Aminoacyl-tRNA synthetases (ARSs) catalyze the attachment of specific amino acids to cognate tRNAs. Although the accuracy of this process is critical for overall translational fidelity, similar sizes of many amino acids provide a challenge to ARSs. For example, prolyl-tRNA synthetases (ProRSs) mischarge alanine and cysteine onto tRNAPro. Many bacterial ProRSs possess an alanine-specific proofreading domain (INS) but lack the capability to edit Cys-tRNAPro. Instead, Cys-tRNAPro is cleared by a single-domain homolog of INS, the trans-editing YbaK protein. A global bioinformatics analysis revealed that there are six types of “INS-like” proteins. In addition to INS and YbaK, four additional single-domain homologs are widely distributed throughout bacteria: ProXp-ala (formerly named PrdX), ProXp-x (annotated as ProX), ProXp-y (annotated as YeaK), and ProXp-z (annotated as PA2301). The last three are domains of unknown function. Whereas many bacteria encode a ProRS containing an INS domain in addition to YbaK, many other combinations of INS-like proteins exist throughout the bacterial kingdom. Here, we focus on Caulobacter crescentus, which encodes a ProRS with a truncated INS domain that lacks catalytic activity, as well as YbaK and ProXp-ala. We show that C. crescentus ProRS can readily form Cys- and Ala-tRNAPro, and deacylation studies confirmed that these species are cleared by C. crescentus YbaK and ProXp-ala, respectively. Substrate specificity of C. crescentus ProXp-ala is determined, in part, by elements in the acceptor stem of tRNAPro and further ensured through collaboration with elongation factor Tu. These results highlight the diversity of approaches used to prevent proline mistranslation and reveal a novel triple-sieve mechanism of editing that relies exclusively on trans-editing factors.

During translation of the genetic code, aminoacyl-tRNA synthetases (ARSs) are responsible for pairing amino acids with their corresponding tRNA isoacceptors. The synthesis of aminoacyl-tRNAs (aa-tRNAs) occurs in a two-step reaction. First, the amino acid is “activated” upon hydrolysis of ATP to form an aminoacyl-adenylate intermediate. The second step involves the esterification of the amino acid to the 3′-end adenosine of the tRNA. Although this reaction generally occurs with high specificity, synthetases can misactivate amino acids with similar molecular structures, leading to the formation of mismatched aa-tRNAs. If left uncorrected, the delivery of mispaired tRNAs to the ribosome leads to mutations in newly synthesized proteins that might compromise cell viability. Among the 20 synthetases found in most living organisms (one per proteinogenic amino acid), approximately 10 have shown promiscuity (1). Consequently, these ARSs have expanded their catalytic function to include “proofreading” or “editing” activities that prevent formation and/or accumulation of mispaired tRNAs. Two major editing mechanisms are used by ARSs for this purpose. “Pre-transfer” editing involves the hydrolysis of the aminoacyl-adenylate formed in the first step of aminoacylation via a variety of mechanisms (1). In contrast, “post-transfer” editing, which occurs in a domain distinct from the aminoacylation active site, results in the cleavage of the ester bond between the mispaired amino acid and tRNA. Synthetases can employ pre- or post-transfer editing, or in some cases both mechanisms are used (1).

Prolyl-tRNA synthetase (ProRS) is unable to effectively differentiate cognate proline from noncognate alanine and cysteine. This promiscuity is characteristic of ProRSs from all domains of life. Several mechanisms have evolved to avoid proline mistranslation, including increased amino acid specificity at the activation step, pre- and post-transfer editing activities of ProRS, and the use of ARS-like trans-acting factors. The strategy used varies between species. For example, Escherichia coli ProRS catalyzes pre-transfer editing of alanyl-
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adenylate and possesses a cis-editing domain (INS) responsible for Ala-tRNAPro clearance (7, 8, 11). E. coli also encodes a single-domain INS homolog known as YbaK, which deacylates Cys-tRNAPro in trans (10, 12). Therefore, a triple-sieve editing mechanism operates in E. coli, wherein the aminocytosine site of ProRS acts as the coarse sieve to reject amino acids larger than proline allowing the synthesis of Ala- and Cys-tRNAPro, in addition to cognate Pro-tRNAPro. The cis-editing activity of INS is responsible for clearing Ala-tRNAPro in the second or fine sieve. Finally, in the third sieve, YbaK edits Cys-tRNAPro in trans. The latter has been shown to occur via chemical discrimination using the unique sulfhydryl-specific chemistry of the cysteine substrate side chain (13). This mechanism explains how E. coli and many bacteria likely achieve high fidelity in proline codon translation. Although the INS domain is found widely distributed throughout bacteria, a significant number of bacteria encode a ProRS lacking a full-length INS. Thus, the triple-sieve mechanism that is operational in E. coli is not applicable in these cases.

