

Release of Nonstop Ribosomes Is Essential

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ABSTRACT Bacterial ribosomes frequently translate to the 3' end of an mRNA without terminating at a stop codon. Almost all bacteria use the transfer-messenger RNA (tmRNA)-based *trans*-translation pathway to release these “nonstop” ribosomes and maintain protein synthesis capacity. *trans*-translation is essential in some species, but in others, such as *Caulobacter crescentus*, *trans*-translation can be inactivated. To determine why *trans*-translation is dispensable in *C. crescentus*, a Tn-seq screen was used to identify genes that specifically alter growth in cells lacking *ssrA*, the gene encoding tmRNA. One of these genes, *CC1214*, was essential in Δ *ssrA* cells. Purified *CC1214* protein could release nonstop ribosomes *in vitro*. *CC1214* is a homolog of the *Escherichia coli* ArfB protein, and using the *CC1214* sequence, ArfB homologs were identified in the majority of bacterial phyla. Most species in which *ssrA* has been deleted contain an ArfB homolog, suggesting that release of nonstop ribosomes may be essential in most or all bacteria.

IMPORTANCE Genes that are conserved across large phylogenetic distances are expected to confer a selective advantage. The genes required for *trans*-translation, *ssrA* and *smpB*, have been found in >99% of sequenced bacterial genomes, suggesting that they are broadly important. However, these genes can be deleted in some species without loss of viability. The identification and characterization of *C. crescentus* ArfB reveals why *trans*-translation is not essential in *C. crescentus* and suggests that many other bacteria are likely to use ArfB to survive when *trans*-translation is compromised.

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The efficient translation cycle of initiation, elongation, termination, and recycling allows rapid protein synthesis and cellular growth. Most ribosomes pass through this cycle many times every cell generation, so breaks in the cycle can lead to rapid depletion of protein synthesis capacity. A major source of translation stress in bacteria is truncated mRNA. Because bacterial ribosomes do not require any information from the 3' end of an mRNA for initiation, they can start translating mRNAs that are incompletely transcribed or damaged (1). If the mRNA does not have a stop codon or if the ribosome reads through the stop codon and reaches the 3' end of the mRNA without terminating, the translation cycle will be broken. A stop codon in the decoding center is required for release factors RF1 or RF2 to hydrolyze the peptidyl-tRNA and allow the ribosome to be recycled (2). When the ribosome is at the 3' end of the mRNA but there is no stop codon in the decoding center, *trans*-translation is used to resolve the “nonstop” translation complex and allow the translation cycle to resume (3). During *trans*-translation, transfer-messenger RNA (tmRNA), encoded by the *ssrA* gene, and SmpB, a small protein, recognize a nonstop translation complex and divert the ribosome onto a reading frame within tmRNA. A stop codon at the end of the tmRNA reading frame allows translation termination and ribosome recycling. *trans*-translation also targets the nascent polypeptide and problematic mRNA for rapid degradation, clearing the cell of all components of the nonstop translation complex (4, 5).

Genes encoding tmRNA and SmpB are found in >99% of se-

quenced bacterial genomes (6), suggesting that *trans*-translation confers a selective advantage under most environmental conditions. In culture, *trans*-translation is essential in some species. However, *ssrA* can be deleted in some bacteria without causing a severe phenotype (7).

The apparent contradiction between the universal conservation of *trans*-translation and the mild phenotype caused by removing this system in *Escherichia coli* was explained by the discovery of ArfA (alternative rescue factor A). The *arfA* gene was identified in a screen for mutations that were synthetically lethal with an *ssrA* deletion (8). ArfA is a small protein that allows RF2 to hydrolyze peptidyl-tRNA on nonstop translation complexes (9, 10). ArfA is a true backup system for *trans*-translation, because ArfA itself is made from a truncated mRNA (11, 12). When *trans*-translation is functional, ArfA is tagged by *trans*-translation and degraded. If *trans*-translation is disabled or overwhelmed, untagged ArfA is produced to release the accumulating nonstop translation complexes. ArfA activity allows *E. coli* to live without *trans*-translation, but in Δ *arfA* cells, *ssrA* is essential (8). Likewise, *ssrA* is essential in *Shigella flexneri*, which does not have *arfA*, but *ssrA* can be deleted in *S. flexneri* cells expressing the *E. coli arfA* gene (13). *arfA* genes have been identified in many *Gammaproteobacteria* and some *Betaproteobacteria*, and this may explain why *ssrA* can be deleted in species such as *Salmonella enterica* and *Yersinia pestis* (7, 8, 11). Other bacteria, such as *Caulobacter crescen-*

TABLE 1 Selected genes identified by Tn-seq

Gene	No. of insertions in indicated background ^a		Log ₁₀ ratio ^b
	Δ <i>ssrA</i>	Wild type	
CC1214	1	564	-2.45
tRNA ^{Arg} (CCU)	0	214	-2.33
<i>rluD</i>	39	2,930	-1.87
<i>lepA</i>	90	5,478	-1.78
<i>Efp</i>	3	139	-1.54
<i>pth</i>	0	24	-1.39
CC1215	85,851	1,011	1.93
<i>smpB</i>	1,644	38	1.62

^a Number of insertions normalized with respect to total number of insertions in Δ *ssrA* background.

^b Ratio as calculated after adding 1 to the number of insertions.

tus, do not have an *arfA* homolog but still do not require *trans*-translation for viability.

This paper describes the results of a screen using transposon mutagenesis followed by sequencing (Tn-Seq) to identify genetic interactions with *ssrA* in *C. crescentus*. One gene, *CC1214*, was shown to be essential in Δ *ssrA* cells but not in wild-type cells. The *CC1214* protein could hydrolyze peptidyl-tRNA in nonstop translation complexes *in vitro* and had homology to *E. coli* ArfB. The phylogenetic distribution of *CC1214* homologs raises the possibility that all or most bacteria require at least one system to resolve nonstop translation complexes.

