loxP system as described previously with some modification. The B. subtilis strains were transformed to a pLOSS*-based Ts plasmid harboring cec. The resulting strain was grown at 37°C overnight in LB agar medium supplemented with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and 100 μg/mL spectinomycin to excise the drug marker flanked by lopP7. The strain was then grown at 37°C overnight to drop off pmK2 on LB agar medium without spectinomycin. The absence of the drug resistance confirmed the absence of plasmid pmK2. The B. subtilis strain KFB792 was constructed by transformation of PY79 with a DNA fragment prepared by Gibson assembly with three PCR fragments, one of which was amplified from pCH1142 using a pair of primers SP89/SP90, and the other two of which were amplified from PY79 genomic DNA using pairs of primers SP91/SP92 and SP93/SP94, respectively.

Growth conditions and general procedures. For western blotting in Fig. 2c, B. subtilis cells were cultured at 37°C in LB medium with or without 0.5% xylene until OD600 reached ~0.5. Bacterial culture (1 mL) was treated with 5% trichloroacetic acid (TCA), and precipitates were formed with 0.75 mL of 1 M Tris-HCl (pH 8.0) and resuspended in 50 μL of buffer I (33 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 1 mg/mL lysozyme, followed by incubation at 37°C for 10 min. Proteins were then solubilized with an equal volume of 2× SDS-loading buffer containing 5 mM dithiothreitol (DTT) with incubation at 65°C for 5 min and subjected to SDS-PAGE and immunoblotting. One hundred microliters portions of bacterial cultures were used for assay of β-galactosidase activity presented in Fig. 3d as follows and also as described previously. The cultures were transferred to individual wells of another 96-well plate for a Thermo Scientific Multiskan Go microplate spectrophotometer and OD600 was recorded. Cells were then lysed by adding 50 μL of Y-PEF reagent (Thermo Scientific) and incubating for 20 min at room temperature. Thirty microliters of o-nitrophenyl-β-D-galactoside (ONPG) (in Z-buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4, 38 mM β-mercaptoethanol) was added to each well. The reaction solution was mixed thoroughly and then OD420 and OD550 were measured every 5 min over 60 min at 28°C. Arbitrary units [AU] of β-galactosidase activity were calculated by the formula ([1000 × Vave × 1.3 × V600/OD600], where V420 and V550 are the first-order rate constants, OD420/min and OD550/min, respectively.

Synthetic lethal screening using the BKE strain library. To isolate B. subtilis mutants whose viability depends on trans-translation, we used the BKE library (a collection of single-gene knockout mutants covering the 3968 non-essential genes, which had been disrupted by replacement with the erythromycin resistance marker) as the source of gene knockouts. We pooled the BKE strains and prepared a genomic DNA mixture using Wizard genome DNA purification kit (Promega). We used this DNA preparation to transform an smpB-deleted strain of B. subtilis that harbored a rescue plasmid carrying lacZ′ and lacZβ (pNAB1286), which was constructed from pLOSSβ with a temperature sensitive (Ts) replication system. The transformants were then cultured at 30°C overnight but the Ts plasmid from bacteria that did not need the rescue plasmid, followed by further growth at 37°C overnight and plating on LB agar containing 40 μg/mL X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), 1 mM IPTG, 12.5 μg/mL lincomycin, and 1 μg/mL erythromycin, and we picked up blue (lacZ′) colonies to obtain transformants that retained the smpB′–lacZβ rescue plasmid even after the high-temperature incubation and prepared chromosomal DNA from these. Genes that had been disrupted by the erythromycin resistance marker were determined by
PCR amplification and DNA sequencing of the mutant-specific barcode sequence, using appropriate primers.

CRISPR interference. The CRISPRi was performed as described previously[56] with some modification. A gene encoding dCas9 under an inducible promoter was integrated into the lacO site on the chromosome of a Brb-deleted B. subtilis strain, into which an srrA-targeted or an smpB-targeted sgRNA gene under a constitutive promoter was further integrated at the amyE site. The guide sequences for CRISPRi were designed on the basis of information provided by the previous study[56] as well as CHOPCHOP, a web tool for the CRISPR/Cas9 experiments[57,58]. The target gene knock-down was induced by addition of 1% xylose.