INS and YbaK belong to a family of homologous INS-like proteins that appears to include four other single-domain proteins previously named PrdX, ProX, Yeak, and PA2301 (9, 10, 12–15). In vitro activity studies have been reported for Clostridium sticklandii PrdX, which was demonstrated to be a robust Ala-tRNAPro deacylase (9, 12). Based on this activity we now rename this protein as ProXP-ala. Because C. sticklandii ProRS lacks an INS domain, it was proposed that some organisms use trans-editing factors to compensate for the lack of a cis-editing domain (9). The function of the other INS-like domains is unknown, and their phylogenetic distribution has not been comprehensively investigated. Here, we perform a global phylogenetic analysis to better understand the distribution of INS-like proteins in bacteria and to gain potential insights into their functional roles. This analysis revealed that a significant number of bacterial species lack INS in the context of ProRS, but contain a wide variety of alternative combinations of INS-like proteins. Caulobacter crescentus was chosen for experimental analysis as this organism encodes a ProRS with a severely truncated INS, in addition to YbaK and ProXP-ala. We show that a novel triple-sieve editing mechanism that makes exclusive use of trans-editing factors for the deaclylation of Ala- and Cys-tRNAPro exists in this species. Moreover, C. crescentus ProXP-ala recognizes elements in the acceptor stem of tRNAPro and collaborates with elongation factor Tu (EF-Tu) to avoid hydrolysis of correctly charged Ala-tRNAAla.

EXPERIMENTAL PROCEDURES

Materials—All amino acids and chemicals were purchased from Sigma unless otherwise noted. [3H]Alanine (54 Ci/mmol), [3H]proline (99 Ci/mmol), [α-32P]PPp, and [α-32P]ATP and [35S]cysteine (1075 Ci/mmol) were from PerkinElmer Life Sciences.

Enzyme Preparation—C. crescentus proS, ybaK, and prdX (NCBI GenBank accession numbers: NP420738, NP_419779, and NP_418930, respectively) genes encoding ProRS, YbaK, and ProXP-ala, respectively, were PCR-amplified from C. crescentus CB15 genomic DNA (provided by Dr. Kenneth Keller, Pennsylvania State University) using primers that included flanking restriction sites for BamHI and Ndel endonucleases (New England Biolabs). Each gene was cloned into pCR2.1-TOPO (Invitrogen) and later subcloned into pET15b (Novagen). Protein expression was carried out in E. coli BL21 (DE3) RIL cells. C. crescentus ProRS and ProXP-ala overexpression was induced with 0.1 mm isopropyl β-D-1-thiogalactopyranoside for ~12 h at room temperature. C. crescentus YbaK overexpression was induced with 1 mm β-D-1-thiogalactopyranoside for 4 h at 37 °C. The histidine-tagged proteins were purified using HIS-select nickel resin (Sigma-Aldrich). Wild-type (WT) E. coli alanyl-tRNA synthetase (AlaRS) (16), WT ProRS (17), E. coli K279A ProRS (17), E. coli EF-Tu (18), and E. coli tRNA nucleotidyltransferase (19) were prepared as described previously. Concentrations of E. coli AlaRS, E. coli K279A ProRS, C. crescentus YbaK, C. crescentus ProXP-ala, E. coli EF-Tu, and E. coli tRNA nucleotidyltransferase were determined by the Bradford assay (20). The concentrations of C. crescentus and E. coli ProRS were determined by active site titration (21).