RESULTS

Identification of genetic interactions with *ssrA*. Genetic interactions with *ssrA* were probed using Tn-seq. Wild-type and Δ *ssrA* cultures were individually mutagenized with the *himar1* transposon and plated on selective medium to allow the growth of cells with an integrated transposon. Colonies were pooled and sequenced to identify the location of transposon insertions. In this experiment, the number of times each sequence is recovered depends on the frequency of transposon insertions at that site and the amount of colonies grown from the mutant cells. Because the frequency of transposition at each site is a function of the DNA sequence and chromosomal context, which are the same in both strains, the ratio of the number of times a sequence is recovered in Δ *ssrA* and wild-type cultures provides a measure of the relative fitness of the mutant in each strain background. Mutants with a low ratio are relatively more fit in the wild-type background, whereas mutants with a high ratio are relatively more fit in Δ *ssrA* cells. Sequence data were pooled to generate a ratio for each gene (Table 1; see also Table S1 in the supplemental material).

Genes with a low ratio include *rluD*, *lepA*, *efp*, *pth*, and tRNA^{Arg}(CCU), which have genetic or functional interactions with *trans*-translation in *E. coli*. Deletion of *rluD* increases the frequency of stop codon readthrough and increases the tagging of *E. coli* proteins (14). LepA (EF4) is a translation elongation factor, and overexpression of *lepA* inhibits *trans*-translation (15). EF-P promotes translation through polyproline sequences and other sequences that stall translation elongation (16). Deletion of *rluD*, *lepA*, or *efp* is likely to generate more substrates for *trans*-translation, so it is not unexpected that mutations in these genes would have a synthetic phenotype in the Δ *ssrA* background. Multiple genetic interactions between *pth* and *ssrA* have been reported in *E. coli*, including exacerbation of *pth* mutant phenotypes in

Δ *ssrA* cells, so it is not surprising that mutation of *pth* in *C. crescentus* Δ *ssrA* cells results in relatively lower fitness (17). tRNA^{Arg}(CCU) is a rare tRNA, and the amount of *trans*-translation of genes with an AGG codon in *E. coli* is negatively correlated with the abundance of tRNA^{Arg}(CCU) (18).

CC1214, annotated as a peptidyl-tRNA hydrolase family protein, had the lowest ratio of any gene (Fig. 1, Table 1). Sequence homology searches suggested that *CC1214* encoded a homolog of *E. coli* ArfB, a gene identified as a multicopy suppressor of lethality in *E. coli* cells lacking both *ssrA* and *arfA* (19). *E. coli* *arfB* cannot support viability from its chromosomal context (8). Nevertheless, the sequence similarity to ArfB made *CC1214* a candidate for releasing nonstop complexes in the absence of *trans*-translation in *C. crescentus*.

Genes with a high ratio include *smpB* and *CC1215* (Table 1). Deletion of *smpB* in wild-type cells causes slow growth, but deletion of *smpB* in Δ *ssrA* cells does not cause any additional growth defect, resulting in a relatively greater fitness in the Δ *ssrA* background. Because a large number of insertions in *CC1215* were recovered in both strains, the high ratio for *CC1215* is likely due to increased fitness in Δ *ssrA* cells. Because *CC1214* and *CC1215* are in an operon, the interactions of both these genes with *ssrA* were investigated.

***CC1214* deletion is synthetically lethal with *ssrA* deletion.** The Tn-seq data suggest that cells lacking both *trans*-translation and *CC1214* are at a severe competitive disadvantage. To characterize the phenotype of cells lacking *CC1214*, the gene was replaced with an Ω cassette encoding spectinomycin resistance, using 2-step recombination (20). The *CC1214:: Ω* strain grew at the same rate as the wild type (Table 2) and showed no morphological defects. In contrast, *CC1214* could not be deleted in Δ *ssrA* cells using the same procedure, suggesting that *CC1214* is essential in the absence of *trans*-translation.

A cotransduction experiment was used to confirm that loss of *CC1214* is lethal in the Δ *ssrA* background. A gene conferring kanamycin resistance was inserted in the chromosome of *C. crescentus* *CC1214:: Ω* ~22 kb from the *CC1214* locus to make strain KCK428. Based on their separation on the chromosome, the kanamycin and spectinomycin resistance markers would be expected to cotransduce using phage ϕ CR30 at a frequency of 45%. Consistent with this prediction, when a ϕ CR30 lysate was prepared from KCK428 cells and used to infect wild-type *C. crescentus*, 52% of kanamycin-resistant transductants were also spectinomycin resistant (Table 3). In contrast, when the ϕ CR30 lysate was used to infect Δ *ssrA* cells, none of the 150 kanamycin-resistant colonies were spectinomycin resistant. When Δ *ssrA* cells containing a plasmid-borne copy of *ssrA* were used as the recipients, the cotransduction frequency was restored to 51%. Likewise, when Δ *ssrA* cells containing a plasmid-borne copy of *CC1214* were used as the recipients, the cotransduction frequency was 55%. These results indicate that *C. crescentus* cells lacking both *ssrA* and *CC1214* cannot grow under typical culture conditions.

