Trans-kingdom rescue of Gln-tRNA$^{\text{Gln}}$ synthesis in yeast cytoplasm and mitochondria

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ABSTRACT

Aminoacylation of transfer RNA$^{\text{Gln}}$ (tRNA$^{\text{Gln}}$) is performed by distinct mechanisms in different kingdoms and represents the most diverged route of aminoacyl-tRNA synthesis found in nature. In *Saccharomyces cerevisiae*, cytosolic Gln-tRNA$^{\text{Gln}}$ is generated by direct glutaminylation of tRNA$^{\text{Gln}}$ by glutaminyl-tRNA synthetase (GlnRS), whereas mitochondrial Gln-tRNA$^{\text{Gln}}$ is formed by an indirect pathway involving charging by a non-discriminating glutamyl-tRNA synthetase and the subsequent transamidation by a specific Glu-tRNA$^{\text{Gln}}$ amidotransferase. Previous studies showed that fusion of a yeast non-specific tRNA-binding cofactor, Arc1p, to *Escherichia coli* GlnRS enables the bacterial enzyme to substitute for its yeast homologue *in vivo*. We report herein that the same fusion enzyme, upon being imported into mitochondria, substituted the indirect pathway for Gln-tRNA$^{\text{Gln}}$ synthesis as well, despite significant differences in the identity determinants of *E. coli* and yeast cytosolic and mitochondrial tRNA$^{\text{Gln}}$ isoacceptors. Fusion of Arc1p to the bacterial enzyme significantly enhanced its aminoacylation activity towards yeast tRNA$^{\text{Gln}}$ isoacceptors *in vitro*. Our study provides a mechanism by which trans-kingdom rescue of distinct pathways of Gln-tRNA$^{\text{Gln}}$ synthesis can be conferred by a single enzyme.

INTRODUCTION

Faithful decoding of messenger RNA (mRNA) into protein relies on accurate aminoacylation of transfer RNA (tRNA) by aminoacyl-tRNA synthetases (aaRSs) and specific codon/anticodon base pairing. AaRSs are a structurally diverse group of enzymes, each of which ligates a specific amino acid to its cognate tRNA. The resultant aminoacyl-tRNA is then delivered to ribosomes

for mRNA translation. Typically, there are 20 aaRSs in prokaryotes, 1 for each amino acid (1–3). In contrast, eukaryotes, such as yeast, possess two distinct sets of aaRSs, one confined to the cytoplasm and the other to mitochondria. Except yeast glutaminyl-tRNA synthetase (GlnRS), which is distributed in both the cytoplasm and mitochondria (4), each set aminoacylates the isoaccepting tRNAs within its respective cellular compartment, and is sequestered from the isoacceptors confined in other compartments. In addition, cytoplasmic and mitochondrial forms of a given aaRS are nearly always encoded by two distinct nuclear genes, regardless of the cellular compartment in which they are active. However, four *Saccharomyces cerevisiae* genes, *ALA1* (encoding alanyl-tRNA synthetase) (5), *GRS1* (encoding glycyl-tRNA synthetase [GlyRS]) (6), *HTS1* (encoding histidyl-tRNA synthetase) (7) and *VAS1* (encoding valyl-tRNA synthetase) (8), specify both mitochondrial and cytoplasmic activities. In addition, cytosolic glutamyl-tRNA synthetase (GluRS$_{\text{c}}$) is localized in both compartments (9).

Many yeast cytoplasmic aaRSs possess an N- or C-terminal polypeptide extension, known as an appended domain, which is absent from their prokaryotic counterparts (10). Many of these domains are rich in lysine residues and are involved in tRNA binding, examples of which include glutaminyl- (GlnRS) (11), arginyl- (12) and valyl-tRNA synthetases (13). These domains act in *cis* as an auxiliary tRNA-binding domain and enhance the tRNA-binding affinity of the enzymes. In contrast, appended domains of some yeast cytoplasmic aaRSs participate in protein–protein interactions, examples of which include GluRS$_{\text{c}}$, methionyl- (MetRS) and seryl-tRNA synthetases. GluRS$_{\text{c}}$ and MetRS form a ternary complex with an aaRS cofactor, Arc1p (a non-specific tRNA-binding protein encoded by *ARC1*), through their N-terminal appended domains (14), while seryl-tRNA synthetase forms a binary complex with the peroxisome biogenesis-related factor Pex21p through its C-terminal appended domain (15). These interactions were shown to promote the tRNA-binding and aminoacylation activities of the associated enzymes as well. The N-terminal domain

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of Arc1p (N(Arc1p)) (amino acid residues 1–131) interacts with the N-terminal domains of MetRS and GluRS$_c$, while its middle (M(Arc1p)) (amino acid residues 132–201) and C-terminal (C(Arc1p)) (amino acid residues 202–376) domains together form a non-specific tRNA-binding domain (16). Moreover, many of the appended domains contain one or more nuclear localization signals (17), which are believed to direct synthetases to nuclei. It was shown that nuclear aminoacylation serves as a functional checkpoint for the maturation or integrity of tRNAs to be exported to the cytosol (18,19).

Despite most aa-tRNA species for mRNA decoding being synthesized by direct aminoacylation of tRNAs by cognate aaRSs, a few aa-tRNA species are generated by much more sophisticated processes. At least four aa-tRNA species were shown to be synthesized by indirect pathways (20). Among them, Gln-tRNA^{Gln} has attracted much of the attention, mostly because of its kingdom-specific pathways or enzymes (21). In eukaryotic cytoplasm and a small subset of bacteria, Gln-tRNA^{Gln} is synthesized by direct glutaminylation of tRNA^{Gln} by GlnRS (22), while in the majority of bacteria and all known archaea, Gln-tRNA^{Gln} is synthesized by an indirect pathway, where a non-discriminating GluRS (ND-GluRS) first attaches Glu to tRNA^{Gln}, forming a mischarged Glu-tRNA^{Gln} (23), which is then transamidated to Gln-tRNA^{Gln} by Glu-tRNA^{Gln} amidotransferase (GluAdT). In most eukaryotic organelles, it is still a mystery which pathway is used to generate this aa-tRNA species.

