CCA-Addition Gone Wild: Unusual Occurrence and Phylogeny of Four Different tRNA Nucleotidyltransferases in *Acanthamoeba castellanii*

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Abstract

tRNAs are important players in the protein synthesis machinery, where they act as adapter molecules for translating the mRNA codons into the corresponding amino acid sequence. In a series of highly conserved maturation steps, the primary transcripts are converted into mature tRNAs. In the amoebozoan *Acanthamoeba castellanii*, a highly unusual evolution of some of these processing steps was identified that are based on unconventional RNA polymerase activities. In this context, we investigated the synthesis of the 3′-terminal CCA-end that is added posttranscriptionally by a specialized polymerase, the tRNA nucleotidyltransferase (CCA-adding enzyme). The majority of eukaryotic organisms carry only a single gene for a CCA-adding enzyme that acts on both the cytosolic and the mitochondrial tRNA pool. In a bioinformatic analysis of the genome of this organism, we identified a surprising multitude of genes for enzymes that contain the active site signature of eukaryotic/eubacterial tRNA nucleotidyltransferases. In vitro activity analyses of these enzymes revealed that two proteins represent bona fide CCA-adding enzymes, one of them carrying an N-terminal sequence corresponding to a putative mitochondrial target signal. The other enzymes have restricted activities and represent CC- and A-adding enzymes, respectively. The A-adding enzyme is of particular interest, as its sequence is closely related to corresponding enzymes from Proteobacteria, indicating a horizontal gene transfer. Interestingly, this unusual diversity of nucleotidyltransferase genes is not restricted to *Acanthamoeba castellanii* but is also present in other members of the *Acanthamoeba* genus, indicating an ancient evolutionary trait.

Key words: *Acanthamoeba castellanii*, CCA-adding enzyme, CC-adding enzyme, A-adding enzyme, horizontal gene transfer, tRNA nucleotidyltransferase phylogeny.

Introduction

In terms of tRNA maturation, *Acanthamoeba castellanii* is one of the most interesting organisms. Several of its mitochondrial tRNAs are subjected to a series of editing events, where encoded mismatches in the acceptor stem are removed by replacing nucleotides in the 5′-part. In this event, a specialized RNA polymerase uses the 3′-part as a template and synthesizes the new base-paired 5′-region of the acceptor stem in an unconventional 3′–5′ direction (Lonergan and Gray 1993; Price and Gray 1999; Lohan and Gray 2007; Betat et al. 2014; Long and Jackman 2015). Similar unusual tRNA editing events are described for *Dictyostelium discoideum*, another amoebozoan species (Abad et al. 2014). Recently, we identified that the latter organism exhibits an additional highly unusual feature in tRNA maturation, as it carries two fully active ATP(CTP):tRNA nucleotidyltransferases (Erber, Hoffmann et al. 2020). Hence, we investigated whether such an unusual evolutionary constellation is also present in *A. castellanii*.

To be charged with the cognate amino acid, tRNAs carry a single-stranded CCA-triplet at their 3′-end. In Eukaryotes, this CCA-triplet is not encoded in the tRNA genes but is added

posttranscriptionally in an essential step of the tRNA maturation process (Marck and Grosjean 2002; Chan and Lowe 2009; Jühling et al. 2009). The responsible enzyme is ATP(CTP):tRNA nucleotidyltransferase (Sprinzl and Cramer 1979; Deutscher 1990; Betat et al. 2010). This polymerase adds the CCA-triplet in a highly accurate but template-independent manner (Yue et al. 1998; Li et al. 2000; Weiner 2004). Due to its activity, this polymerase is called CCA-adding enzyme. According to the composition of the catalytic core, CCA-adding enzymes are divided into two classes. Class I is found in Archaea, whereas class II is present in Eukaryotes and Bacteria (Yue et al. 1996; Aravind and Koonin 1999; Martin and Keller 2007). The class II enzymes are identified by a set of highly conserved motifs, forming the catalytic core located in the N-terminal part of the protein (fig. 1A) (Li et al. 2002; Augustin et al. 2003; Tomita et al. 2004; