

# Evolutionary Basis of Converting a Bacterial tRNA Synthetase into a Yeast Cytoplasmic or Mitochondrial Enzyme\*

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Previous studies showed that cytoplasmic and mitochondrial forms of yeast valyl-tRNA synthetase (ValRS) are specified by the *VASI* gene through alternative initiation of translation. Sequence comparison suggests that the yeast cytoplasmic (or mature mitochondrial) ValRS contains an N-terminal appendage that acts in *cis* as a nonspecific tRNA-binding domain (TRBD) and is absent from its bacterial relatives. We show here that *Escherichia coli* ValRS can substitute for the mitochondrial and cytoplasmic functions of *VASI* by fusion of a mitochondrial targeting signal and a TRBD, respectively. In addition, the bacterial ValRS gene can be converted into a dual functional yeast gene encoding both cytoplasmic and mitochondrial activities by fusion of a DNA sequence specifying both the mitochondrial targeting signal and TRBD. *In vitro* assays suggested that fusion of a nonspecific TRBD to the bacterial enzyme significantly enhanced its yeast tRNA-binding and aminoacylation activities. These results not only underscore the necessity of retaining a TRBD for functioning of a tRNA synthetase in yeast cytoplasm, but also provide insights into the evolution of tRNA synthetase genes.

Aminoacyl-tRNA synthetases (aaRSs)<sup>2</sup> are a group of ancient enzymes responsible for protein translation, each of which catalyzes the attachment of a specific amino acid to its cognate tRNAs, forming aminoacyl-tRNAs. These charged tRNAs are then delivered by elongation factor-1 to ribosomes for protein translation. In prokaryotes, there are typically 20 aminoacyl-tRNA synthetases, one for each amino acid (1–4). In eukaryotes, protein synthesis occurs not only in the cytoplasm, but also in organelles, such as mitochondria and chloroplasts (5). Thus, eukaryotes, such as yeast, commonly have two genes that encode distinct sets of proteins for each aminoacylation activity, one localized in the cytoplasm and the other in the mitochondria. Each set aminoacylates the isoaccepting tRNAs within its respective cell compartment and is sequestered from the isoacceptors confined in other compartments. In most cases, cytoplasmic and mitochondrial synthetase activities are encoded by two distinct nuclear genes,

regardless of the cell compartments to which they are confined. However, in some cases, cytoplasmic and mitochondrial forms of a tRNA synthetase with a given amino acid specificity are encoded by the same nuclear gene through alternative initiation of translation, examples of which include *ALAI* (coding for alanyl-tRNA synthetase) (6, 7), *GRS1* (coding for glycyl-tRNA synthetase) (8), *HTS1* (coding for histidyl-tRNA synthetase) (9), and *VASI* (coding for valyl-tRNA synthetase (ValRS)) (10). Because the isozymes are targeted to different compartments, the two isoforms of ValRS, for example, cannot be substituted for each other *in vivo*. A similar scenario has been observed for genes encoding mitochondrial and cytoplasmic forms of *Arabidopsis thaliana* alanyl-tRNA synthetase, threonyl-tRNA synthetase, and ValRS (11).

Many yeast cytoplasmic tRNA synthetases contain an N- or C-terminal polypeptide extension that is absent from their bacterial homologs (12). A well studied example is the appended domain (Ad) of yeast glutamyl-tRNA synthetase (GlnRS), which binds crude yeast tRNAs, single-stranded RNA, and pseudoknot RNA with comparable affinities; the  $K_d$  values are  $\sim 0.6 \mu\text{M}$  (13, 14). Similar examples have been reported in yeast ValRS (15) and tRNA synthetases of higher eukaryotes, such as the EMAPII-like domain of plant methionyl-tRNA synthetase (16), the repeat domain of human methionyl-tRNA synthetase (17), and the N-terminal domain of mammalian lysyl-tRNA synthetase (18, 19). In addition to serving as a *cis*-acting tRNA-binding domain (TRBD), the Ads of some yeast tRNA synthetases were found to participate in protein-protein interactions, such as those of yeast glutamyl-, methionyl- (20), and seryl-tRNA synthetases (21). These interactions were shown to enhance their tRNA binding and aminoacylation (20, 21).

Interestingly, many of the Ads of yeast tRNA synthetases contain one or several canonical nuclear localization signals (22) that are thought to play a role in the nuclear import of these otherwise “cytoplasmic” proteins. Nuclear aminoacylation of tRNAs is thought to serve as a functional checkpoint for the maturity of tRNAs before they are exported from the nucleus (23, 24). By contrast, in higher eukaryotes, nine aminoacyl-tRNA synthetases and three auxiliary proteins (p43, p38, and p18) form a multienzyme complex through interactions of their hydrophobic Ads (25). In addition, ValRS from mammalian cells is exclusively isolated as a high molecular mass complex with the elongation factor EF-1H (26–28). Recently, several tRNA synthetases from prokaryotes and eukaryotes have been shown to take part in functions beyond aminoacylation, including roles in mitochondrial RNA splicing, transcriptional and translational regulation, cytokine-like activity, and amino acid biosynthesis (29, 30).

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<sup>2</sup> The abbreviations used are: aaRS, aminoacyl-tRNA synthetase; Ad, appended domain; Ec, *E. coli*; EF, elongation factor; 5-FOA, 5-fluoroorotic acid; GlnRS, glutamyl-tRNA synthetase; MTS, mitochondrial targeting signal; Sc, *Saccharomyces cerevisiae*; TRBD, tRNA-binding domain; ValRS, valyl-tRNA synthetase; YPG, yeast extract peptone glycerol.