There are two distinct types of tRNA-dependent amidotransferase: the heterotrimeric GatCAB and heterodimeric GatDE. In bacteria lacking GlnRS, GatCAB functions as a GluAdT, while in bacteria lacking asparaginyl-tRNA synthetase (AsnRS), GatCAB functions as an AspAdT that transamidates Asp-tRNA^{Asn} to Asn-tRNA^{Asn} (24,25). In archaea, GatCAB is found only in species lacking AsnRS, and functions as an AspAdT (21), while GatDE is archaea specific and acts exclusively as a GluAdT. The debate over which route is really used to generate yeast mitochondrial Gln-tRNA^{Gln} was finally resolved. It was shown that yeast mitochondria use the indirect pathway to generate Gln-tRNA^{Gln} (9). However, contrary to our anticipation, ND-GluRS used in this pathway is not the mitochondrion-specific GluRS (GluRS$_m$) that charges mitochondrial tRNA^{Glu}, but, instead, the cytosolic GluRS (GluRS$_c$) exported from the cytosol. Moreover, yeast GluAdT is a novel type of tRNA-dependent amidotransferase, a heterotrimeric GatFAB (Figure 1A).

Although a small portion of cytosolic GlnRS is targeted to the mitochondrial matrix (4), the imported enzyme poorly aminoacylates tRNA$_m^{Gln}$ (mitochondrial genome-encoded tRNA^{Gln}) and therefore cannot generate a sufficient amount of Gln-tRNA$_m^{Gln}$ to satisfy mitochondrial protein synthesis (9). This effect is not surprising, considering the fact that the identity determinants of cytosolic and mitochondrial tRNA^{Gln} isoacceptors are so different. From this perspective, it is truly remarkable to find that fusion of Arc1p or the appended domain of yeast GlnRS to *Escherichia coli* GlnRS (EcGlnRS) enables the bacterial enzyme to overcome the species barrier and rescue the growth defect of a yeast *GLN4* (gene encoding GlnRS) knockout strain (11,26). We were prompted to ask whether the same set of fusion enzymes can substitute the indirect pathway for Gln-tRNA$_m^{Gln}$ synthesis in mitochondria as well. Even though many examples of cross-species or cross-compartmental complementation were reported in yeast (27–29), they are mostly, if not all, confined to complementation by orthologous or paralogous enzymes. To our knowledge, trans-kingdom rescue by a non-homologous aaRS was never previously reported. Advances in this study may recapitulate the evolutionary footprints leading to functional replacement of the indirect pathway for Gln-tRNA^{Gln} synthesis by the direct pathway in certain bacterial lineages.

MATERIALS AND METHODS

Construction of plasmids

Cloning of the EcGlnRS gene and its fusion genes into pADH (a high-copy-number yeast shuttle vector with a constitutive *ADH* promoter, a multiple cloning site and a short sequence coding for a His$_6$ tag) was previously described (13). To fuse a mitochondrial targeting signal (MTS) to the N-terminus of EcGlnRS or its derivatives, a DNA sequence encoding the MTS of the mitochondrial form of yeast valyl-tRNA synthetase (base pairs +1 to +138 relative to ATG1) was polymerase chain reaction (PCR) amplified as an EagI–SpeI fragment and fused in-frame at the 5′-end of the target genes. Mutagenesis was performed following a protocol provided by the manufacturer (Stratagene, La Jolla, CA, USA). A green fluorescence protein (GFP) assay by fluorescence microscopy followed a previously described protocol (14). The western blot analysis followed a protocol described earlier (27).

Fluorescence microscopy

Yeast cells were grown to OD$_{600}$ $\sim$ 0.6 in SD/-Leu selective medium. Cells were pretreated with MitoTracker ($\sim$300 nM) for 30 min. The samples were then examined by fluorescence microscopy (Axio observer.A1; Carl Zeiss, Inc.) using a 100$\times$ objective at 25°C and images were captured with a CCD camera (Axiocam MRm; Carl Zeiss, Inc.). Merged images and mitochondrial tracks were generated with AxioVision Rel. 4.8 software, and then subjected to 2D deconvolution with AutoQuant X2.

Complementation assays for cytoplasmic GlnRS activity

The yeast *GLN4* knockout strain, YR2 (*MATa, his3-Δ1, leu2-Δ0, ura3-Δ0, lys2-Δ0, gln4::kanMX4*), was maintained by a plasmid carrying the wild-type *GLN4* gene and a *URA3* marker. Complementation assays for cytoplasmic GlnRS activity were performed by introducing a test plasmid carrying the gene of interest and a *LEU2* marker into YR2, and the ability of the transformants to grow in the presence of 5-fluoroorotic acid (5-FOA) was determined. Starting from a cell density of 4.0 A_{600}, cell cultures were 5-fold serially diluted and 10 μl aliquots

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B

E. coli tRNA^{Gln} (CUG)   Yeast tRNA$_n^{Gln}$ (CUG)   Yeast tRNA$_m^{Gln}$ (UUG)
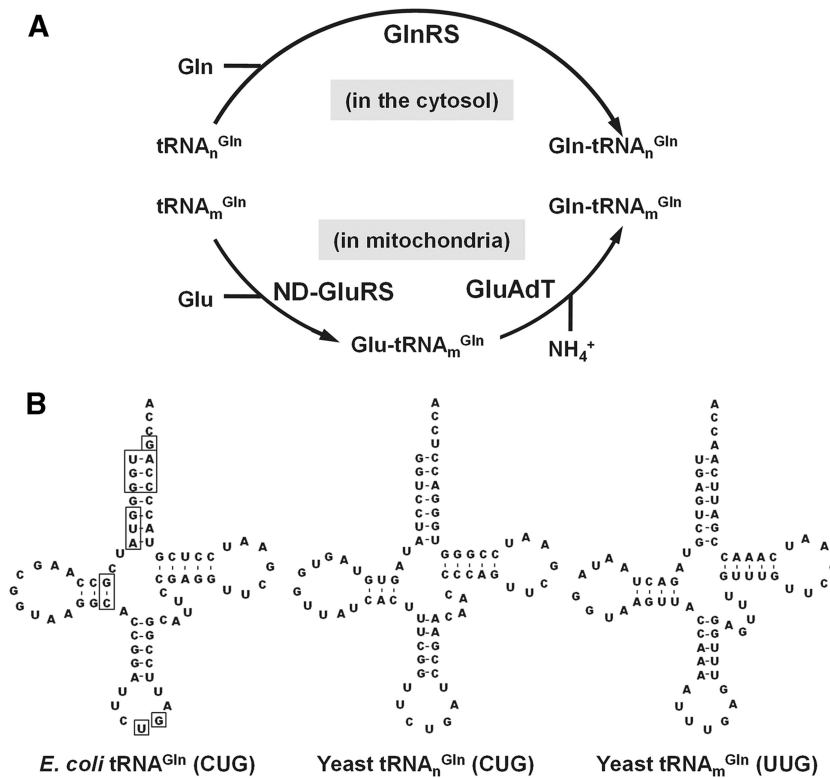
Figure 1. Translation of glutamine codons in yeast. (**A**) The direct and indirect pathways for Gln-tRNA^{Gln} synthesis in yeast. In yeast cytoplasm, Gln-tRNA^{Gln} is synthesized by direct coupling of Gln to its cognate tRNA^{Gln} by GlnRS. In contrast, in yeast mitochondria, Gln-tRNA^{Gln} is formed by an indirect pathway, where Glu is first ligated to tRNA^{Gln} by ND-GluRS, forming a mischarged Glu-tRNA^{Gln}, which is then converted to Gln-tRNA^{Gln} by GluAdT. (**B**) Comparison of *E. coli* and yeast cytoplasmic and mitochondrial tRNA^{Gln} isoacceptors. Secondary structures of tRNAs^{Gln} are shown in cloverleaf form. Nucleotides and base pairs that were shown to be important for recognition by EcGlnRS are boxed.