Preparation of tRNAs and Aminoacyl-tRNA Substrates—WT E. coli tRNAPro, G1:C72/U70 E. coli tRNAPro (7), C70U E. coli tRNAPro, and WT E. coli tRNAAla (22) were prepared by in vitro transcription using T7 RNA polymerase as described previously (7). E. coli [35S]Cys-tRNAPro was prepared as described (13). Aminoacylation of tRNA substrates with alanine was carried out in buffer A (50 mM HEPES, pH 7.5, 4 mM ATP, 25 mM MgCl2, 0.1 mg/ml BSA, 20 mM β-mercaptoethanol, 20 mM KCl) by incubating 4 μM enzyme (E. coli AlaRS for tRNAAla and C. crescentus ProRS and ProXP-ala) for 30 min at room temperature. Prior to aminocytosine, tRNA substrates were 32P-labeled using tRNA nucleotidyltransferase as described previously (23). Following aminoacylation, aa-tRNAs were phenol-chloroform-extracted followed by ethanol precipitation. Substrates for decay assays were stored at −80 °C.

Aminoacylation Assays—Aminoacylation reactions to determine kinetic parameters (for the amino acid) were performed in buffer A at 37 °C using trace amounts of 32P-labeled tRNA, 20 mM unlabeled tRNA, and variable concentrations of alanine (10–300 μM) or proline (0.01–1 mM). Reactions were initiated by addition of C. crescentus ProRS to a final concentration of 0.1 μM for proline charging or 0.5 μM for alanine charging. Alanine mischarging of tRNAPro by C. crescentus ProRS was carried out in buffer A using 1 μM ProRS, 5 μM E. coli tRNAPro, and 250 mM alanine. E. coli AlaRS charging of tRNAAla with alanine was accomplished with 50 mM AlaRS, 5 μM E. coli tRNAAla, and 50 mM alanine. Reactions were initiated by addition of 4 mM ATP and incubated at 37 °C. In some reactions, 1 μM C. crescentus PrdX and 10 μM E. coli EF-Tu were included. Prior to use in aminocytosine assays EF-Tu was activated as reported previously (24). Briefly, purified E. coli EF-Tu-GDP (10 μM) was incubated for 1 h at 37 °C in buffer containing 50 mM HEPES, pH 7.5, 1 mM dithiothreitol, 68 mM KCl, 6.7 mM MgCl2, 0.5 mM GTP, 2.5 mM phosphoenolpyruvate, and 30 μg/ml pyruvate kinase. Graphs for all assays were prepared using SigmaPlot.
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(Systat Software, San Jose, CA) with error bars representing the S.D. of triplicate data.

ATP:PP Exchange Assays—ATP:PP exchange assays were carried out using published conditions (8) with the following amino acid concentrations: 0.025–5 mM proline, 0.1–15 mM cysteine, and 50–1000 mM alanine. C. crescentus ProRS (10 nM) was used for proline activation, and 250 nM enzyme was used for cysteine and alanine activation.

Pre-transfer Editing Assays—ATP hydrolysis assays were performed as described previously (8). The C. crescentus ProRS concentration was 0.5 μM, and the amino acid concentrations were as follows: 3 mM proline, 3 mM cysteine, and 500 mM alanine. Assays were performed both in the absence and presence of 10 μM E. coli tRNAPro.

Decaylation Assays—Cys- and Ala-tRNA decaylation reactions were performed using published conditions at 37 °C and 25 °C, respectively. Briefly, reactions containing ~0.7 μM aminoacyl-tRNA and buffer B (300 mM KPO4, pH 7, 0.2 mg/ml BSA, and 9.6 mM MgCl2) were initiated by addition of enzyme. For Cys-tRNAPro decaylation, 0.4 μM C. crescentus YbaK, 5 μM C. crescentus ProRS, and 5 μM C. crescentus ProRp-ala were used. For Ala-tRNA decaylation, 5 μM C. crescentus ProRS, 1 μM C. crescentus ProRp-ala, and 3 μM E. coli ProRS were used. At the indicated time points, reaction aliquots (2 μl) were quenched into 6 μl of a solution containing 0.4 unit/μl P1 nuclease in 200 mM NaOAc, pH 5. Decaylation levels were monitored using polyethyleneimine-cellulose TLC and analyzed as described previously (23).