In this report, we focused on the cross-species and cross-compartmental complementation activities of a bacterial tRNA synthetase in an attempt to understand further the evolutionary pathway that has converted a bacterial tRNA synthetase gene into a dual functional yeast gene possessing both cytoplasmic and mitochondrial activities. Our results show that although *Escherichia coli* ValRS *per se* cannot substitute for the cytoplasmic activity of yeast *VAS1*, fusion of a nonspecific TRBD (such as Arc1p or the Ads of yeast GlnRS and ValRS) to the bacterial enzyme enabled the otherwise nonfunctional enzyme to rescue the growth defect of a *VAS1* knock-out strain on 5-fluoroorotic acid (5-FOA). In contrast, the *E. coli* enzyme, when targeted to mitochondria, could substitute for the mitochondrial activity of *VAS1* without the assistance of a *cis*-acting nonspecific TRBD. These results, together with others, suggest that acquiring a *cis*- or *trans*-acting TRBD might be necessary and sufficient for functioning by a yeast cytoplasmic tRNA synthetase, which might explain why so many yeast cytoplasmic aaRSs contain an N- or C-terminal Ad (12). In contrast, obtaining a *cis*- or *trans*-acting TRBD does not appear to be necessary for functioning of most yeast mitochondrial aaRSs.

## EXPERIMENTAL PROCEDURES

**Construction of Plasmids**—Cloning of the wild-type yeast *VAS1* gene into the high copy number yeast shuttle vector pADH was previously described (31). A short DNA duplex coding for a FLAG (for Western blotting) or His<sub>6</sub> tag (for protein purification) was inserted in-frame at the 3' end of the *VAS1* gene. To clone the *E. coli valS* gene, the open reading frame of this gene was first amplified by PCR using bacterial genomic DNA as the template. The forward (with an inserted SpeI site, underlined) and reverse (with an inserted XbaI site, underlined) primers used contained sequences complementary to base pairs -15 to +18 (5'-AACCTGGAAACTAGTATGGAAAA-GACATATAAC-3') and +2842 to +2871 (5'-ATCACTGT-GTTTTCTAGACAGCGCGGCGAT-3') of *E. coli valS*, respectively. After the PCR, the amplified DNA products were digested with the restriction enzymes SpeI and XbaI and then cloned into the appropriate sites of pADH for expression. Cloning of the *Bacillus subtilis valS* gene into pADH followed a similar protocol.

For fusion of the Ad of yeast ValRS (residues 1–97), the Ad of yeast GlnRS (residues 1–228), or Arc1p to *E. coli* ValRS, DNA sequence coding for the respective protein domain was PCR-amplified as an SpeI-SpeI fragment and inserted in-frame into the SpeI site at the 5' end of the open reading frame of *E. coli valS*, yielding Ad(ScValRS)-EcValRS, Ad(ScGlnRS)-EcValRS, and Arc1p-EcValRS. For fusion of a mitochondrial targeting signal (MTS) to *B. subtilis* and *E. coli* ValRSs, DNA sequence coding for the MTS of the mitochondrial precursor form of yeast ValRS (residues 1–46) was PCR-amplified as an SpeI-SpeI fragment and inserted in-frame into the SpeI site at the 5' end of the open reading frames of the *B. subtilis* and *E. coli valS* genes. The orientation of the SpeI-SpeI fragment was subsequently verified by DNA sequencing. Expressions of these constructs were under the control of a constitutive *ADH* promoter. Fusion of the DNA segment containing the promoter and sequence

coding for the MTS and Ad of yeast ValRS to *E. coli valS* followed a similar protocol, except that the vector used for cloning and expression was pRS425 in this case.

**Complementation Assays for Cytoplasmic Function**—The yeast *VAS1* knock-out strain, CW1, was described previously (31). This strain is maintained by a plasmid containing the wild-type *VAS1* gene and a *URA3* marker. Complementation assays for cytoplasmic ValRS activity were carried out by introducing a test plasmid carrying the gene of interest and a *LEU2* marker into CW1, and the ability of the transformants to grow in the presence of 5-FOA was determined. Starting from a cell density of 4.0  $A_{600}$ , cell cultures were 5-fold serially diluted, and 10- $\mu$ l aliquots of each dilution were spotted onto the designated plates containing 5-FOA. Plates were incubated at 30 °C for 3–5 days. The transformants evicted the maintenance plasmid with a *URA3* marker in the presence of 5-FOA and thus could not grow on the selection medium unless a functional cytoplasmic ValRS was encoded by the test plasmid.

**Complementation Assays for Mitochondrial Function**—CW1 was cotransformed with a test plasmid (carrying a *LEU2* marker) and a second maintenance plasmid (carrying a *HIS3* marker) that expresses only the cytoplasmic form of ValRS (due to a mutation in the ATG1 initiator codon). In the presence of 5-FOA, the first maintenance plasmid (carrying a *URA3* marker) was evicted from the cotransformants, whereas the second maintenance plasmid was retained. Thus, all cotransformants survived 5-FOA selections due to the presence of the cytoplasmic ValRS derived from the second maintenance plasmid. The mitochondrial phenotypes of the cotransformants were further tested on YPG plates at 30 °C, with results documented on day 3 after plating. Because a yeast cell cannot survive on glycerol without functional mitochondria, the cotransformants did not grow on the YPG plates unless a functional mitochondrial ValRS was generated from the test plasmid.

**Western Blot Analysis**—The protein expression patterns of the constructs used in the complementation assays were determined by a chemiluminescence-based Western blot analysis. INVSc1 (Invitrogen) was first transformed with the constructs of interest, and total protein extracts were prepared from each transformant. Aliquots of the protein extracts (40  $\mu$ g) were loaded onto a mini gel (8  $\times$  10 cm) containing 10% polyacrylamide and electrophoresed at 100 V for 1–2 h. After electrophoresis, the resolved proteins were transferred using a semi-dry transfer device to a polyvinylidene fluoride membrane in a buffer containing 30 mM glycine, 48 mM Tris base (pH 8.3), 0.037% SDS, and 20% methanol. The membrane was probed with a horseradish peroxidase-conjugated anti-FLAG tag antibody (Sigma) and then exposed to x-ray film after the addition of the appropriate substrates.

**Aminoacylation Assay**—Aminoacylation reactions were carried out at 25 °C in a buffer containing 10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 4 mM ATP, 0.1 mg/ml bovine serum albumin, 0.1 mM *E. coli* or brewers' yeast tRNA (Hoffmann-La Roche, Basel, Switzerland), and 30  $\mu$ M valine (1.7  $\mu$ M [<sup>3</sup>H]valine; Moravsek Biochemicals, Brea, CA). The specific activity of [<sup>3</sup>H]valine used was 35.0 Ci/mmol. Purification of the His<sub>6</sub>-tagged proteins was as described previ-

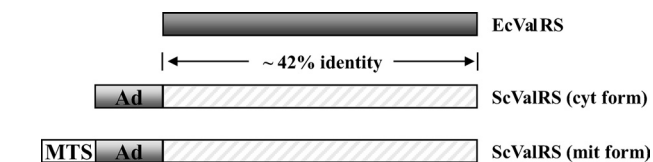
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ously (32). Determination of active protein concentrations by active-site titration was as described (33). The final concentration of ValRS used in the reaction was 5, 10, or 50 nM. Reactions were quenched by spotting 10- $\mu$ l aliquots of the reaction mixture onto Whatman filters soaked in 5% trichloroacetic acid and 1 mM valine. The filters were washed three times, for 15 min each, in ice-cold 5% trichloroacetic acid before liquid scintillation counting. Data were obtained from at least three independent experiments and averaged. Determination of the kinetic

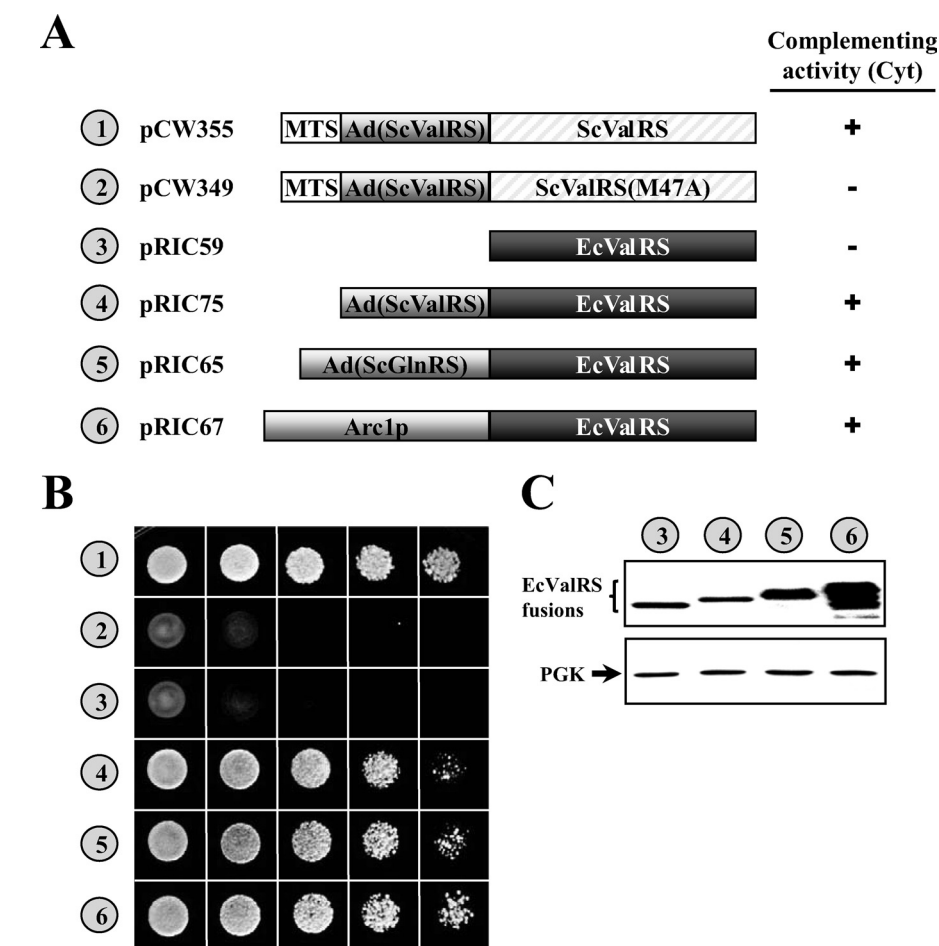
parameters,  $K_m$  and  $k_{cat}$ , of the purified enzymes for yeast tRNA<sup>Val</sup> was as described (15).