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 GlnRS was encoded by the test plasmid.

Complementation assays for mitochondrial GluAdT activity

The *Ymr293c* (gene encoding GatA) knockout strain was purchased from Open Biosystems (Lafayette, CO, USA) and maintained by a plasmid carrying the wild-type GatA gene and a *URA3* marker. Complementation assays for mitochondrial GatA activity were performed by introducing a test plasmid (carrying a *LEU2* marker and the gene of interest) into the knockout strain, and the resultant transformants were selected on 5-FOA lacking leucine. In the presence of 5-FOA, the maintenance plasmid (carrying a *URA3* marker) was evicted from the transformants. Transformants that survived 5-FOA selection were further tested on yeast extract peptone glycerol (YPG) plates at 30°C, with results documented on Day 3 following plating. As a yeast cell cannot survive on glycerol, a non-fermentable carbohydrate, without functional mitochondria, the transformants did not grow on the YPG plates unless a functional GatA was generated

from the test plasmid. Complementation assays for GatB (encoded by *Pet112*) and GatF (encoded by *Ygr102c*) knockout strains followed a similar protocol.

Degradation assay

To determine the turnover of proteins of interest, a cycloheximide-chase assay was performed as previously described (30). Genes encoding MTS-EcGlnRS and MTS-Arc1p-EcGlnRS were first cloned in pGAL1 (a high-copy-number yeast shuttle vector with an inducible *GAL1* promoter and a short sequence coding for a His$_6$ tag), and the resultant constructs were then transformed into INVSc1 (*MATa, his3Δ1, leu2, trp1-289, ura3-52; MATα, his3Δ1, leu2, trp1-289* and *ura3-52*) (Invitrogen, Carlsbad, CA, USA). Transformants containing these constructs were grown in medium lacking leucine with 2% raffinose to a cell density of ~1.0 A_{600} and were then induced with 2% galactose for 2 h. Then, cells were washed twice and grown in medium containing 2% glucose and $100\,\mu g\,ml^{-1}$ cycloheximide but lacking leucine. Cells were harvested at 0, 0.5, 1, 2, 4 and 8 h after the addition of cycloheximide and lysed. Forty microgram samples of the cell extracts were resolved on 10% polyacrylamide gels and electrophoresed at 100 V for ~1 h, and the proteins were transferred to a polyvinylidene fluoride membrane and immunoblotted with a horseradish peroxidase-conjugated anti-His$_6$ tag

antibody (Sigma, St Louis, MO, USA). Quantitative data were obtained from three independent experiments and normalized to phosphoglycerate kinase (PGK).

Aminoacylation assay

Aminoacylation reactions were performed at $25°C$ in a buffer containing 30 mM HEPES (pH 7.5), 25 mM KCl, 15 mM $MgCl_2$, 5 mM dithiothreitol, 4 mM adenosine triphosphate (ATP), 5 μM *in vitro*–transcribed yeast cytoplasmic or mitochondrial tRNA^{Gln} and 10 μM glutamine (4 μM ^{3}H-glutamine; Moravek Biochemicals, Brea, CA, USA). The specific activity of ^{3}H-glutamine used was 50.3 Ci/mmol. Purification of His$_6$-tagged GlnRS or its derivatives was as previously described (31). Reactions were quenched by spotting 10 μl aliquots of the reaction mixture onto Whatman filters (Maidstone, UK) soaked in 5% trichloroacetic acid and 1 mM glycine. The filters were washed three times for 15 min each in ice-cold 5% trichloroacetic acid before liquid scintillation counting. Data were obtained from three independent experiments and averaged.

Determination of active protein concentrations by active site titration was as previously described (32). For active site titration of EcGlnRS (or its derivatives), 30 mM HEPES (pH 7.5), 25 mM KCl, 15 mM $MgCl_2$, 5 mM dithiothreitol, 10 μM ATP, 2.5 mM Gln, 0.2 U/ml inorganic pyrophosphatase, γ-[^{32}P]-ATP (1000 CPM/μl final), 10 μM *E. coli* tRNA^{Gln} and appropriate concentration of EcGlnRS (or its derivatives) were incubated at $25°C$. For active site titration of yeast GlnRS, 10 μM yeast tRNA^{Gln} was used instead.

Genes coding for yeast cytoplasmic and mitochondrial tRNAs^{Gln} were PCR amplified using genomic DNA as the template and cloned in pBluescript II KS (+/−) (Agilent, Santa Clara, CA, USA). A T7 promoter and a BstNI site were, respectively, inserted at the 5′- and 3′-ends of the gene. tRNA transcripts were obtained by *in vitro* run-off transcription of the BstNI-linearized plasmid DNA, purified on a 12% polyacrylamide gel containing 8 M urea, excised from the gel and eluted from the sliced gel at $37°C$ with elution buffer (0.5 M NH_4OAc at pH 7.5 and 1 mM ethylenediaminetetraacetic acid). Concentrations of tRNAs were determined by the absorbance at 260 nm using calculated extinction coefficients based on the base compositions. Kinetic parameters for the purified enzymes were determined by directly fitting the data points to the Michaelis–Menten equation. Initial rates of aminoacylation were determined at $25°C$ with tRNA^{Gln} concentrations ranging from 1 to 20 μM and enzyme concentrations ranging from 15 to 300 nM.