Bioinformatics—Bacterial organisms with partial or complete sequenced genomes (>2500) were used to identify ProRS genes (NCBI GenBank). Sequence alignments using ClustalW2 (25) were carried out to categorize the 1657 ProRS genes identified into one of three classes depending on whether they (i) contain a full-length INS, (ii) lack INS but instead contain a distinct C-terminal extension (C-term), or (iii) contain a truncated INS domain (mini-INS) (supplemental Fig. 1). In cases where multiple strains existed in the database, redundant sequences were removed. These 1657 organisms were then used to identify INS-like genes using a cut-off of 25% sequence identity for gene classification. The candidate INS-like genes were aligned using ClustalW2 with sequences representing the previously reported INS homologs: Haemophilus influenzae, Rhodospseudomonas palustris, C. crescentus, and E. coli YbaK; Aeropyrum pernix, R. palustris, C. sticklandii, and Thermus thermophilus ProX; C. sticklandii, Homo sapiens, C. crescentus, and Agrobacterium tumefaciens PrdX; E. coli, Bordetella avium, and Streptomycyes griseus YeaK; and Bordetella parapertussis, Streptomycyes albus, and Frankia alni PA2301 (9, 13, 26–29). Based on homology to ProRS and known function, we have renamed PrdX as ProRp-ala, as mentioned above. We also renamed ProX, YeaK, and PA2301, homologs of unknown function, as ProX-p, ProX-p-y, and ProX-p-z, respectively.

The resulting phylogenetic tree was constructed on the phylogeny.fr platform (30) by aligning 120 sequences (20 sequences from each of the six subfamilies) using MUSCLE 3.7. MultiSeq 2.0 included in the VMD software was used to isolate the INS domain sequences from full-length of ProRS (31). The tree was generated using PhyML (32) using default settings. Branch support was provided by bootstraps of 100 replicates. The tree was displayed and edited using the “interactive Tree Of Life” (iTOl) tool (33).

RESULTS

Bacterial ProRSs and the INS Superfamily—ProRSs are class II synthetases that all contain the consensus motifs 1, 2, and 3 that constitute the core catalytic domain. However, several architectures for “extra” domains have been identified for ProRSs throughout the three domains of life (34, 35). In bacteria, three distinct ProRS species have been previously reported. The predominant form of bacterial ProRS contains a full-length INS editing domain inserted between motifs 2 and 3. A subset of bacteria contain a severely truncated INS (mini-INS) domain that lacks catalytic activity (supplemental Fig. 1) (6). Finally, some bacterial species lack an INS domain altogether, and instead contain a smaller C-terminal extension (C-term) with a distinct architecture. The function of the C-terminal domain is believed to be structural (36, 37). To determine the distribution of the different architectures in bacteria, we gathered ProRS sequence information from 1657 bacteria with partially or complete sequenced genomes and used multiple sequence alignments to classify a particular ProRS into one of the three groups (INS, mini-INS, or C-term). Based on this analysis, 64% of bacteria encode an INS-containing ProRS, 10% have a mini-INS, and 22% possess a C-term domain (supplemental Table 1). Interestingly, mini-INS ProRSs are mostly found in organisms from the α-proteobacteria phylum (and some ε- and β-proteobacteria). In contrast, the C-term and INS ProRSs are widely distributed in the rest of bacterial phyla.

Our phylogenetic analysis strongly supports the existence of six distinct INS-like protein domains (Fig. 1). INS and ProXp-ala sequences cluster together in accordance with their alanine-specific editing activities. In contrast, ProXp-ala is more distantly related to ProXp-x, ProXp-y, and ProXp-z, which is in agreement with the lack of robust Ala-tRNAPro editing by the latter enzymes from R. palustris, E. coli, and B. parapertussis, respectively.3 We found that 64% of bacteria that lack INS but instead encode a mini-INS- or C-term-containing ProRS, also encode ProXp-ala. Therefore, although the correlation is high, the occurrence of ProXp-ala does not strictly correlate with the absence of INS, suggesting the possibility of functional redundancy. The well studied Cys-tRNA deacylase, YbaK, is the most abundant INS-like domain, present in 57% of bacteria. The most common combination of domains is the INS/YbaK pairing, which occurs in 25% of bacterial genomes. However, a wide variety of other combinations are also observed, as summarized in supplemental Table 2. Interestingly, 55 organisms encode two ProRS genes, one with an INS and one lacking INS. Fifteen percent lack an INS or INS-like domain altogether, suggesting that they do not require tRNAPro proofreading or have evolved an alternative pathway for that function. Sixty-seven organisms that lack INS encode both YbaK and ProXp-ala (and in some cases ProXp-x as well), suggesting that they may rely exclusively on trans-editing factors to avoid proline mistranslation. To test

3 J. M. Bacusmo, Z. Liu, R. Simari, O. Vargas-Rodriguez, and K. Musier-Forsyth, unpublished observations.
this hypothesis, we chose to study the α-proteobacterium C. crescentus, which encodes a mini-INS, YbaK, and ProXp-ala.