## RESULTS

**Converting *E. coli* ValRS into a Functional Yeast Cytoplasmic Enzyme**—Previous studies showed that a single *VAS1* gene specifies both the mitochondrial and cytoplasmic forms of ValRS through alternative use of two in-frame AUG initiator codons, AUG1 and AUG47 (10, 31). Hence, the mitochondrial precursor form essentially possesses the same polypeptide sequence as its cytoplasmic counterpart, except for an N-terminal MTS that is subsequently cleaved away by a matrix-processing peptidase upon being imported into mitochondria. Comparison of *E. coli* and yeast (the cytoplasmic form or the mature mitochondrial form) ValRSs showed that the catalytic cores of these two enzymes are significantly homologous to each other ( $\sim$ 42% identity), but the yeast enzyme has an N-terminal polypeptide extension of  $\sim$ 97 residues that is absent from its *E. coli* counterpart (Fig. 1). This Ad has recently been shown to act in *cis* as a nonspecific TRBD to facilitate tRNA binding and aminoacylation by the enzyme (15).

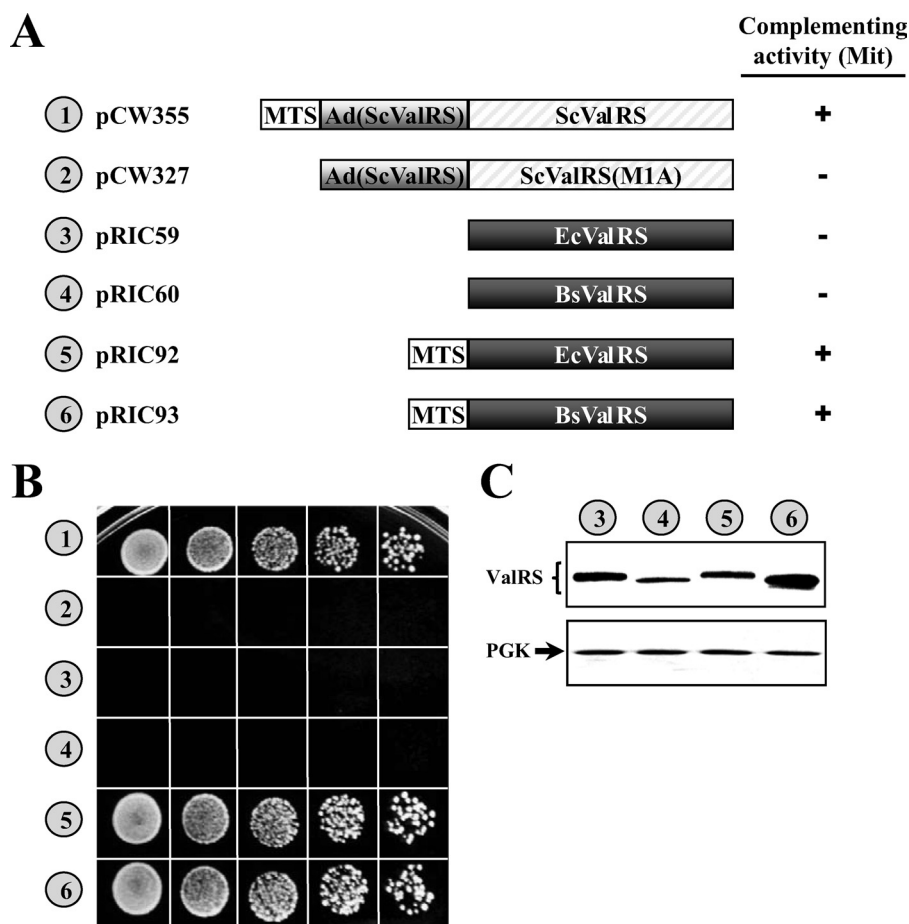


**FIGURE 1. Comparison of *E. coli* and yeast ValRS.** Although the catalytic cores of *E. coli* and yeast ValRSs are significantly homologous to each other, the yeast cytoplasmic (cyt) protein contains an N-terminal domain of  $\sim$ 97 residues that is absent from its *E. coli* counterpart. The polypeptide sequence of the mitochondrial (mit) precursor form of yeast ValRS is essentially identical to that of its cytoplasmic isoform except for a mitochondrial targeting signal attached at its N terminus.



**FIGURE 2. Converting *E. coli* ValRS into a functional yeast cytoplasmic enzyme.** CW1, a yeast *VAS1* knock-out strain, was transformed with constructs encoding various ValRSs, and the ability of these constructs to rescue the cytoplasmic defect of the knock-out strain was tested. *A*, schematic summary of constructs and their complementation activities. The symbols + and - indicate positive and negative complementation, respectively. *Cyt*, cytoplasmic. *B*, complementation assays for cytoplasmic ValRS activity on a 5-FOA plate. *C*, assay of protein expression by Western blotting. *Upper panel*, ValRS; *lower panel*, phosphoglycerate kinase (*PGK*) (as a loading control). *Numbers 1–6 (circled) in B and C* represent the constructs shown in *A*.