RESULTS

Escherichia coli and yeast cytoplasmic and mitochondrial tRNA^{Gln} isoacceptors are highly divergent

Previous studies on tRNA recognition by EcGlnRS showed that the identity elements of tRNA^{Gln} reside mainly in the discriminator base, acceptor stem and anticodon loop (33) (Figure 1B). Particularly, the first three base p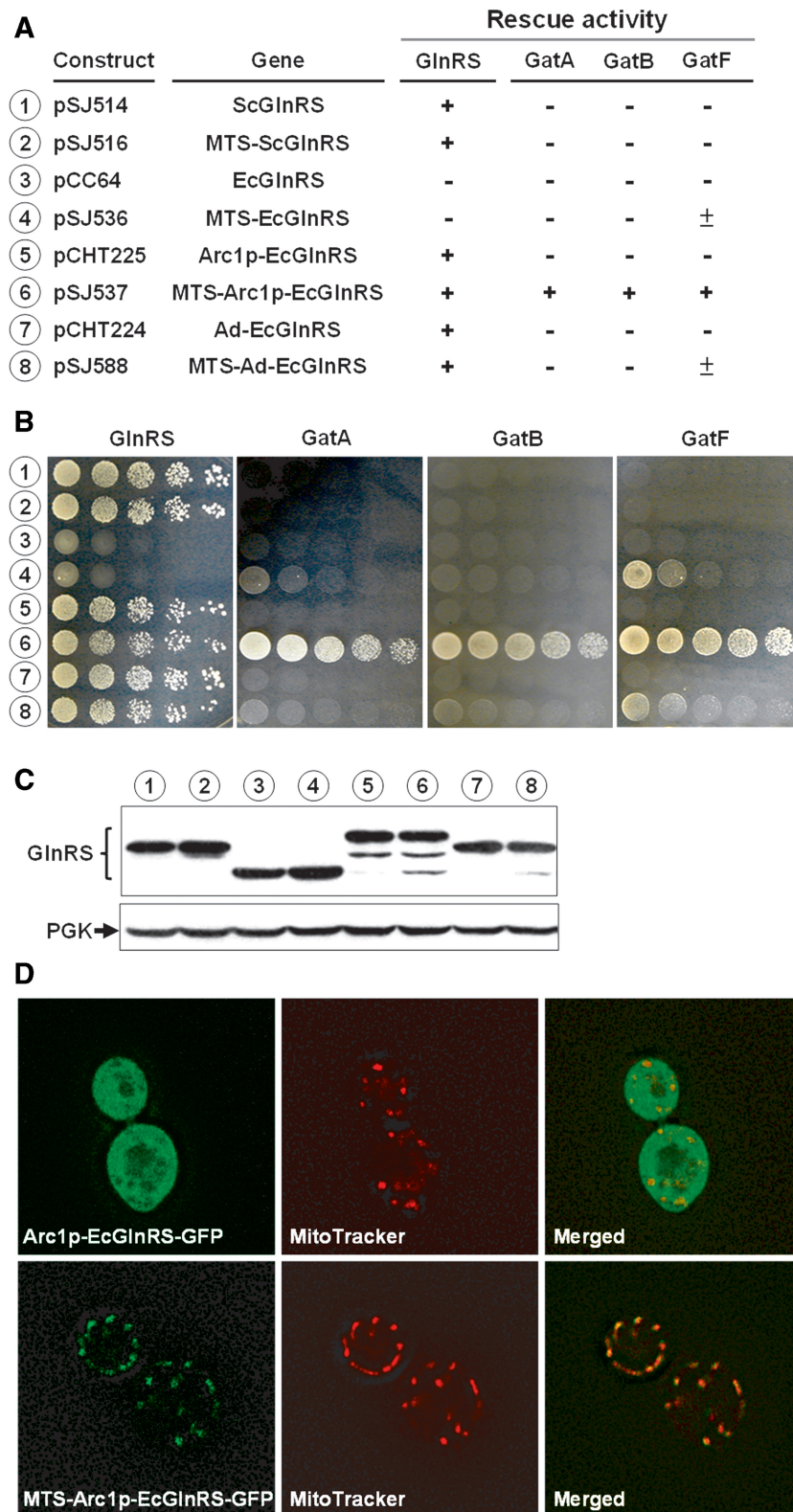airs (U1:A72, G2:C71 and G3:C70) of the acceptor stem, the discriminator base (G73), G10 in the D-stem and the second and third nucleotides of the anticodon (U35 and G36) significantly contribute to the efficiency of tRNA^{Gln} aminoacylation by GlnRS. Analysis of the nucleotide sequences and secondary structures of tRNA^{Gln} isoacceptors from *E. coli* and yeast cytoplasm and mitochondria revealed that except for G2:C71, G10, U35 and G36, almost all other identity elements have diverged (Figure 1B). Most surprisingly, the discriminator base differs in all of these tRNA isoacceptors: a G in *E. coli* tRNA^{Gln}, a U in yeast tRNA$_n$^{Gln} (nuclear genome-encoded cytosolic tRNA^{Gln}) and an A in yeast tRNA$_m$^{Gln}. As a result of these differences, yeast tRNA$_n$^{Gln} and tRNA$_m$^{Gln} are expected to be poor substrates for EcGlnRS.

The fusion enzyme, Arc1p-EcGlnRS, can substitute for the indirect pathway of mitochondrial Gln-tRNA^{Gln} synthesis

It was recently established that yeast mitochondrial Gln-tRNA^{Gln} is generated by the indirect pathway. Although a small fraction of yeast GlnRS (ScGlnRS) is imported into mitochondria, it poorly aminoacylates mitochondrial tRNA^{Gln} (9). The question arose as to whether this enzyme can substitute for the indirect pathway if it is highly expressed from a strong promoter and forced into mitochondria. A *GLN4* knockout strain was used as a host to test the ability of a target gene to substitute the direct pathway for Gln-tRNA$_n$^{Gln} synthesis; three GluAdT knockout strains that are, respectively, defective in GatA, GatB and GatF were used as hosts to test the ability of a target gene to substitute the indirect pathway for Gln-tRNA$_m$^{Gln} synthesis. To import an enzyme of interest into mitochondria, an MTS of yeast valyl-tRNA synthetase was fused to its N-terminus. Unless otherwise indicated, all genes used here were cloned in pADH, a high-copy-number yeast shuttle vector with a constitutive *ADH* promoter and a short sequence coding for a His$_6$ tag.

As shown in Figure 2, ScGlnRS with an N-terminal MTS could not rescue the growth defects of the GluAdT knockout strains, suggesting that tRNA$_m$^{Gln} is indeed a poor substrate for ScGlnRS such that no substantial amount of Gln-tRNA$_m$^{Gln} was generated by the overexpressed enzyme. Paradoxically, this construct still retained its ability to rescue the growth defect of the *GLN4* knockout strain, suggesting that, even with an MTS, a minor portion of MTS-ScGlnRS was left behind in the cytoplasm, presumably due to overexpression of the enzyme and overloading of the mitochondrial import machinery (34).