***CC1215* interactions with *ssrA* are due to polar effects on *CC1214*.** The Tn-seq data indicated that *himar1* insertions in *CC1215* confer a competitive advantage in Δ *ssrA* cells. *CC1215* could be replaced with the *cat* gene, encoding chloramphenicol resistance, in both wild-type and Δ *ssrA* cells. Insertions of *cat* in the sense and antisense orientation relative to *CC1215* were recovered in both strains. Deletion of *CC1215* did not alter the growth rate of strains in liquid culture or on plates (Table 2), indicating

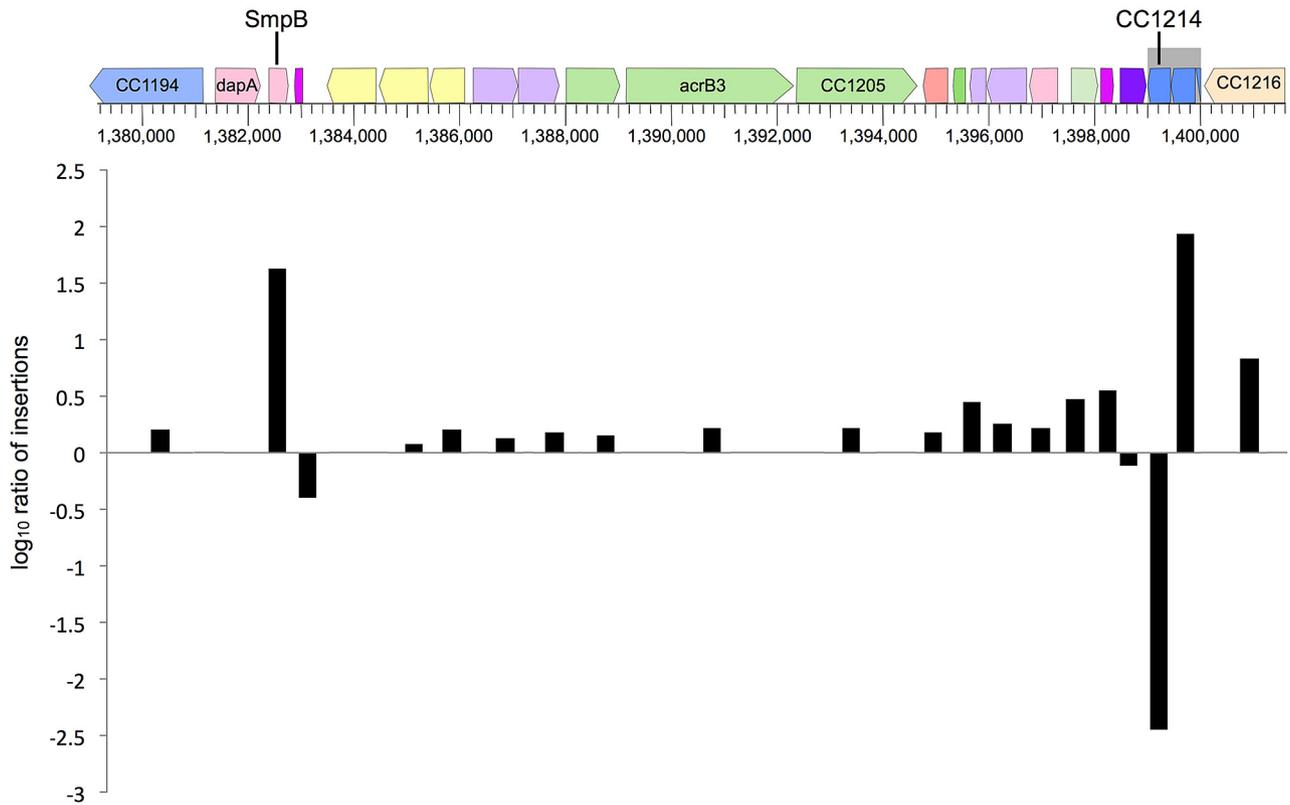


FIG 1 Tn-seq identification of genes interacting with *ssrA*. After Tn-seq, the \log_{10} ratio of the normalized number of transposon insertions in $\Delta ssrA$ cells to the number in wild-type cells was calculated and plotted for a portion of the *C. crescentus* chromosome. The organization of genes and the direction of transcription are represented with colored arrows.

that the increased recovery of *himar1* insertions in $\Delta ssrA$ cells in the Tn-seq experiment was not due to loss of the *CC1215* gene product. One likely explanation for the observed interaction between *CC1215* and *ssrA* is that *himar1* insertion resulted in increased expression of *CC1214*, which is immediately downstream

TABLE 2 Doubling times of mutant strains

Strain description	Doubling time \pm SD (min)
Wild type	103 \pm 1
$\Delta ssrA$	127 \pm 1
<i>CC1214::</i> Ω	103 \pm 1
<i>CC1215::cat</i> (sense)	101 \pm 1
<i>CC1215::cat</i> (antisense)	99 \pm 5
$\Delta ssrA$ <i>CC1215::cat</i> (sense)	122 \pm 1
$\Delta ssrA$ <i>CC1215::cat</i> (antisense)	114 \pm 4

TABLE 3 Cotransduction of *CC1214* alleles with Kan^r^a

Description of recipient strain	% of transductants with <i>CC1214::</i> Ω	% of transductants with <i>CC1214</i>
Wild type	52	48
$\Delta ssrA$	0	100
$\Delta ssrA$ <i>pssrA</i>	51	49
$\Delta ssrA$ <i>pCC1214</i>	55	45
$\Delta ssrA$ <i>pCC1214G30A</i>	0	100

^a Kan^r, kanamycin resistance.

from *CC1214*, making the $\Delta ssrA$ cells more fit. Consistent with that hypothesis, *CC1215* could not be replaced with an Ω cassette in $\Delta ssrA$ cells, but in wild-type cells, the *CC1215::* Ω mutation was viable. Because the Ω cassette contains transcriptional terminators flanking the antibiotic resistance gene, it produces strong polar effects (21).

CC1214 hydrolyzes peptidyl-tRNA on nonstop translation complexes. *CC1214* was annotated as a peptidyl-tRNA hydrolase, but it has greater sequence similarity to *E. coli arfB*. Because purified *E. coli* ArfB can release peptidyl-tRNA from nonstop complexes *in vitro*, the *CC1214* protein was purified and tested for nonstop ribosome release activity. *In vitro* transcription/translation assays were programmed with a dihydrofolate reductase (DHFR) gene lacking a stop codon (DHFR-ns) and resolved on bis-Tris-polyacrylamide gels that preserve the peptidyl-tRNA bond. Ribosomes translating DHFR-ns reach the 3' end of the mRNA and cannot be released by RF1 or RF2, so the percentage of DHFR protein in the peptidyl-tRNA band is a measure of the ribosome release activity. For untreated reaction mixtures, 52% of the DHFR protein was in the peptidyl-tRNA band (Fig. 2). When puromycin, a drug that hydrolyzes peptidyl-tRNA in the ribosome, was added to the reaction mixtures prior to electrophoresis, 16% of the DHFR protein remained as peptidyl-tRNA. When purified *CC1214* was added to the reaction mixtures, 11% of the DHFR was in the peptidyl-tRNA, indicating that *CC1214* can hydrolyze ~80% of peptidyl-tRNA from nonstop complexes under these conditions. This amount of release activity was similar to the

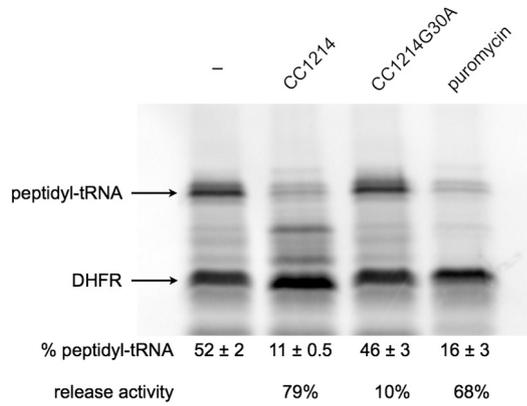


FIG 2 CC1214 hydrolyzes peptidyl-tRNA on nonstop translation complexes. *In vitro* transcription/translation reactions were programmed with a DHFR gene lacking a stop codon, treated with CC1214, CC1214G30A, or puromycin, and resolved on a bis-Tris gel. The positions of free DHFR protein and DHFR peptidyl-tRNA are indicated. The average (± standard deviation) percentages of DHFR in the peptidyl-tRNA band from 3 experiments and the percent release activities with respect to the release activity in the untreated control are shown.

release activity observed for RF1 and RF2 when the DHFR template contained a TAA stop codon (not shown).

Bacterial factors that hydrolyze peptidyl-tRNA on the ribosome, including RF1, RF2, and ArfB, have a GGQ motif that is important for activity (8, 22), but this motif is not found in Pth enzymes that hydrolyze free peptidyl-tRNA. A GGQ motif is conserved in CC1214. A variant of CC1214 in which the GGQ motif was replaced with GAQ (CC1214G30A) was purified and tested for nonstop ribosome release activity. In these reactions, 48% of the DHFR remained in the peptidyl-tRNA band, indicating that the GGQ motif is important for CC1214 activity. Consistent with that hypothesis, a plasmid-borne copy of CC1214G30A would not allow transduction of CC1214::Ω into Δ*ssrA* cells (Table 3).

Multiplicity expression of CC1214 suppresses the Δ*ssrA* growth defect. *C. crescentus* strains lacking *ssrA* or *smgB* grow slowly (23). To determine whether overexpression of CC1214 could suppress this phenotype, CC1214 was cloned under the control of a xylose-inducible promoter on a multicopy plasmid. During exponential growth phase in liquid culture, wild-type cells overexpressing CC1214 had a doubling time of 94 ± 3 min (mean ± standard deviation) (Fig. 3). Wild-type cells with an empty

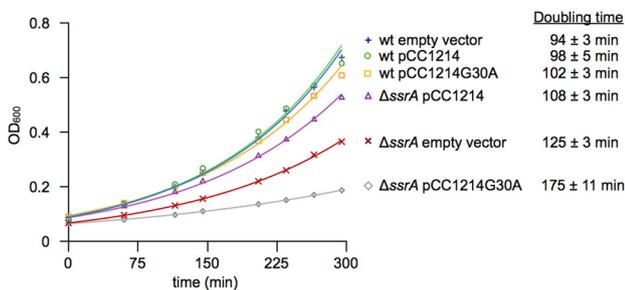


FIG 3 Overexpression of CC1214 suppresses the slow growth phenotype in Δ*ssrA* cells. The growth of *C. crescentus* strains was monitored by measuring the optical density at 600 nm and plotted versus time. The doubling time (± standard deviation) of each strain is indicated. wt, wild type.

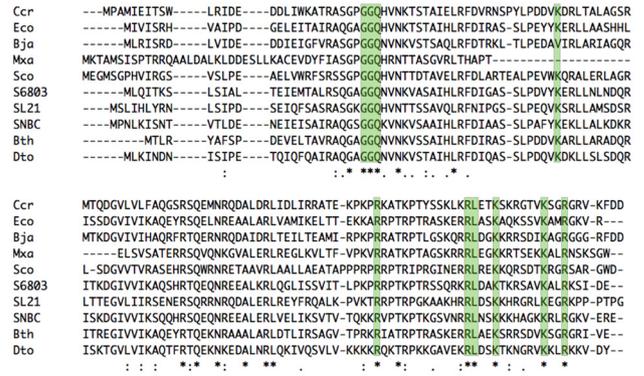


FIG 4 ArfB homologs are found across the bacterial kingdom. ArfB sequences were aligned with Clustal Omega. Asterisks indicate conserved residues, colons indicate residues with strongly similar properties, and dots indicate residues with weakly similar properties. Residues which have been shown experimentally to be important for hydrolysis activity in *E. coli* are highlighted in green. Species abbreviations are as follows: Ccr, *Caulobacter crescentus*; Eco, *Escherichia coli*; Bja, *Bradyrhizobium japonicum*; Mxa, *Myxococcus xanthus*; Sco, *Streptomyces coelicolor*; S6803, *Synechocystis* sp. strain PCC6803; SL21, *Spiriochaeta* sp. strain L21-RPul-D2; SNBC, *Sulfurovum* sp. strain NBC37-1; Bth, *Burkholderia thailandensis*; and Dto, *Desulfobacula toluolica* strain Tol2.

vector had a doubling time of 98 ± 5 min, indicating that overexpression of CC1214 does not have a large effect on growth rate when *trans*-translation is functional. Consistent with previously published data (23), the doubling time in Δ*ssrA* cells with an empty vector was 125 ± 3 min. However, when CC1214 was overexpressed, the doubling time decreased to 108 ± 3 min. This partial suppression of the slow growth phenotype in Δ*ssrA* cells suggests that release of nonstop complexes by CC1214 can compensate for the absence of *trans*-translation.