Amino Acid Specificity and Misacylation by C. crescentus ProRS—ATP-PPi exchange experiments were carried out to probe the amino acid specificity of C. crescentus ProRS. A ~3-fold higher \( K_m \) was measured for cysteine relative to cognate proline (Table 1). This observation is similar to previous reports using E. coli and other bacterial ProRSs (6, 8). However, the turnover number \( (k_{cat}) \) for cysteine activation is ~240-fold lower relative to cognate proline. Thus, overall, the relative efficiency of cysteine activation by C. crescentus ProRS is ~780-fold reduced relative to proline. The \( K_m \) for alanine is substantially higher than that of proline (~1000 mM), and overall alanine activation by C. crescentus ProRS is at least ~10^4-fold less efficient relative to proline.

The activation levels measured for cysteine are above the threshold of 1/3000 where editing is predicted to be required (38). Despite the relatively low alanine activation by C. crescentus ProRS, the parameters reported here are similar to those previously reported for E. coli ProRS (7). Although to our knowledge the relative amino acid levels in C. crescentus have not been reported, when taking into account the relative concentration of proline and alanine in E. coli cells (39), the “effective discrimination factor” in E. coli is reduced to 1200 (7), and this value is expected to be similarly lowered in C. crescentus. In vitro, C. crescentus ProRS mischarges alanine onto tRNA^Pro to levels comparable with the post-transfer editing-deficient E. coli K279A ProRS (Fig. 2A). In contrast, the robust post-transfer editing activity of WT E. coli ProRS prevents accumulation of Ala-tRNA^Pro (Fig. 2A).

To further understand the mischarging capabilities of C. crescentus ProRS, we determined the steady-state parameters for the amino acids in the overall aminoacylation reaction. \(^{32}\)P-Labeled tRNA^Pro was used in these assays, which allowed the use of saturating amino acid concentrations (23). In vitro transcribed E. coli tRNA^Pro/UGG, which is 82% identical to C. crescentus tRNA^Pro/UGG (40) and contains all of the elements essential for aminoacylation by ProRS (41), was used in these assays. Aminoacylation data indicate that C. crescentus ProRS has an elevated \( K_m \) for alanine compared with proline (~5000-fold difference), but similar \( k_{cat} \) values (only ~2-fold reduced). Thus, the overall in vitro discrimination factor for alanine compared with proline in this assay is ~10^3 (Table 1). Overall, our results indicate that C. crescentus ProRS can mischarge tRNA^Pro with both cysteine and alanine to levels where proofreading activity is likely to be necessary to prevent mistranslation.
Pre- and Post-transfer Editing by C. crescentus ProRS—We next investigated the pre- and post-transfer editing activities of *C. crescentus* ProRS. Accumulation of AMP in the presence of ATP and amino acid reflects the presence of pre-transfer editing activity. In the case of *E. coli* ProRS, this activity is independent of the presence of tRNA (8). Therefore, we monitored the formation of AMP in the absence of tRNA in reactions containing proline, cysteine, or alanine. As expected, no accumulation of AMP is observed in reactions containing cognate proline (Fig. 2B). Similarly, cysteine does not stimulate significant levels of ATP hydrolysis. In contrast, substantial ATP hydrolysis is observed when alanine is present. As previously observed for *E. coli* ProRS (8), this activity was not stimulated by the addition of tRNA<sup>Pro</sup> (data not shown). These results demonstrate that *C. crescentus* ProRS possesses pre-transfer editing activity toward alanine but not cysteine.