To test whether the *E. coli* enzyme can functionally substitute for its yeast homolog *in vivo*, the wild-type *E. coli valS* gene (encoding ValRS) was cloned into a yeast shuttle vector, and the ability of the resultant construct to rescue the growth defect of a yeast *vas1*<sup>-</sup> strain on 5-FOA was tested. As shown in Fig. 2, the construct containing *E. coli valS* failed to restore the growth phenotype of the knock-out strain CW1 on 5-FOA (Fig. 2*B*, number 3), suggesting that the cytoplasmic activity of yeast *VAS1* cannot be substituted with the bacterial enzyme *in vivo*. However, fusion of a nonspecific TRBD, such as Arc1p or the Ads of yeast GlnRS and ValRS, to the *E. coli* enzyme enabled the otherwise nonfunctional bacterial enzyme to act as a yeast enzyme; each of these fusions effectively rescued the growth defect of the knock-out strain on 5-FOA (Fig. 2*B*, numbers 4–6). Constructs pCW355 and pCW349 served as positive and negative controls, respectively, in the assay (Fig. 2*B*, numbers 1 and 2). Construct pCW355 carries a wild-type *VAS1* gene that expresses both cytoplasmic and mitochondrial forms of yeast ValRS, whereas construct pCW349 carries an initiator



**FIGURE 3. Conversion of *E. coli* ValRS into a functional yeast mitochondrial enzyme.** CW1, a yeast *VAS1* knock-out strain, was transformed with constructs encoding various ValRSs, and the ability of these constructs to rescue the mitochondrial defect of the knock-out strain was tested. *A*, schematic summary of constructs and their complementation activities. *Mit*, mitochondrial. ScValRS(M1A) specifies only the cytoplasmic form of yeast ValRS due to a mutation at the AUG1 initiator codon. *B*, complementation assays for mitochondrial ValRS activity on a YPG plate. *C*, assay of protein expression by Western blotting. *Upper panel*, ValRS; *lower panel*, phosphoglycerate kinase (PGK) (as a loading control). *Numbers 1–6 (circled) in B and C represent the constructs shown in A.*

mutant of *VAS1* (ATG47 to GCG) that expresses only the mitochondrial form of yeast ValRS.

To examine whether *E. coli* ValRS and its fusions are properly expressed in yeast, we next examined the relative expression levels of these constructs by Western blotting using an anti-FLAG tag antibody. As shown in Fig. 2C, *E. coli* ValRS and its fusions were all properly expressed in yeast. Fusion of Arc1p or the Ad of yeast GlnRS to *E. coli* ValRS significantly enhanced its expression level (2- and 7-fold, respectively), whereas fusion of the Ad of yeast ValRS to the *E. coli* enzyme slightly reduced its expression level (1.5-fold). Evidently, not all TRBDs enhanced the protein expression level of *E. coli* ValRS in yeast. This observation also reinforces the idea that the negative phenotype of *E. coli* ValRS in the complementation assay (Fig. 2, number 3) was not caused by a lower level of protein expression.

**Converting *E. coli* ValRS into a Functional Yeast Mitochondrial Enzyme**—In contrast to the cytoplasmic aaRSs, the mitochondrial enzymes are generally believed to be more bacterium-like in terms of their genetic origin and sequence homology. To investigate whether the mitochondrial activity of yeast *VAS1* can be functionally substituted with *E. coli* *valsS*, a

DNA segment coding for the MTS of the mitochondrial precursor form of yeast ValRS (base pairs +1 to +138 relative to the ATG1 initiator codon) was inserted in-frame at the 5' end of *E. coli* *valsS* to facilitate the mitochondrial import of the encoded bacterial protein, and the mitochondrial complementation activity of the resulting construct was tested. As shown in Fig. 3, although the *E. coli* enzyme *per se* (without fusion of an MTS) failed to restore the growth phenotype of CW1 on YPG, fusion of an MTS to the bacterial enzyme enabled it to rescue the mitochondrial defect of the knock-out strain (Fig. 3B, numbers 3 and 5). Interestingly, ValRS of *B. subtilis*, a Gram-positive bacterium that is genetically highly divergent from *E. coli*, could also be made a functional yeast mitochondrial enzyme by fusion of the MTS (Fig. 3B, numbers 4 and 6).

Because the MTS of the fusion enzymes was cleaved away upon being imported into mitochondria (Fig. 3C, numbers 3–6), it is therefore likely that the MTS did not participate in the aminoacylation reactions catalyzed by the bacterial enzymes. Constructs pCW355 and pCW327 served as positive and negative controls, respectively, in the assay (Fig. 3B, numbers 1 and 2). Construct pCW355 carries a wild-

type *VAS1* gene that expresses both cytoplasmic and mitochondrial forms of yeast ValRS, whereas construct pCW327 carries an initiator mutant of *VAS1* (ATG1 to GCG) that expresses only the cytoplasmic form of yeast ValRS.

**Enhancing the Aminoacylation Activity of *E. coli* ValRS with a TRBD**—To investigate whether the TRBDs shown in Fig. 2A enhance the aminoacylation activity of *E. coli* ValRS *in vitro*, His<sub>6</sub>-tagged *E. coli* ValRS, Ad(ScValRS)-EcValRS, and yeast ValRS were purified to homogeneity using nickel-nitrilotriacetic acid column chromatography and then assayed using unfractionated *E. coli* and yeast tRNAs as the substrates. Aminoacylation was carried out at 25 °C at a final concentration of 5, 10, or 50 nM ValRS. As shown in Fig. 4A, yeast ValRS could barely charge *E. coli* tRNA *in vitro* compared with the *E. coli* enzyme under the conditions used. Likewise, *E. coli* ValRS had a relatively poor charging activity against yeast tRNA *in vitro* compared with the yeast enzyme (Fig. 4B). This finding is not surprising, considering the fact that the *E. coli* and yeast enzymes have coevolved with their respective cognate tRNAs and therefore developed certain levels of species specificity or barriers in tRNA recognition.