Protein purification. Hexahistidine-tagged proteins (B. subtilis BrfA62-His6, RF1-His6, RF2-His6, E. coli His6-ArfA60, and their derivatives) were expressed in E. coli BL21 (DE3) from the pET28b-based plasmid. E. coli cells were grown in LB kanamycin (25 μg/mL) medium. At a mid-log phase, IPTG (final concentration, 1 mM) was added, and cells were grown for an additional 3 h to express and accumulate the target protein. Cells were then harvested, washed with ice-cold 50 mM HEPES-KOH pH 7.6 buffer, and stored at −80 °C. They were suspended in binding buffer (50 mM HEPES-KOH pH 7.6, 5 mM imidazole, 300 mM NaCl, 1 mM MgCl2, 1 mg/mL Pefabloc) and disrupted by passing through a microflow cell lysis器 (Microfluidics) at 16,000 psi three times. After removal of debris by centrifugation (4 °C, 15,000 rpm for 15 min), Ni-NTA agarose was added to the sample, which was then incubated at 4 °C for 1 h. The Ni-NTA agarose was loaded on a spin column and washed seven times with wash buffer (50 mM HEPES-KOH pH 7.6, 20 mM imidazole, 0.1% Triton-X). Protein was eluted with the elution buffer (20 mM HEPES-KOH pH 7.6, 300 mM NaCl, 300 mM imidazole). Purified RFs were dialyzed against dialysis buffer B (50 mM HEPES-KOH pH 7.6, 100 mM potassium acetate, 1 mM DTT, 30% glycerol). Purified BrfA62-His6 and His6-ArfA (2–60) were dialyzed against dialysis buffer B (50 mM HEPES-KOH pH 7.6, 100 mM potassium acetate, 1 mM DTT, 300 mM NaCl, 30% glycerol).

For the structural analysis, the wild-type RF2 and variant RF2-GGP protein were expressed from PET11a vectors incorporating a C-terminal hexahistidine tag (His6) for purification and detection purposes. The inactive RF2-GGP mutant was generated by site-directed mutagenesis. The wild-type RF2 and variant RF2-GGP proteins were over-expressed in E. coli BL21(DE3) in a T7 expression system with purified E. coli T7 RNA polymerase (Takara) further to the reaction mixture. Whereas the original reaction produced a ns70S complex was loaded onto a linear sucrose gradient (10-40% (w/v) sucrose in 40% (w/v) sucrose in 40% (w/v) sucrose) in buffer B250 (50 mM HEPES-KOH pH 7.2, 250 mM potassium acetate, 25 mM magnesium acetate, 250 mM imidazole, 0.1% DDM). The eluted ns70S complex was loaded onto an analytical sucrose gradient (10–40% (w/v) sucrose in 40% (w/v) sucrose) in buffer B250 (50 mM HEPES-KOH pH 7.2, 250 mM potassium acetate, 25 mM magnesium acetate, 250 mM imidazole, 0.1% DDM) and analysis confirmed by gel filtration using a Superdex 75 column (GE Healthcare) luminoimager. Uncropped immunoblotting data are shown in the Supporting Information. Images were obtained and analyzed using an Amersham Imager 600 (GE Healthcare) luminoimager. Uncropped immunoblotting data are shown in the Supporting Information.

In vitro translation using PURE system. The E. coli-based coupled transcription–translation system with purified components (PURExpress 1.0; Gene- Frontier) was used for in vitro translation with some modifications. To enhance translation, we added 2.5 μL of T7 RNA polymerase (Takara) further to the reaction mixture. Whereas the original reaction mixture contained an inactive RF2-GGP mutant, we added 154,405 particles containing stoichiometric amounts of BrfA, P-site tRNA, and an inactive RF2-GGP mutant produced using HHPred65 based on an E. coli RF2 template (PDB ID 5MGP[9], domains 1 and 3 of RF2 were less well-resolved due to high flexibility (Supplementary Fig. 4e) and a final cryo-EM map was also generated using the Vitrobot Mark IV (FEI, Holland). Data collection was performed using EM-TOOLS (TVIPS GmbH) on a TECNAI Krios transmission electron microscope equipped with a Falcon III direct electron detector (FEI, Holland) at 300 keV at a pixel size of 1.065 Å and a defocus range of 0.4–2.2 μm. Forty frames (dose per frame of 2e− Å⁻²) were aligned using Motion Correction software[59]. Power spectra and defocus values were determined using the GCTF software[60]. Micrographs showing thin rings beyond 3.2 Å were manually inspected for good areas and automatic particle picking was performed using the Gautomatch software (http://www.mrcmb.cam.ac.uk/kzhang/). Single particles were then imported and processed in Relion 3 (ref. 81). In total, 314,119 particles were first subjected to 2D classification (60 classes for 100 rounds) and 389,381 particles showing ribosome-like features were then selected for 3D refinement using an E. coli 70S ribosome as a reference structure (Supplementary Fig. 4a). 3D classification was then performed, resulting in 317,095 particles containing P-site tRNA and RF2 (Supplementary Fig. 4b) that were further selected for focus sorting on the RF2 (Supplementary Fig. 4c). Focus sorting yielded a major population of 154,605 particles containing stoichiometric amounts of BrfA, P-site tRNA, and an inactive RF2-GGP mutant which after CTF refinement and a final cryo-EM refinement produced a final cryo-EM reconstruction with an average resolution of 3.06 Å according to FSC143 criterion (Supplementary Fig. 4d). The final cryo-EM maps were sharpened by dividing the maps by the modulation transfer function of the detector and applying an automatically determined negative B factor in Relion 3 (ref. 81). The final cryo-EM map was also filtered according to local resolution using SPHIRE[82].