Suppression by CC1214 required nonstop ribosome release, because overproduction of the inactive CC1214G30A variant resulted in a decrease in growth rate instead of an increase. In wild-type cells overexpressing CC1214G30A, the doubling time was 102 ± 3 min, and in Δ*ssrA* cells overexpressing CC1214G30A, the doubling time was 175 ± 11 min. These results suggest that the inactive mutant can compete with wild-type CC1214 for binding to nonstop ribosomes and inhibit nonstop ribosome release in the absence of *trans*-translation. Consistent with this interpretation, mutants of *E. coli* ArfB, RF1, and RF2 with mutations that alter the GGQ motif can bind nonstop ribosomes (19).

Genes encoding ArfB are widely distributed in bacteria. Because CC1214 has significant sequence similarity to the *E. coli* ArfB gene, including the catalytic GGQ motif and positively charged residues in the C-terminal tail that are required for release of nonstop ribosomes (Fig. 4), and because CC1214 can release nonstop ribosomes *in vitro* and can support the viability of strains in which *ssrA* is deleted, we propose that CC1214 and its homologs should be renamed ArfB. To determine how widely distributed genes encoding ArfB are within the *Alphaproteobacteria*, 236 completed alphaproteobacterial genomes were searched using BLAST (24) with the *C. crescentus* ArfB sequence. No homologs were identified in the rickettsial branch (including *Rickettsia*, *Ehrlichia*, *Wolbachia*, *Candidatus*, and *Anaplasma* species), but of the remaining 171 species, 80% encoded ArfB (Fig. 5). To determine whether ArfB is abundant throughout other lineages of bacteria, BLAST searches were performed on the NCBI set of representative ge-