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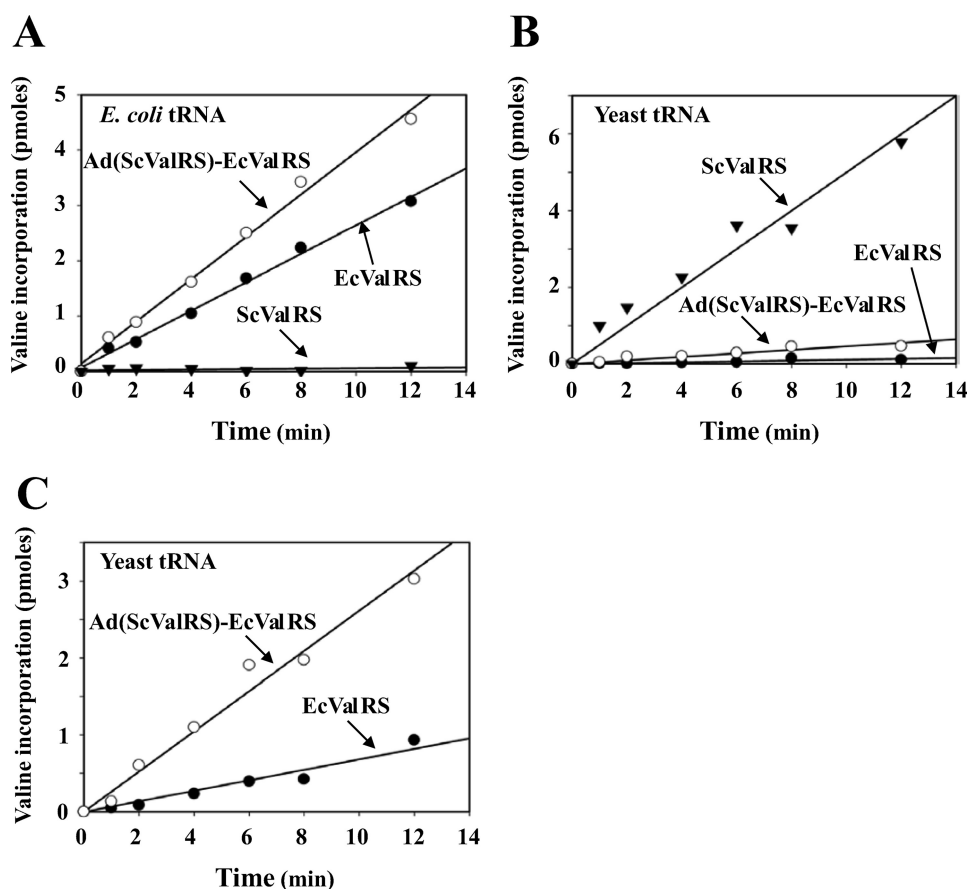


FIGURE 4. **Aminoacylation assays.** *A*, aminoacylation assay using crude *E. coli* tRNA as the substrate. The ability of ScValRS, EcValRS, and Ad(ScValRS)-EcValRS to incorporate [ $^3\text{H}$ ]valine into *E. coli* tRNA<sup>Val</sup> was assayed at a final concentration of 5 nM. The relative amounts of [ $^3\text{H}$ ]valine that were incorporated into tRNA were subsequently determined by a liquid scintillation counter. *B*, aminoacylation assay using crude yeast tRNA as the substrate. The aminoacylation activities of the ValRSs were assayed at a final concentration of 10 nM. *C*, aminoacylation assay using crude yeast tRNA as the substrate. The aminoacylation activities of the ValRSs were assayed at a final concentration of 50 nM.

**TABLE 1**  
Kinetic parameters for aminoacylation of tRNA<sup>Val</sup> by EcValRS and Ad(ScValRS)-EcValRS

Each value is determined from a hyperbolic fit of two independent data sets.

ValRS variant	$K_m$ $\mu\text{M}$	$k_{\text{cat}}$ $\text{s}^{-1}$	$k_{\text{cat}}/K_m$ $\text{M}^{-1}\text{s}^{-1}$	Relative efficiency
EcValRS	$2.08 \pm 0.08$	$(2.9 \times 10^{-2}) \pm 0.007$	$1.39 \times 10^4$	1.0
Ad(ScValRS)-EcValRS	$0.39 \pm 0.05$	$(2.7 \times 10^{-2}) \pm 0.002$	$6.92 \times 10^4$	5.0

As for the aminoacylation activity of the fusion enzyme (Ad(ScValRS)-EcValRS), our results showed that fusion of the Ad of yeast ValRS to the *E. coli* enzyme enhanced its aminoacylation activity against yeast tRNA by  $\sim 3.8$ -fold (Fig. 4B) but had only a minor effect on its aminoacylation activity against *E. coli* tRNA ( $\sim 1.4$ -fold increase) (Fig. 4A). To illustrate better the difference in aminoacylation activities against yeast tRNA between *E. coli* ValRS and the fusion enzyme, the protein concentration used for the assay was increased by 5-fold (from 10 to 50 nM). As shown in Fig. 4C, the fusion enzyme showed significantly higher aminoacylation activity against yeast tRNA than did *E. coli* ValRS, which might explain why the fusion enzyme was able to rescue the growth defect of CW1 on 5-FOA, whereas *E. coli* ValRS was not (Fig. 2). It should be noted that

although the fusion enzyme had a significantly higher aminoacylation activity against yeast tRNA than did the *E. coli* enzyme, this level of activity was still far lower than that of the yeast enzyme (Fig. 4B).