Previous studies showed that fusion of Arc1p or the appended domain of yeast GlnRS (Ad(ScGlnRS)) to EcGlnRS enables the bacterial enzyme to substitute the direct pathway for Gln-tRNA$_n$^{Gln} synthesis (11,26). We wondered whether these fusion enzymes, once being targeted into mitochondria, can substitute the indirect pathway for Gln-tRNA$_m$^{Gln} synthesis. The aforementioned MTS was fused to the N-termini of these fusion enzymes, and the ability of the resultant constructs

A

	Construct	Gene	Rescue activity			
			GlnRS	GatA	GatB	GatF
①	pSJ514	ScGlnRS	+	-	-	-
②	pSJ516	MTS-ScGlnRS	+	-	-	-
③	pCC64	EcGlnRS	-	-	-	-
④	pSJ536	MTS-EcGlnRS	-	-	-	±
⑤	pCHT225	Arc1p-EcGlnRS	+	-	-	-
⑥	pSJ537	MTS-Arc1p-EcGlnRS	+	+	+	+
⑦	pCHT224	Ad-EcGlnRS	+	-	-	-
⑧	pSJ588	MTS-Ad-EcGlnRS	+	-	-	±

B

C

D

Figure 2. Complementation assays for EcGlnRS and its derivatives. Constructs were transformed into various knockout strains of *S. cerevisiae*, and the ability of the constructs to rescue growth defects of the knockout strains was tested. The experiments were repeated three times. (**A**) Schematic summary of the GlnRS constructs and their complementation activities. The symbols '+' and '−', respectively, denote positive and negative complementation. (**B**) Complementation assays. Complementation assays for the direct and indirect pathways of Gln-tRNA^{Gln} synthesis were, respectively, tested on FOA and YPG media. (**C**) Western blotting. Upper panel, GlnRS; lower panel, PGK (as a loading control). (**D**) Analysis of cellular localization of Arc1p-EcGlnRS and MTS-Arc1p-EcGlnRS by fluorescence microscopy. As a reference, MitoTracker was used to label mitochondria. Numbers 1–8 (circled) in (A)–(C) denote the constructs shown in (A).

(MTS-Arc1p-EcGlnRS and MTS-Ad(ScGlnRS)-EcGlnRS) to rescue growth defects of the GluAdT knockout strains was tested. As controls, EcGlnRS and MTS-EcGlnRS were also tested. As shown in Figure 2B, EcGlnRS failed to restore the growth phenotypes of any of the knockout strains tested, while MTS-EcGlnRS slightly rescued the growth defect of the GatF knockout strain on YPG. On the other hand, MTS-Arc1p-EcGlnRS robustly substituted both pathways of Gln-tRNA^{Gln} synthesis, suggesting that Arc1p-EcGlnRS can recognize both cytoplasmic and mitochondrial tRNA^{Gln} isoacceptors. Conceivably, a minor portion of MTS-Arc1p-EcGlnRS was left behind in the cytoplasm, despite the introduction of a strong MTS. We next tested the MTS-Ad(ScGlnRS)-EcGlnRS construct for its ability to rescue the growth defects of knockout strains. Contrary to our anticipation, this fusion enzyme barely substituted the GluAdT activity of GatFAB. A growth curve assay in liquid medium essentially confirmed the observations made in complementation assays (Supplementary Figure S1).

To examine whether these constructs, especially those with negative complementary activity, were properly expressed in yeast, protein extracts were prepared from the transformants and then subjected to sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and western blot analyses. As shown in Figure 2C, all constructs used in the complementation assays were well expressed in yeast, regardless of whether they were confined to the cytoplasm or mitochondria. In addition, the MTS moiety attached to the fusion enzymes appeared to be cleaved off upon being imported into mitochondria, as they showed similar sizes to their counterparts lacking an MTS (compare odd with even lanes in Figure 2C). To provide further evidence that the MTS used actually directed the fusion enzymes into mitochondria, cellular distributions of two GFP fusion constructs, Arc1p-EcGlnRS-GFP and MTS-Arc1p-EcGlnRS-GFP, were analyzed by fluorescence microscopy. As a reference, MitoTracker was used to label mitochondria. Figure 2D shows that the majority, if not all, of MTS-Arc1p-EcGlnRS-GFP was localized in mitochondria (Figure 2D), indicating that the MTS chosen is very efficient in mitochondrial importation (30). In contrast, Arc1p-EcGlnRS-GFP was exclusively localized in the cytoplasm (Figure 2D). It is speculated that the dual-functional phenotype of MTS-Arc1p-EcGlnRS was attributed to overexpression of the enzyme and overloading of the mitochondrial import machinery.

The N-terminal domain of Arc1p is dispensable for the functioning of Arc1p-EcGlnRS

A recent study showed that GluRS$_c$ can be imported into mitochondria and acts as a ND-GluRS mischarging Glu to tRNA$_m^{Gln}$ (9). This means that tRNA$_m^{Gln}$ is a natural substrate of this enzyme. Along with the fact that GluRS$_c$ strongly interacts with Arc1p, the question arose as to whether the imported GluRS$_c$ mediates the interaction of MTS-Arc1p-EcGlnRS with tRNA$_m^{Gln}$ in mitochondria or the fusion enzyme binds and aminoacylates tRNA$_m^{Gln}$
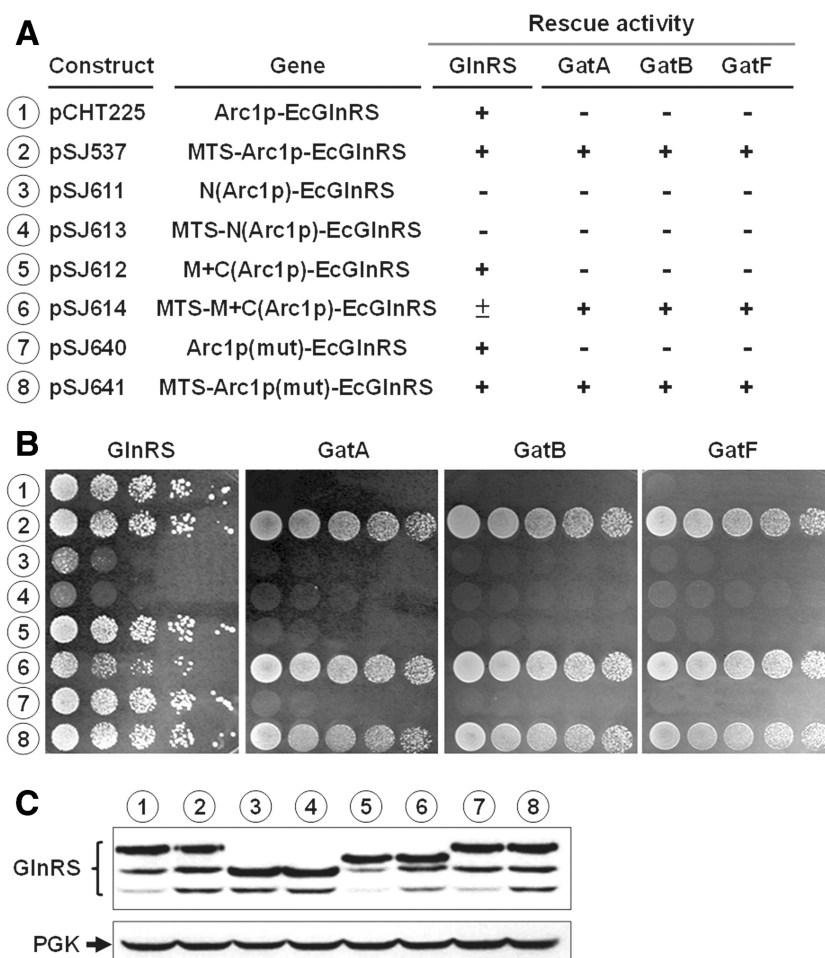
by itself. To shed light on this question, various constructs were made that fused either the N-terminal (N(Arc1p)) or middle plus C-terminal (M+C(Arc1p)) domain of Arc1p to EcGlnRS (respectively, yielding N(Arc1p)-EcGlnRS and M+C(Arc1p)-EcGlnRS), and the ability of the resultant constructs to restore the growth phenotype of the knockout strains was tested. Note that the M+C domain of Arc1p is a non-specific tRNA-binding domain.

As shown in Figure 3, M+C(Arc1p)-EcGlnRS complemented cytoplasmic GlnRS activity, while MTS-M+C(Arc1p)-EcGlnRS complemented both cytoplasmic GlnRS and mitochondrial GluAdT activities. In contrast, N(Arc1p)-EcGlnRS and MTS-N(Arc1p)-EcGlnRS failed to substitute for either of the two pathways. This result suggests that the N-domain of Arc1p is dispensable for functioning of the fusion enzyme in the cytoplasm and mitochondria, and the M+C domain of Arc1p is sufficient and necessary for the task. This conclusion was reinforced by the observation that an Arc1p double mutant (T55R and Y104A), which was previously shown to have lost its ability to interact with GluRS$_c$ (35), successfully converted EcGlnRS into an active yeast enzyme. As shown in Figure 3, Arc1p(mut)-EcGlnRS complemented the *GLN4* knockout strain, while MTS-Arc1p(mut)-EcGlnRS complemented both the gln4$^-$ strain and the knockout strains defective in GluAdT activity. Figure 3 also shows that all constructs used in the complementation assays were well expressed in yeast, regardless of whether they possessed positive complementation activity. Minor protein degradation was observed for all Arc1p fusion constructs in the western blots, indicating that these fusion proteins are rather sensitive to degradation.

Fusion of Arc1p to EcGlnRS does not enhance its protein stability *in vivo*

To explore the possibility that fusion of Arc1p enhances the protein stability of EcGlnRS, which in turn increases its steady-state level and complementary activity, a cycloheximide-chase assay was performed. Genes encoding MTS-EcGlnRS and MTS-Arc1p-EcGlnRS were first cloned in pGAL1, a high-copy-number yeast shuttle vector with an inducible *GAL1* promoter and a short sequence coding for a His$_6$ tag. The resultant constructs were transformed into a yeast strain (INVSc1), and cultures of the transformants were induced with galactose for 2 h, followed by the addition of cycloheximide, a protein synthesis inhibitor. Cells were harvested at various intervals following cycloheximide treatment and prepared for western blot analyses using an anti-His$_6$ tag antibody. As shown in Figure 4, both proteins were considerably stable under the conditions used. Protein levels of MTS-EcGlnRS and MTS-Arc1p-EcGlnRS remained almost unchanged throughout the time period tested. Less than 15% of EcGlnRS and Arc1p-EcGlnRS were degraded even after 8 h of cycloheximide treatment. Thus, Arc1p did not enhance the protein stability of EcGlnRS, and the positive phenotype of Arc1p-EcGlnRS observed in the complementation assays was not attributed to changes in protein stability.

A

	Construct	Gene	Rescue activity			
			GlnRS	GatA	GatB	GatF
①	pCHT225	Arc1p-EcGlnRS	+	-	-	-
②	pSJ537	MTS-Arc1p-EcGlnRS	+	+	+	+
③	pSJ611	N(Arc1p)-EcGlnRS	-	-	-	-
④	pSJ613	MTS-N(Arc1p)-EcGlnRS	-	-	-	-
⑤	pSJ612	M+C(Arc1p)-EcGlnRS	+	-	-	-
⑥	pSJ614	MTS-M+C(Arc1p)-EcGlnRS	±	+	+	+
⑦	pSJ640	Arc1p(mut)-EcGlnRS	+	-	-	-
⑧	pSJ641	MTS-Arc1p(mut)-EcGlnRS	+	+	+	+

Figure 3. Complementation assays for Arc1p-EcGlnRS and its derivatives. Constructs were transformed into various knockout strains of *S. cerevisiae*, and the ability of the constructs to rescue growth defects of the knockout strains was tested. The experiments were repeated three times. (**A**) Schematic summary of the GlnRS constructs and their complementation activities. The symbols '+' and '−', respectively, denote positive and negative complementation. (**B**) Complementation assays. (**C**) Western blotting. Upper panel, GlnRS; lower panel, PGK (as a loading control). Numbers 1–8 (circled) in (A–C) denote the constructs shown in (A).

Arc1p-EcGlnRS can charge both cytoplasmic and mitochondrial tRNA^{Gln} isoacceptors *in vitro*

To compare the *in vitro* aminoacylation activities of ScGlnRS, EcGlnRS and Arc1p-EcGlnRS, recombinant GlnRS-His$_6$ enzymes were purified from *E. coli* or yeast transformants by Ni-NTA column chromatography to homogeneity, and *in vitro*–transcribed yeast tRNA$_n^{Gln}$ and tRNA$_m^{Gln}$ were used as the substrates. Figure 5 shows that the yeast enzyme was much more active than both the bacterial enzyme and Arc1p fusion enzyme towards yeast tRNA$_n^{Gln}$ (Figure 5A). This effect is not surprising, considering the fact that the identity determinants of yeast tRNA$_n^{Gln}$ drastically differ from those of the *E. coli* tRNA^{Gln} isoacceptor (Figure 1B). Evidently, yeast tRNA$_n^{Gln}$ is a poor substrate for EcGlnRS and the Arc1p fusion enzyme.