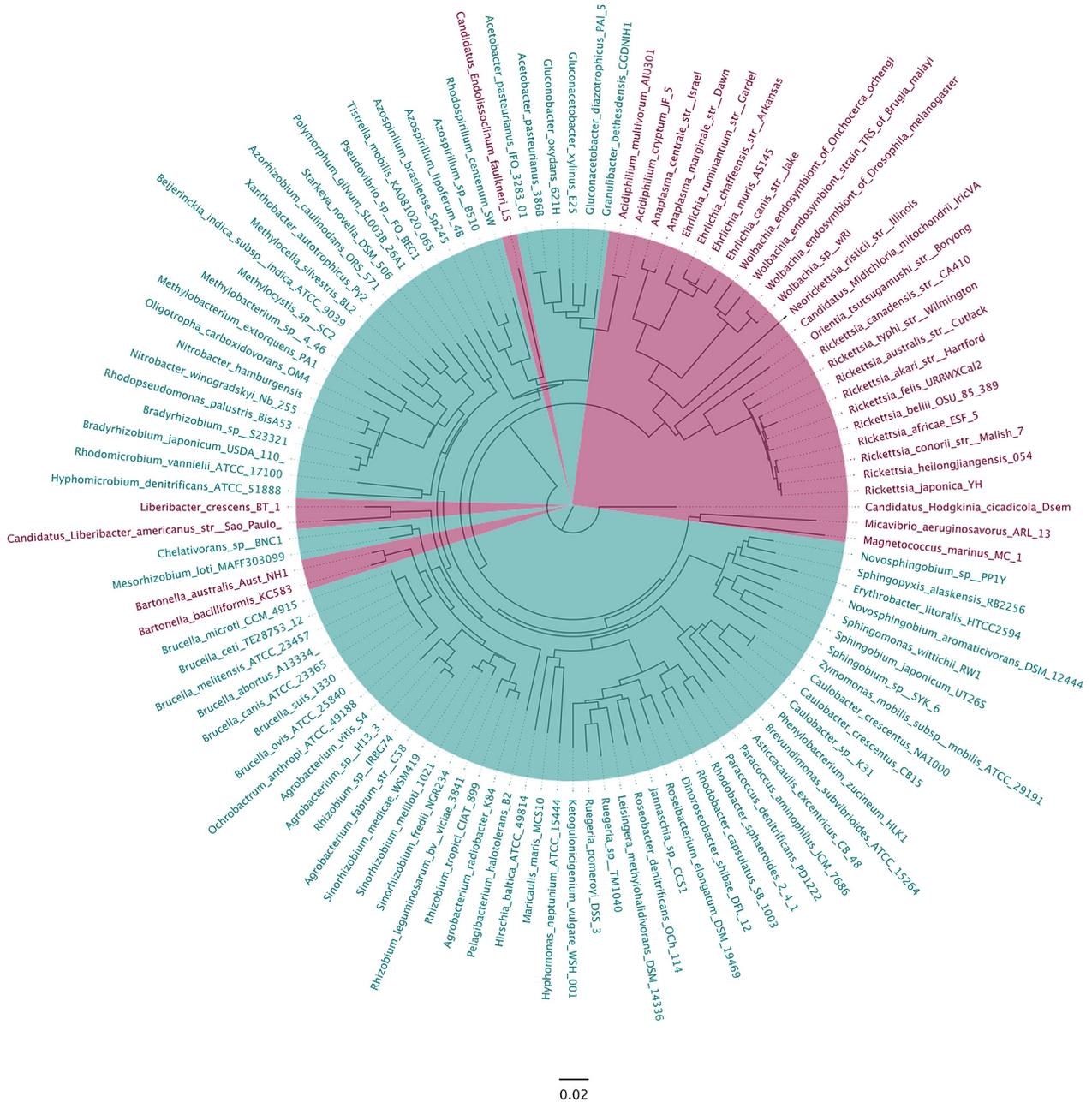


FIG 5 ArfB homolog distribution in *Alphaproteobacteria*. A tree of alphaproteobacterial species generated using 16S rRNA gene sequences is shown, with species encoding ArfB homologs highlighted in turquoise and species that do not encode an ArfB homolog highlighted in magenta.

names for the 21 different bacterial phyla. Homologs were found in 11 phyla (Table 4). More than 60% of *Planctomycetes*, *Chlorobi*, and *Betaproteobacteria* species encoded homologs, and >40% of *Cyanobacteria*, *Bacteroidetes*, *Deltaproteobacteria*, and *Actinobacteria* species had recognizable homologs.

DISCUSSION

The results described here show that *C. crescentus* can survive without *trans*-translation only because it has ArfB to release nonstop translation complexes. ArfB can hydrolyze peptidyl-tRNA on nonstop translation complexes *in vitro*, and *C. crescentus* cells re-

quire either *trans*-translation or ArfB activity for viability. Although *E. coli* ArfB was identified in a multicopy suppressor screen as a gene that would allow both *ssrA* and *arfA* to be deleted, *E. coli* ArfB will not support viability as the only nonstop complex release activity from its chromosomal locus (19). In contrast, the *C. crescentus* ArfB in its wild-type locus can act as a backup system for *trans*-translation during growth in culture. *E. coli* and *C. crescentus* ArfB have similar release activities *in vitro*, so it is likely that *E. coli* ArfB does not act as a backup system for *trans*-translation solely because it is expressed at low levels.

Homology searches using the *C. crescentus* ArfB sequence re-

TABLE 4 Phylogenetic distribution of ArfB homologs

Phylum	No. of representative genomes	No. with <i>arfB</i>	% with <i>arfB</i>
<i>Actinobacteria</i>	174	83	48
<i>Aquificae</i>	9	0	0
<i>Cyanobacteria</i>	55	25	45
<i>Chlorobi/Bacteroidetes</i>	96	48	50
<i>Firmicutes</i>	268	0	0
<i>Alphaproteobacteria</i>	178	102	57
<i>Betaproteobacteria</i>	102	75	74
<i>Epsilonproteobacteria</i>	29	3	10
<i>Gammaproteobacteria</i>	271	101	37
<i>Deltaproteobacteria</i>	54	32	59
<i>Fusobacteria</i>	7	0	0
<i>Spirochaetes</i>	41	7	17
<i>Chlamydiae/Verrucomicrobia</i>	19	0	0
<i>Planctomycetes</i>	7	6	86
<i>Tenericutes</i>	48	0	0
<i>Synergistetes</i>	7	0	0
<i>Thermotogae</i>	17	0	0
<i>Deinococcus</i>	7	0	0
<i>Chloroflexi</i>	16	1	6
<i>Armatimonadetes</i>	1	0	0
<i>Caldiserica</i>	1	0	0
<i>Chrysiogenetes</i>	1	0	0
<i>Deferribacteres</i>	4	0	0
<i>Dictyoglomi</i>	2	0	0
<i>Elusimicrobia</i>	2	0	0
<i>Fibrobacteres/Acidobacteria</i>	9	0	0
<i>Gemmatimonadetes</i>	1	1	100
<i>Nitrospirae</i>	3	1	33
<i>Thermodesulfobacteria</i>	2	0	0
Total	1,431	383	34

vealed that several other species that do not require *trans*-translation encode ArfB, including *Bradyrhizobium japonicum* and *Streptomyces coelicolor* (25, 26). With the addition of ArfB homologs similar to the *C. crescentus* protein, all species in which *ssrA* or *smpB* have been deleted encode either ArfB or ArfA, with the exception of *Francisella tularensis* and *Bacillus subtilis*. Tn-seq experiments or other synthetic-lethal screens should reveal whether *F. tularensis* and *B. subtilis* have a different backup system for *trans*-translation or whether resolution of nonstop complexes is not essential in these species.

C. crescentus cells lacking *trans*-translation grow slowly and have a cell cycle defect, and this phenotype is not complemented by *ssrA-DD*, a variant that releases nonstop complexes but does not target the nascent polypeptide for rapid degradation (23). These results were interpreted to indicate that degradation of one or more tagged proteins was required for normal growth in *C. crescentus*. However, this hypothesis is not consistent with the observation that overexpression of *arfB* in Δ *ssrA* cells restored the wild-type growth rate. Instead, it appears that the slow growth phenotype in cells lacking *trans*-translation is caused by failure to release nonstop translation complexes, similar to phenotypes observed for *ssrA* deletions in other species.

Does *C. crescentus* ArfB perform unique functions for the cell, or is its only role to fill in when *trans*-translation activity is overwhelmed? This question is particularly interesting in *C. crescentus*, because the levels of tmRNA and SmpB fluctuate as a function of

the cell cycle, with both molecules dropping to very low levels in early S phase (27, 28). Nevertheless, ArfB does not play a critical role in the cell cycle, because strains in which *arfB* is deleted grow at the same rate as the wild type. Moreover, transcriptome sequencing (RNA-seq) and ribosome profiling experiments indicated that *arfB* is not highly transcribed or translated during growth in culture (21), and experiments on synchronized cultures indicated that *arfB* transcription is not cell cycle regulated (29). Any role for ArfB other than acting as an emergency backup for *trans*-translation remains to be identified.