Direct measurement of post-transfer editing activity is possible using deacylation assays. Cys- and Ala-tRNA<sup>Pro</sup> were prepared using post-transfer editing-deficient *E. coli* K279A ProRS. Deacylation assays demonstrate that *C. crescentus* ProRS does not possess post-transfer editing activity, consistent with the presence of a mini-INS domain that lacks catalytic function (Fig. 3). Interestingly, even though *C. crescentus* ProRS can hydrolyze alanyl-AMP via pre-transfer editing, this proof-reading mechanism is insufficient to prevent formation of Ala-tRNA<sup>Pro</sup> (Fig. 2A). This result is consistent with the fact that high levels of alanine mischarging are observed for *E. coli* K279A ProRS, despite the fact that this enzyme still carries out pre-transfer editing (8). Taken together, our data indicate that *C. crescentus* ProRS cannot prevent formation of mispaired Ala- and Cys-tRNA<sup>Pro</sup> and is likely to rely on alternative mechanisms to clear these mischarged species.

*C. crescentus* YbaK and ProXp-ala Deacylation Activities—We next cloned, expressed, and purified *C. crescentus* YbaK and ProXp-ala and characterized their post-transfer deacylation activities in vitro. These experiments confirmed robust deacylation activity of *C. crescentus* YbaK and ProXp-ala for Cys-tRNA<sup>Pro</sup> and Ala-tRNA<sup>Pro</sup>, respectively (Fig. 3). Moreover, deacylation by ProXp-ala is not affected by the presence of *C. crescentus* ProRS (data not shown). These results support the formation of an alternative triple-sieve editing mechanism that operates in *C. crescentus*, which relies on the trans-editing activities of YbaK and ProXp-ala.

tRNA Specificity of *C. crescentus* ProXp-ala—A previous in vitro study indicated that *C. sticklandii* ProXp-ala deacylates correctly charged Ala-tRNA<sub>Ala</sub> albeit with a reduced hydrolysis rate (12). We also observed weak Ala-tRNA<sub>Ala</sub> deacylation by *C. crescentus* ProXp-ala, with the rate of hydrolysis ~28-fold reduced relative to Ala-tRNA<sup>Pro</sup> deacylation (Fig. 4A). Sequence comparison of bacterial tRNA<sub>Ala</sub> and tRNA<sup>Pro</sup>
reveals differences at the top of the acceptor stem domain (Fig. 4B) (40). Although both tRNAs share an A73 “discriminator” base, bacterial tRNAPro has a unique C1:G72 base pair, which is important for ProRS recognition (41). In contrast, tRNAAla contains a G1:C72 base pair at the end of the acceptor stem, and a unique G3:U70 wobble base pair, which is essential for AlaRS recognition (42, 43). The G3:U70 base pair is not only critical for AlaRS aminoacylation, but also for editing by AlaRS, as well as by the trans-editing homolog, AlaXp (44). To establish whether C. crescentus ProXp-ala recognizes acceptor stem sequences, we transplanted the G1:C72 and U70 elements of tRNAAla into the framework of tRNAPro (Fig. 4B). The incorporation of these elements into tRNAPro reduced the rate of ProXp-ala deacylation by 12-fold, a level similar to that of tRNAAla (Fig. 4A). When only the G3:U70 base pair was introduced into tRNAPro the rate of hydrolysis was reduced 4-fold. These results indicate that ProXp-ala is sensitive to the identity of the first and third base pairs of tRNAPro.