To further compare the activities of EcGlnRS and Arc1p-EcGlnRS towards this tRNA species, enzyme concentrations used in the assays were increased 25-fold to 50 nM. Under these conditions, the fusion enzyme exhibited significantly higher aminoacylation activity than

that of the *E. coli* enzyme (Figure 5B), which might explain why the Arc1p fusion enzyme, but not the wild-type *E. coli* enzyme, complemented the *gln4*$^-$ strain when they were overexpressed from a strong promoter (Figure 2). We next tested whether these enzymes could efficiently charge *in vitro*–transcribed yeast tRNA$_m^{Gln}$. As shown in Figure 5C, the wild-type bacterial and yeast enzymes poorly aminoacylated the mitochondrial tRNA^{Gln}, while fusion of Arc1p to the *E. coli* enzyme significantly improved its aminoacylation activity towards this tRNA species (Figure 5C). This improvement may account for the positive phenotype of Arc1p-EcGlnRS in rescuing knockout strains defective in GluAdT activity (Figure 2).

Kinetic parameters for these enzymes were subsequently determined using unfractionated yeast tRNA as the substrate. As shown in Table 1, the K_M and k_{cat} values for ScGlnRS were, respectively, 1.92 μM and 0.82 s^{-1}, while the K_M and k_{cat} values for EcGlnRS were, respectively, 5.20 μM and 0.02 s^{-1} under the same condition. Apparently, yeast tRNA was a poor substrate for

A

B
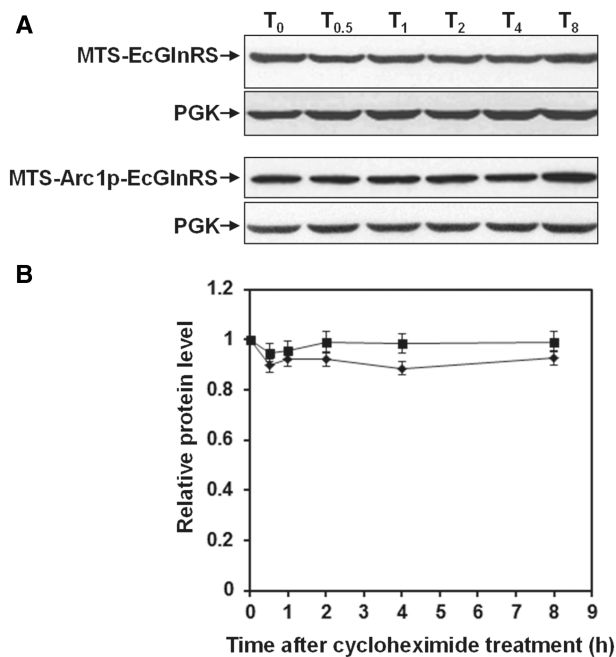
Figure 4. Degradation assays for MTS-EcGlnRS and MTS-Arc1p-EcGlnRS. Transformants were grown to a cell density of ~1.0 A_{600}, and then induced with galactose for 2 h before the addition of cycloheximide. Cells were harvested at various time points following treatment with cycloheximide and lysed. T_0, T_2, T_4, T_8 and T_{12}, respectively, denote 0, 2, 4, 8 and 12 h post-induction. PGK serves as a loading control. Quantitative data for the relative levels of MTS-EcGlnRS and MTS-Arc1p-EcGlnRS are shown in a separate diagram below the western blots. The 'filled squares' and 'filled diamonds', respectively, denote MTS-Arc1p-EcGlnRS and MTS-EcGlnRS. The experiments were repeated three times, and data were averaged.

EcGlnRS. Fusion of Arc1p to the *E. coli* enzyme significantly reduced its K_M for yeast tRNA^{Gln} (5.7-fold) and slightly enhanced its k_{cat} (2.5-fold). These results suggested that Arc1p enhances not only the apparent affinity of the bacterial enzyme for yeast tRNA^{Gln} but also the catalytic rate, albeit to a lesser extent.

DISCUSSION

In the work reported here, we attempted to substitute the indirect transamidation pathway for Gln-tRNA^{Gln} synthesis in yeast mitochondria with a direct glutaminylation pathway. Our results show that EcGlnRS poorly charged yeast cytoplasmic and mitochondrial tRNAs^{Gln}. But, fusion of Arc1p to the bacterial enzyme significantly improved its aminoacylation activity towards these tRNA species (Figure 5 and Table 1). As a result, the fusion enzyme rescued the growth defect of a *GLN4* knockout strain, and, upon being imported into mitochondria, the growth defects of knockout strains defective in GluAdT activity (Figure 2). The Arc1p moiety of the fusion enzyme acted in *cis* as an auxiliary tRNA-binding domain increasing the catalytic efficiency of the bacterial enzyme towards yeast tRNAs^{Gln} (Figure 5 and Table 1). Paradoxically, the MTS-Arc1p-EcGlnRS

fusion construct rescued both the direct and indirect pathways of Gln-tRNA^{Gln} synthesis *in vivo* (Figures 2 and 3), suggesting that the fusion enzyme was distributed (and thus functional) in both cellular compartments. In addition, our results showed that tRNA$_n$^{Gln} is a better substrate than tRNA$_m$^{Gln} for the bacterial fusion enzyme (Figure 5). It is yet to be determined whether certain post-transcriptional modifications are required for more-efficient aminoacylation of tRNA$_m$^{Gln} (36). Since the fusion enzyme successfully complemented knockout strains deficient in GluAdT activity, it is conceivable that synthesis of mitochondrial Gln-tRNA^{Gln} is the only essential mitochondrial function of the GluAdT subunits. It is noted that GatA was shown to be involved in endoplasmic reticulum remodelling (37).

The specificity of an aminoacylation reaction is achieved by direct recognition of tRNA by its cognate aaRS. In some instances, recognition depends primarily on the anticodon loop, while in others, it relies more on sequences or structures in the acceptor stem (38). In the instance of tRNA^{Ala}, the primary identity determinant is a G3:U70 base pair, which is strictly conserved in the acceptor stems of almost all known tRNA^{Ala} isoacceptors from prokaryotes, archaea, eukaryote cytoplasm and chloroplasts (39,40). Such a unique feature may account for the high efficiency of cross-species and cross-compartmental complementation (5,27,41). In contrast, the identity determinants of tRNA^{Gln} are not restricted to a single base pair or domain, but, instead, are widely distributed in the acceptor stem, discriminator base, D-loop and anticodon loop (42) (Figure 1). These determinants all exhibit strong effects on the efficiency of tRNA^{Gln} aminoacylation by EcGlnRS (33). In particular, unlike most other aaRSs, amino acid recognition and activation by EcGlnRS appear to be tRNA-dependent (43). Consequently, specific GlnRS/tRNA^{Gln} interactions affect esterification of tRNA^{Gln} and also recognition/activation of the cognate amino acid (44). With these considerations in mind, it was surprising to find that the identity determinants were not conserved in the isoaccepting tRNAs^{Gln} of *E. coli* and yeast cytoplasm and mitochondria (21). Even more surprisingly was the finding that the discriminator base differed in all these tRNA isoacceptors (Figure 1). Perhaps for this reason, yeast cytosolic GlnRS, even when overexpressed and forced into mitochondria, failed to rescue the indirect pathway (Figure 2). In contrast, the Arc1p-EcGlnRS fusion enzyme charged both the cytoplasmic and mitochondrial tRNA^{Gln} isoacceptors to appreciable levels *in vitro* (Figure 5), and substituted both the direct and indirect pathways of Gln-tRNA^{Gln} synthesis *in vivo* (Figures 2 and 3). Conceivably, the fusion enzyme possesses more-relaxed tRNA specificity, which enables the enzyme to recognize both yeast tRNA^{Gln} isoacceptors.