Mitochondria contain a protein, ICT1, that has 33% sequence identity with *C. crescentus* ArfB, and ICT1 has been shown to hydrolyze peptidyl-tRNA on nonstop translation complexes, as well as at stop codons (30). ICT1 is clearly important for mitochondria, because it is conserved in all mitochondrial genomes and is essential in HeLa cells (30, 31). Mitochondria are thought to derive from *Alphaproteobacteria*, likely from the rickettsial lineage (32), and the distribution of tmRNA and ArfB in current *Alphaproteobacteria* suggests that early *Alphaproteobacteria* had both *trans*-translation and ArfB. In fact, mitochondria from some single-celled eukaryotes retain tmRNA and SmpB in addition to ArfB (6). Why did the rickettsial lineage keep *trans*-translation and lose ArfB, while mitochondria in higher eukaryotes kept the ArfB homolog and lost *trans*-translation? Presumably, bacteria retain *trans*-translation because it targets the nascent polypeptide and nonstop mRNA for degradation in addition to releasing the ribosome. Mitochondria contain homologs of bacterial ClpXP, the protease that degrades most tmRNA-tagged proteins, so proteolysis of tagged proteins should have occurred in early mitochondria. It is possible that mitochondria have a different pathway to remove incomplete proteins, limiting the advantage of *trans*-translation. Alternatively, the simplicity of the single-chain ArfB might have been advantageous during genome reduction.

MATERIALS AND METHODS

Bacterial growth. Strains are described in Table 5. *C. crescentus* strains were grown at 30°C in peptone-yeast extract (PYE) medium, supplemented with tetracycline (2 µg/ml), streptomycin (50 µg/ml), spectinomycin (100 µg/ml), or kanamycin (20 µg/ml) where appropriate. Culture growth was monitored by measuring the optical density at 600 nm.

Plasmid construction. Oligonucleotide sequences are shown in Table S2 in the supplemental material. Plasmid pBBR-PBN was constructed by amplifying plasmid pBBR1-tet by PCR using primers BBRtetPBN1 and BBRtetPBN2, digesting the product with BamHI, and ligating to circularize the plasmid. Plasmid pCC1214 was constructed by amplifying the *CC1214* gene by PCR using primers 1214_His6F and 1214_Rev, digesting the resulting DNA with NdeI and HindIII, and ligating the product into pML81 cut with the same enzymes to create plasmid pML81-His6-1214. The xylose promoter and *CC1214* were then amplified from pML81-His6-1214 by PCR using primers PstXyl224F and 1214PstIR and ligated into plasmid pBBR-PBN, cut with PstI, to produce pCC1214. pCC1214G30A was made by primer extension mutagenesis. pML81-His6-1214 was used as the template in two separate PCRs using primers PstXyl224F and G30A_R2 or primers G30A_F2 and 1214PstIR. The products of these reactions were used in a second PCR with primers PstXyl224F and 1214PstIR. The resulting product was cloned into the PstI site of pBN and sequenced to verify the mutation. pET28CC1214 was constructed by amplifying *CC1214* with primers 1214_Fwd and 1214_Rev, digesting with NdeI and HindIII, and ligating into pET28b that had also been digested with NdeI and HindIII.

Tn-seq. Overnight cultures of wild-type and Δ *ssrA* *C. crescentus* strains were grown and mutagenized with a *himar1* transposon. More than

TABLE 5 Strains and plasmids

Strain or plasmid	Description	Reference or source
Strains		
CB15N	Wild-type <i>C. crescentus</i>	38
CB15N Δ <i>ssrA</i>	In-frame deletion of <i>ssrA</i>	27
CB15N <i>CC1214::</i> Ω	<i>CC1214</i> replaced by omega cassette	This study
KCK426	<i>E. coli</i> BL21(DE3) carrying pET28CC1214	This study
KCK428	CB15N <i>CC1214::</i> Ω with Kan ^r marker 22.4 kb downstream	This study
KCK429	CB15N <i>CC1215</i> replaced by <i>cat</i> gene in antisense orientation	This study
KCK430	CB15N <i>CC1215</i> replaced by <i>cat</i> gene in sense orientation	This study
KCK431	CB15N Δ <i>ssrA</i> <i>CC1215</i> replaced by <i>cat</i> gene in antisense orientation	This study
KCK432	CB15N Δ <i>ssrA</i> <i>CC1215</i> replaced by <i>cat</i> gene in sense orientation	This study
Plasmids		
pML81	pJS14-derived plasmid with xylose promoter and NdeI site	M. T. Laub, unpublished
pML81-His6-CC1214	Expresses CC1214 from xylose promoter	This study
pBBR1-tet	pBBR1 plasmid with <i>tetA</i> cloned into NotI site	Gift from Camille Lochte, Pasteur Institute of Lille
pBBR-PBN	pBBR1-tet-derived plasmid with PstI, BamHI, and NdeI cloning sites.	This study
pCC1214	Expresses CC1214 from xylose promoter	This study
pACC1214G30A	Expresses CC1214 active-site mutant (GAQ) from xylose promoter	This study
pSsrA	Expresses <i>ssrA</i> from its native promoter	23
pBGS18T(1349674–1350629)	pBGS18T containing targeting fragment from position 1349674 to 1350629	34
pET28CC1214	Expresses CC1214 from T7 promoter	This study
pDHFR	Expresses DHFR from T7 promoter	New England Biolabs
pMT425	Plasmid with vanillate-inducible promoter, <i>cat</i> gene	39

100,000 colonies were pooled, and chromosomal DNA was extracted and sequenced using an Illumina HiSeq 2000 as previously described (33). Data sets were analyzed in SeqMonk version 0.27.0 by normalizing to the total number of reads in the largest data set, adding 1 to eliminate infinite and zero ratios, and calculating the ratio of insertions in Δ *ssrA* cultures to insertions in wild-type cultures (33).