EF-Tu Discrimination against Mischarged Ala-tRNAPro—Despite a significant preference for the tRNAPro acceptor stem, tRNA specificity of ProXp-ala may not be sufficient to prevent hydrolysis of Ala-tRNAAla in vivo, as weak in vitro deacylation of the correctly charged tRNAAla is observed (Fig. 4A). EF-Tu binds with a wide range of affinities to different aa-tRNAs (45). Dale and Uhlenbeck have shown that the operational binding affinity of EF-Tu depends on the individual thermodynamic contributions of the amino acid moiety and the tRNA. Consequently, a binding compensation occurs, and EF-Tu binds to correctly charged tRNAs with affinities that allow efficient delivery of aa-tRNAs to the ribosome. Because alanine and tRNAPro are considered weak EF-Tu binders (46), we hypothesized that EF-Tu is likely to protect Ala-tRNAAla, but not Ala-tRNAPro from ProXp-ala hydrolysis. To test this hypothesis, we performed in vitro charging and mischarging assays in the presence of both ProXp-ala and E. coli EF-Tu. Addition of EF-Tu to a C. crescentus ProRS misacylation assay does not affect the overall synthesis of Ala-tRNAPro (Fig. 5A).
alanine led to the hydrolysis of cognate Pro-tRNAPro, mutation of a highly conserved histidine residue in residues within a tunable hydrophobic pocket (11). Whereas specificity can therefore be engineered through mutagenesis of specific mechanism and water-mediated hydrolysis. Changes in substrate specificity as INS, which uses a steric exclusion mechanism of ProXp-ala has not yet been explored, it has the same substrate specificity is not tunable (13). Although the detailed mechanism of ProXp-ala has not yet been explored, it has the same substrate specificity as INS, which uses a steric exclusion mechanism and water-mediated hydrolysis. Changes in substrate specificity can therefore be engineered through mutagenesis of specific residues within a tunable hydrophobic pocket (11). Whereas mutation of a highly conserved histidine residue in E. coli INS to alanine led to the hydrolysis of cognate Pro-tRNAPro, mutation of the same histidine residue in human ProX (a member of the ProXp-ala subfamily defined here) did not alter the substrate specificity (29). These results suggest that although ProXp-ala and INS share Ala-tRNA specificity, the details of substrate binding and recognition may be different. Finally, the lack of robust Ala- or Cys-tRNAPro deacylation by ProXp-x, ProXp-y, and ProXp-z3 supports the presence of orthologous groups within the INS superfamily that correlate with well defined clades in the phylogenetic tree (Fig. 1).

In C. crescentus, the activities of YbaK and ProXp-ala establish a novel triple-sieve mechanism that exclusively relies on trans-editing domains for post-transfer editing. In this new model (Fig. 6), the aminocoylation domain of C. crescentus ProRS acts as a fine sieve to reject amino acids larger than proline allowing the formation of Pro-tRNAPro, as well as Cys-tRNAPro and both lack the tRNA binding domain of trans-editing factors. In this new model (10)), the aminoacylation domain of C. crescentus ProRS (1 M) with by C. crescentus ProRS (1 M), ProXp-ala only ( ), and C. crescentus ProXp-ala (1 M) with E. coli EF-Tu (10 μM) (A). A reaction with no enzyme was also performed ( ). Error bars, S.D. of triplicate determinations.

**DISCUSSION**

The wide distribution of INS-like proteins in bacteria highlights the importance of tRNAPro proofreading in most bacteria. The overlapping distribution at the phylum level also suggests that the three INS-like domains of unknown function may have distinct substrate specificities. Indeed, the INS-like trans-editing factors studied experimentally to date, which include YbaK from H. influenzae (10), E. coli (12, 13), and C. crescentus (present work), and ProXp-ala from C. sticklandii (9, 12), H. sapiens (29), and C. crescentus (present work), possess distinct substrate specificities and unique mechanistic features. For example, the mechanism of catalysis of YbaK involves thiol-specific chemistry, and thus, YbaK substrate specificity is not tunable (13). Although the detailed mechanism of ProXp-ala has not yet been explored, it has the same substrate specificity as INS, which uses a steric exclusion mechanism and water-mediated hydrolysis. Changes in substrate specificity can therefore be engineered through mutagenesis of specific residues within a tunable hydrophobic pocket (11). Whereas mutation of a highly conserved histidine residue in E. coli INS to alanine led to the hydrolysis of cognate Pro-tRNAPro, mutation of the same histidine residue in human ProX (a member of the ProXp-ala subfamily defined here) did not alter the substrate specificity (29). These results suggest that although ProXp-ala and INS addition of C. crescentus ProXp-ala (equimolar with C. crescentus ProRS) decreases the formation of Ala-tRNAPro by at least 20-fold both in the absence and presence of EF-Tu (Fig. 5A). In contrast, when cognate charging assays are performed with E. coli AlaRS, the concentration of Ala-tRNAAla only decreases ~3-fold in the presence of a 20:1 ratio of ProXp-ala to AlaRS and E. coli EF-Tu promotes accumulation of Ala-tRNAAla (by ~1.5-fold) (Fig. 5B). In good agreement with the thermodynamic compensation model, EF-Tu binds to Ala-tRNAAla with higher affinity than Ala-tRNAPro from ProXp-ala hydrolysis (Fig. 5).