To gain further insights into the molecular mechanism of enzyme catalysis, the  $K_m$  and  $k_{\text{cat}}$  values of *E. coli* ValRS and Ad(ScValRS)-EcValRS for yeast tRNA<sup>Val</sup> were determined. As it turned out, fusion of the Ad of yeast ValRS to the *E. coli* enzyme significantly enhanced its tRNA-binding affinity ( $\sim 5.3$ -fold increase) and had little effect on its turnover number. Ad(ScValRS)-EcValRS has a  $k_{\text{cat}}/K_m$  value 5-fold higher than that of *E. coli* ValRS (see Table 1). In sum, these results suggest that TRBDs such as the Ad of yeast ValRS can significantly improve the aminoacylation activity of *E. coli* ValRS against yeast tRNA.

*Merging Cytoplasmic and Mitochondrial ValRS Activities into a Single Gene*—Because *E. coli* ValRS can be converted to a functional yeast cytoplasmic or mitochondrial enzyme, we wondered whether the *E. coli* ValRS gene can be converted into a dual functional yeast gene specifying both cytoplasmic and mitochondrial activities. To investigate this possibility, a DNA segment

(base pairs  $-300$  to  $+432$  relative to the ATG1 initiator codon of yeast *VAS1*) that contains the *VAS1* promoter and the sequence coding for the MTS (residues 1–46) and Ad (residues 47–144) of yeast ValRS was fused in-frame to the 5' end of the open reading frame of *E. coli valS*, and the complementation activities of the resultant construct were tested. As shown in Fig. 5, the fusion construct successfully rescued the growth defects of the *VAS1* knock-out strain on both 5-FOA and YPG, suggesting that, as with *VAS1*, the fusion construct specifies both activities, with the mitochondrial activity being provided by the longer form (MTS-Ad(ScValRS)-EcValRS) and the cytoplasmic activity by the shorter form (Ad(ScValRS)-EcValRS). Because the MTS of the mitochondrial precursor form (the longer form) was cleaved away upon being imported into mitochondria, making the processed mitochondrial form indistinguishable in size from its cytoplasmic counterpart, only one protein band with a molecular mass close to Ad(ScValRS)-EcValRS ( $\sim 119$  kDa) was identified by Western blotting (Fig. 5D).

## DISCUSSION

For a given amino acid specificity, there are generally two distinct nuclear aaRS genes in yeast, one encoding the cytoplasmic