Molecular modeling of the BrfA-RF2<sub>ns70S</sub> complex. The molecular model for the ribosomal proteins and rRNA core was based on the molecular model from the B. subtilis 70S ribosome (PDB ID 5AXE[83] and 5MGF[9]), the models were rigid body fitted into the cryo-EM density map using UCSF Chimera followed by refinement in Coot[84]. Proteins L1, L10, L11 protein, and the L7/L12 stalk were not included in the final model due to the poor quality of density in the final cryo-EM map. For B. subtilis RF2, a homology model was generated using I-TASSER[85] based on an E. coli RF2 template (PDB ID 5MGP[9]), Domains 1 and 3 of RF2 were less well-resolved due to high flexibility (Supplementary Fig. 4e) and therefore only the backbone was modeled. Residues 2–55 of BrfA were built de novo using an HHPred model[86] as an initial starting point to determine the placement of the central helical regions. The complete atomic model of the BrfA-RF2-RF1<sub>ns70S</sub> complex was manually adjusted to fit the cryo-EM density with phenix real_space_refine for cryo-EM[87] using restraints obtained by phenix secondary_structure_restraints[88]. The model refinement and statistics of the refined model were obtained using MolProbity[89] (Supplementary Table 1).

Figure preparation. Figures showing electron densities and atomic models were generated using either UCSF Chimera, UCSF ChimeraX[90] or PyMol (Version 1.8 Schrödinger). The comparison of BrfA with full-length mRNA (PDB ID 4V5E[8]) from ArfA (PDB ID 5MGF[9], 5MVD[57], 5UG9[9]), RF2<sub>intron</sub> (PDB ID 1GQE), and RF2<sub>stop</sub> (PDB ID 4V5E[8]) was obtained by alignment of the 16S rRNAs from the respective structures using PyMol (Version 1.8 Schrödinger).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.
Data availability
The cryo-EM map of the BrfA-RF2-nos70S complex is available through the EMDB code EMDB-10353 and the associated molecular model is deposited in the Protein Data Bank with the entry code 6ZS (https://doi.org/10.2210/pdb6ZS/pdb). The data that support the findings of this study are available from the corresponding authors on request. The source data underlying Figs 2c, d and 3a–c and Supplementary Figs 1a, b and 3 are provided as a Source Data file.

Received: 2 August 2019; Accepted: 7 November 2019; Published online: 27 November 2019

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Acknowledgements

We thank Dr. Hyouta Himeno of Hirosaki University for the *B. subtilis* ssrA mutant strain; Dr. Jiří Nováček (CEITEC, Brno, Czech Republic) for help with high-resolution cryo-EM data collection; Dr. Allen Buskirk for discussion; and Yoko Sumino, Saori Amano, Sakika Otani, Toru Irie, S. Rieder, and C. Ungewickell for expert technical support. We are also thankful to the National BioResource Project for providing the BKE strain library. This work was supported by grants from MEXT and JSPS Grant-in-Aid for Scientific Research (Grant No. 25291006 and 16H04788 to S.C., 26116008 to S.C. and K.I., 19K16044 to K.F.) and the Deutsche Forschungsgemeinschaft (SPP1679-WI3285/5-2 to D.N.W.).

Author contributions

N.S.-C., K.F., and S.C. performed biochemical experiments and C.M., B.B., and D.N.W. performed structural experiments. N.S.-C., K.F., K.I., S.C., C.M., B.B., and D.N.W. designed experiments, analyzed data, and were involved in writing the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41467-019-13408-7.

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Peer review information Nature Communications thanks the anonymous reviewers for their contribution to the peer review of this work. Peer reviewer reports are available.

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