**FIGURE 5. Effect of EF-Tu on ProXp-ala activity.** A, alanine mischarging of E. coli tRNAPro (5 μM) by C. crescentus ProRS (1 μM). Reactions were performed in the presence ( ) and absence ( ) of 10 μM E. coli EF-Tu. Aminocoylation was in the presence of 1 μM C. crescentus ProXp-ala only ( ) and C. crescentus ProXp-ala (1 μM) with E. coli EF-Tu (10 μM) ( ). A no enzyme control was also performed ( ). B, alanine aminoacylation of E. coli tRNAPro (5 μM) by 50 mM E. coli AlaRS. Reactions were performed in the presence ( ) and absence of 10 μM E. coli EF-Tu ( ). Aminocoylation was in the presence of 1 μM C. crescentus ProXp-ala only ( ) and C. crescentus ProXp-ala (1 μM) with E. coli EF-Tu (10 μM) ( ). A reaction with no enzyme was also performed ( ). Error bars, S.D. of triplicate determinations.

In C. crescentus, the activities of YbaK and ProXp-ala establish a novel triple-sieve mechanism that exclusively relies on trans-editing domains for post-transfer editing. In this new model (Fig. 6), the aminoacylation domain of C. crescentus ProRS acts as a fine sieve to reject amino acids larger than proline allowing the formation of Pro-tRNAPro, as well as Cys-tRNAPro and Ala-tRNAPro in C. crescentus, respectively. The order in which aa-tRNAPro species are proofread by YbaK and ProXp-ala is still unknown.

Because ProXp-ala and YbaK deacylate cognate Ala-tRNAAla and Cys-tRNAPro, and both lack the tRNA binding domain of ProRS, these enzymes were proposed to act as general deacylases (12, 29). However, we now show that the unique C1:G72 base pair of bacterial tRNAPro contributes to efficient ProXp-ala hydrolysis. Nevertheless, ProXp-ala cannot effectively discriminate against Ala-tRNAAla, and because the cellular concentration of Ala-tRNAAla is likely to be higher than Ala-tRNAPro, a mechanism to prevent hydrolysis of Ala-tRNAAla by ProXp-ala must exist. Our experiments show that the translational factor EF-Tu is responsible for preventing Ala-tRNAAla deacylation by ProXp-ala. Based on these results, we propose that upon synthesis of Ala-tRNAAla by AlaRS, EF-Tu can sequester Ala-tRNAAla and prevent trans-editing by ProXp-ala, which cannot effectively compete against EF-Tu for binding to this substrate. In contrast, the tRNA specificity of ProXp-ala together with the weak affinity of EF-Tu for Ala-tRNAPro prevents accumulation of Ala-tRNAPro. Taken together,
our data suggest that EF-Tu and ProXp-ala collaborate to prevent hydrolysis of Ala-tRNA\textsubscript{Ala} and ultimately mistranslation of proline.

The fact that ProXp-ala and INS coexist in some organisms suggests that the trans-editing function may serve as a redundant mechanism to prevent alanine-for-proline mistranslation. A similar scenario exists with AlaRS and AlaXp, which both prevent serine-for-alanine mistranslation (9, 47). Alternatively, ProRS lacks a full-length INS domain and readily misactivates alanine and cysteine. Two trans-editing domains, ProXp-ala and YbaK, function to deacetylate Ala-tRNA\textsubscript{Ala} and Cys-tRNA\textsubscript{Cys}, respectively. The structures of \textit{R. palustris} ProRS (27), \textit{C. crescentus} ProXp-ala (27) and \textit{H. influenzae} YbaK (28) are shown.

![Diagram of Proline Translation Fidelity Ensured by trans-Editing Domains](image)

FIGURE 6. \textit{Proposed triple-sieve editing mechanism in \textit{C. crescentus}.} \textit{C. crescentus} ProRS lacks a full-length INS domain and readily misactivates alanine and cysteine. Two trans-editing domains, ProXp-ala and YbaK, function to deacetylate Ala-tRNA\textsubscript{Pro} and Cys-tRNA\textsubscript{Cys}, respectively. The structures of \textit{R. palustris} ProRS (27), \textit{C. crescentus} ProXp-ala (27) and \textit{H. influenzae} YbaK (28) are shown.

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