Genetic deletions. Regions of ~1.5 kb flanking *CC1214* were amplified by PCR using primers 1214_UF and 1214_UR or 1214_DF and 1214_DR. The products were cut with SpeI and EcoRI or NheI and EcoRI, respectively, and ligated into the SpeI and NheI sites of integrating plasmid pNPTS138. The omega cassette from pHP45 Ω was inserted at the EcoRI site between the flanking regions. The resulting plasmid was used to construct CB15N*CC1214::* Ω using the two-step recombination method (20). Mutants were selected on spectinomycin, and the *CC1214::* Ω mutation was confirmed by PCR with primers 1214_ScrnF and 1214_ScrnR. CB15N*CC1215::cat* was constructed similarly, using primers 1215_UF and 1215_UR to amplify a region ~1.5 kb upstream from position 1215. A downstream flanking region was amplified using primers 1215_DF and 1215_DR. The PCR fragments were digested with NheI and EcoRI or BamHI and EcoRI and ligated into pNPTS138 digested with NheI and BamHI. The *cat* gene from pMT425 was amplified with primers cat220F and EcoCatR, cut with EcoRI, and ligated into the EcoRI site between the two flanking regions. The direction of the *cat* insertion was determined by PCR, and two-step recombination was used to construct CB15N*CC1215::cat* with the *cat* cassette facing in either the sense (KCK429) or antisense (KCK430) direction with respect to *CC1215*. The presence of the mutation in selected clones was verified by PCR using primers 1215_ScrnF and 1215_ScrnR. To construct strains KCK431 and KCK432, ϕ CR30 phage lysate was prepared from strain KCK429 and KCK430, respectively, and used to infect CB15N Δ *ssrA*. The presence of the mutation was verified using primers 1215_ScrnF and 1215_ScrnR.

Phage transduction. A kanamycin resistance marker was inserted 22.4 kb from *CC1214::* Ω to make strain KCK428 by transforming CB15N *CC1214::* Ω with the nonreplicating plasmid pBGS18T(1349674–1350629) (34). ϕ CR30 phage lysate was prepared from KCK428 and used to infect CB15N or CB15N Δ *ssrA* (35). Transductants were selected on plates containing kanamycin and patched onto plates containing strepto-

mycin and spectinomycin to determine the cotransduction frequency. The expected frequency was predicted by the equation $\log(\text{distance}) = -1.56(\text{ctf}) + 5.06$, where distance represents the number of bases between the two markers and ctf represents cotransduction frequency (34).

Purification of CC1214. KCK426 was grown to an optical density at 600 nm of ≈ 0.8 , and the expression of CC1214 was induced by the addition of isopropyl- β -D-thiogalactopyranoside (IPTG) to 1 mM. Cells were harvested by centrifugation, resuspended in guanidinium lysis buffer (6 M guanidine hydrochloride, 20 mM sodium phosphate [pH 7.8], 400 mM NaCl), and sonicated. The lysate was cleared by centrifugation at $11,000 \times g$ for 10 min. A column was loaded with Ni-nitrilotriacetic acid (NTA) agarose (Qiagen) that had been equilibrated with denaturing binding buffer (8 M urea, 20 mM sodium phosphate [pH 7.8], 500 mM NaCl). The column was loaded with cleared lysate and washed by adding 10 volumes of denaturing binding buffer and rocking for 15 min. The column was then washed with 10 volumes of denaturing wash buffer (8 M urea, 20 mM sodium phosphate [pH 6.0], and 500 mM NaCl) and 10 volumes of denaturing wash buffer equilibrated to pH 5.3. Bound protein was eluted with denaturing elution buffer (8 M urea, 20 mM sodium phosphate [pH 4.0], and 500 mM NaCl) and visualized by SDS-PAGE. Fractions containing 6 \times His-ArfB were dialyzed against buffer H (10 mM HEPES [pH 7.5], 150 mM NaCl).

In vitro translation and peptidyl hydrolysis assays. CC1214 was assayed for peptidyl hydrolysis activity using the PURExpress system (New England Biolabs) similarly to previous assays (36), with some modifications. Nonstop DHFR was amplified using primers HAF_T7 and UTR_D-HFR_FL and used as the template for the reaction. After 1 h of incubation at 37°C, CC1214 or CC1214G30A was added to a final concentration of 200 nM. A control sample was treated with 70 μ g/ml puromycin. After 1 h of incubation at 37°C, total protein was precipitated by the addition of acetone, resuspended in sample loading buffer (5 mM sodium bisulfite, 50 mM MOPS [morpholinepropanesulfonic acid], 50 mM Tris base, 1 μ M EDTA, 0.1% SDS, 5% glycerol, 0.01% xylene cyanol, 0.01% bromophenol blue), and resolved on a bis-Tris gel using MOPS running buffer.

Sequence homology searches. The protein sequence of CC1214 was used in a tblastn search (24) of the June 2014 NCBI database of represen-

tative microbial genomes. An E value cutoff of 10^{-7} and a molecular-mass cutoff of 20 kDa were used to identify homologs. In cases where a homolog was identified for a given phylum, that homolog was then used in a tblastn search of the remaining representative genomes in that phylum. If a homolog was not found, tblastn searches were performed with homologs from closely related phyla. A tree of *Alphaproteobacteria* species was constructed by collecting 16S rRNA gene sequences from PATRIC (37) and aligning them with BLAST. The tree was viewed and colored using Fig tree software (version 1.4.2 [<http://tree.bio.ed.ac.uk/software/figtree/>]).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01916-14/-/DCSupplemental>.

Table S1, XLSX file, 0.4 MB.

Table S2, DOCX file, 0.1 MB.

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