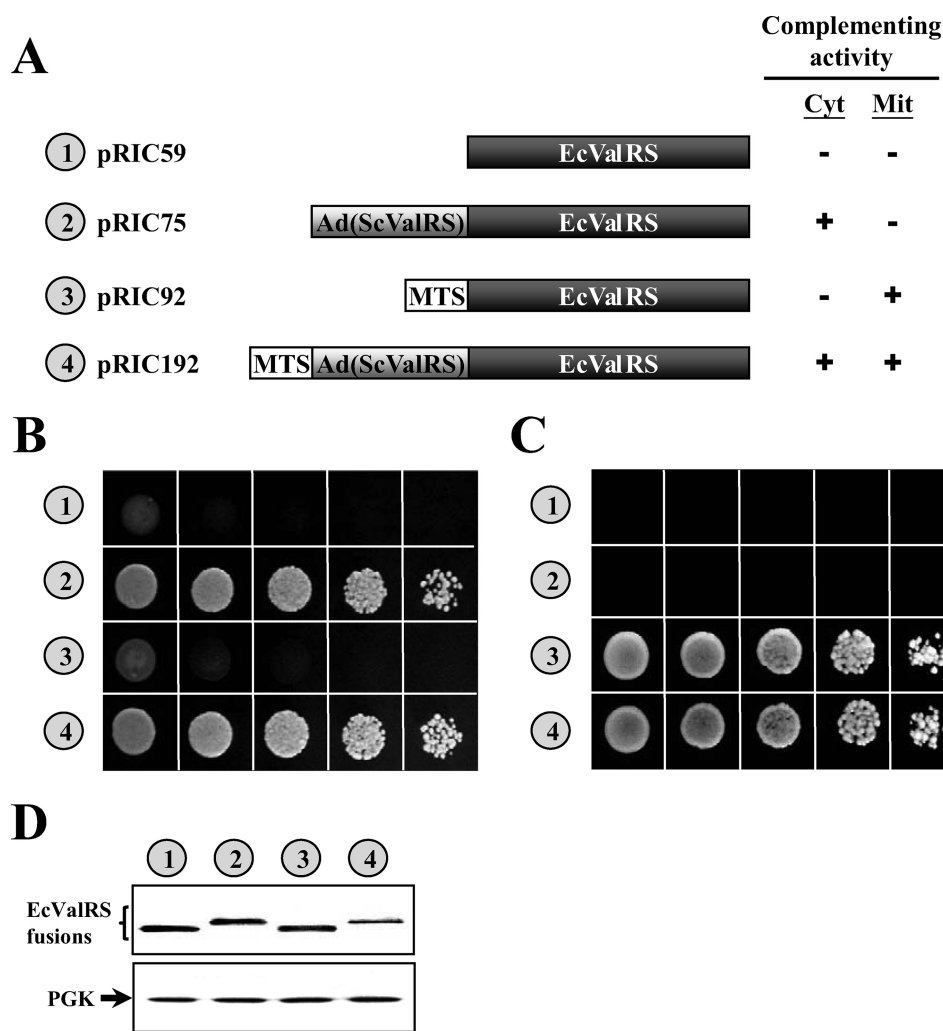


FIGURE 5. Merging cytoplasmic and mitochondrial ValRS activities into a single gene. CW1 was transformed with constructs encoding *E. coli* ValRS and its fusions, and the ability of these constructs to rescue the cytoplasmic and mitochondrial defects of the knock-out strain was tested. *A*, schematic summary of constructs and their complementation activities. *Cyt*, cytoplasmic; *Mit*, mitochondrial. *B*, complementation assays for cytoplasmic ValRS activity on a 5-FOA plate. *C*, complementation assays for mitochondrial ValRS activity on a YPG plate. *D*, assay of protein expression by Western blotting. *Upper panel*, ValRS; *lower panel*, phosphoglycerate kinase (*PGK*) (as a loading control). Numbers 1–4 (circled) in *B–D* represent the constructs shown in *A*.

mic form and the other its mitochondrial counterpart. Occasionally, one of the two homologous genes has been lost during evolution, whereas the other has picked up the lost function and become functional in both compartments. Some examples of this type include *ALAI* (6, 7), *GRS1* (8), *HTS1* (9), and *VAS1* (10). Our results presented herein argue that a bacterial aaRS gene can be converted into a dual functional yeast gene by fusion of a DNA sequence coding for both an MTS and a TRBD (Fig. 5). In addition, our results underline the necessity of obtaining a nonspecific TRBD for efficient binding and aminoacylation of yeast cytoplasmic tRNA by the bacterial enzyme (Fig. 4 and Table 1).

Although the fusion enzyme Ad(ScValRS)-EcValRS has relatively poor aminoacylation activity against yeast tRNA compared with the wild-type yeast enzyme (Fig. 4), the fusion enzyme can still effectively rescue the growth defect of CW1 on 5-FOA (Fig. 2). A similar scenario was observed in *E. coli* GlnRS, where fusion of Arc1p to the *E. coli* enzyme only slightly improved its aminoacylation activity against yeast tRNA yet

enabled this bacterial enzyme to rescue the growth defect of a yeast *GLN4* (encoding GlnRS) knock-out strain (13). Therefore, it appears that a mutant or chimeric enzyme with aminoacylation activity as low as 1–2% relative to the native yeast enzyme is sufficient to retain a near wild-type growth phenotype for the knock-out strain. This finding is particularly interesting in light of the fact that although these TRBDs possess only nonspecific tRNA-binding activity, they can promote the formation of an active complex with yeast cognate tRNA by the prokaryotic enzyme and not an abortive complex that is often seen with non-cognate or mutant tRNA. This observation also reinforces the notion that  $k_{cat}$  discrimination (specificity of tRNA aminoacylation) plays a key role in the formation of Val-tRNA<sup>Val</sup>.

The binding affinity of a tRNA synthetase for its cognate tRNAs is generally characterized by dissociation constants on the order of 0.1–1  $\mu$ M under physiological conditions (34). This relatively poor affinity ensures that the synthetases (or tRNAs) turn over rapidly during aminoacylation. In this sense, it is interesting to point out that the binding affinities of the Ads of yeast GlnRS and ValRS for tRNA also fall into this range, making them useful as a *cis*-acting tRNA-binding cofactor during aminoacylation (15).

Although the Ads of GluRS and MetRS are also rich in positively charged residues and important for aminoacylation, they do not function as a TRBD. Instead, these Ads specifically interact with a tRNA-binding cofactor, Arc1p, which, in turn, recruits tRNA to the vicinity of the enzymes for aminoacylation (35). A functionally similar tRNA-recruiting domain was identified in an auxiliary protein associated with the mammalian multisynthetase complex (36).

ValRS from mammalian cells is exclusively isolated as a high molecular mass complex with the elongation factor EF-1H (26–28). Like yeast ValRS, the mammalian enzyme also contains a strong affinity for the polyanionic carrier, heparin-Ultrogel. However, the mammalian enzyme exhibits additional hydrophobic properties (37). Sequence analysis revealed that mammalian ValRS has conserved the positively charged N-terminal extension (residues 200–298) that distinguishes yeast ValRS from its bacterial counterparts while acquiring an additional hydrophobic domain (residues 1–199) that is responsible for interacting with the four subunits of elongation factor

## Evolution of an aaRS Gene

EF-1H ( $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$  subunits) (38). A very recent report showed that, like the Ad of yeast ValRS, the positively charged N-terminal extension (residues 200–298) of the mammalian enzyme is also a nonspecific TRBD (15).

However, regardless of the detailed interpretation, the most interesting findings reported here are the capability of a nonspecific TRBD to enhance the formation of an active synthetase-tRNA complex and the conversion of an *E. coli* aaRS gene to a dual functional yeast gene encoding both cytoplasmic and mitochondrial activities. To our knowledge, this appears to be the first example wherein an engineered bacterial aaRS gene can provide both cytoplasmic and mitochondrial aminoacylation activities in yeast. In addition, our findings also suggest that a nonspecific TRBD is necessary for proper functioning of eukaryotic cytoplasmic aaRSs but not for functioning of eukaryotic mitochondrial or bacterial enzymes. Perhaps this is one of the major reasons why so many yeast cytoplasmic aaRSs possess an N- or C-terminal appendage, whereas most yeast mitochondrial aaRSs do not.

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