Since all known archaea and most bacteria use the indirect pathway to generate Gln-tRNA^{Gln} (20), it is likely that the indirect pathway is a more ancient route for synthesis of this aa-tRNA species, and that GlnRS is an evolutionarily later addition to the repertoire of aaRSs (45). In yeast mitochondria, Gln-tRNA^{Gln} is, as expected, generated by the indirect pathway. However, ND-GluRS
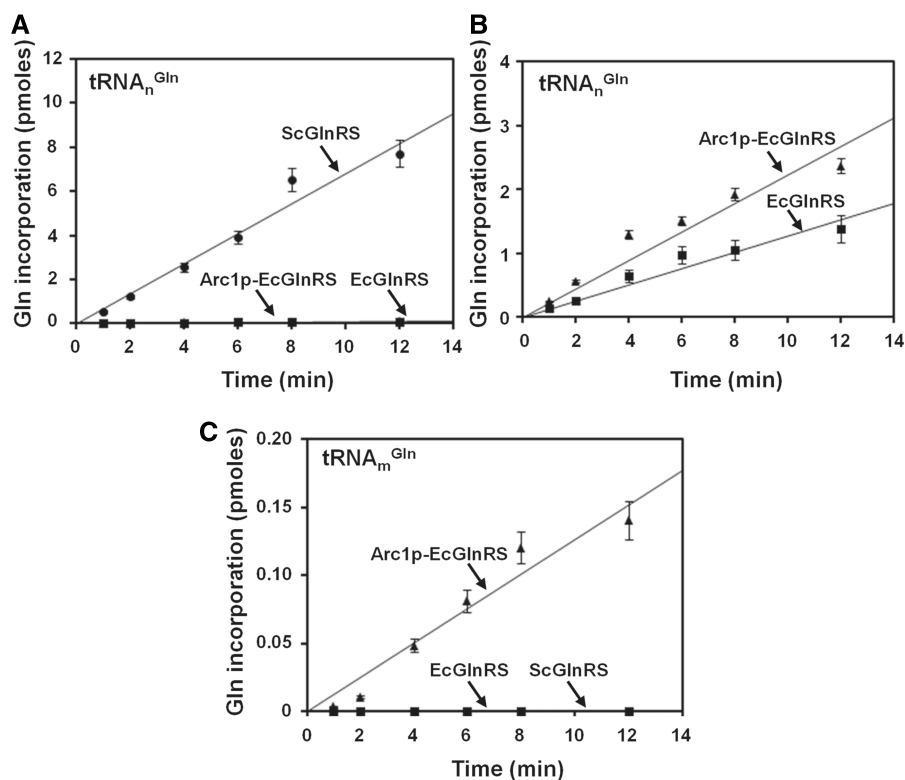
Figure 5. Aminoacylation assays for ScGlnRS, EcGlnRS and Arc1p-EcGlnRS. Aminoacylation activities of purified recombinant GlnRS-His$_6$ enzymes were determined at 25°C by measuring the relative amounts of ^{3}H-Gln that were incorporated into tRNA^{Gln} using a liquid scintillation counter. tRNA$_n^{Gln}$ and tRNA$_m^{Gln}$, respectively, denote cytoplasmic and mitochondrial tRNAs^{Gln}. The experiments were repeated three times, and data were averaged. (**A**) Aminoacylation of tRNA$_n^{Gln}$ using 2 nM GlnRS. (**B**) Aminoacylation of tRNA$_n^{Gln}$ using 50 nM GlnRS. (**C**) Aminoacylation of tRNA$_m^{Gln}$ using 200 nM GlnRS.

Table 1. Kinetic parameters for aminoacylation of yeast total tRNA by ScGlnRS, EcGlnRS and Arc1p-EcGlnRS

GlnRS variant[a]	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
ScGlnRS	1.92 ± 0.40	0.82 ± 0.15	4.27×10^5
EcGlnRS	5.20 ± 0.90	0.02 ± 0.01	0.04×10^5
Arc1p-EcGlnRS	0.92 ± 0.22	0.05 ± 0.01	0.54×10^5

[a]Each value is determined from a hyperbolic fit of three independent datasets.

used in this pathway turns out to be GluRS$_c$ which is exported from the cytosol, and GluAdT is a novel type of tRNA-dependent amidotransferase, GatFAB (9). GatA and GatB of this enzyme, respectively, share significant sequence homology with GatA and GatB of GatCAB. In contrast, GatF appears to be fungus specific. It was speculated that during yeast mitochondrial evolution, the organellar GluRS lost its tRNA^{Gln} specificity for reasons as yet unknown, which prompted the organism to export GluRS$_c$ from the cytosol to replace its function. Under fermentation conditions, GluRS$_c$ is confined to the cytoplasm by forming a complex with Arc1p, while under respiratory conditions, the expression level of Arc1p is reduced, leading to the release of this enzyme from the complex. The released GluRS$_c$ is then imported into mitochondria for functioning (9).

Regardless of the detailed interpretation, our results demonstrated that the indirect transamidation pathway for Gln-tRNA^{Gln} synthesis in yeast mitochondria can be substituted with a direct glutaminylation pathway (Figures 2 and 3). Moreover, dual recognition of the tRNA^{Gln} isoacceptors confined in separate species or cellular compartments by a single GlnRS enzyme may be a prerequisite for achieving such a process (Figures 2 and 5). Together, these findings may shed light on an earlier hypothesis of horizontal gene transfer, in which the indirect pathway for Gln-tRNA^{Gln} synthesis in certain bacterial lineages was functionally replaced by a GlnRS gene of eukaryotic origin during evolution (45–47).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figure 1.

Conflict of interest statement. None declared.

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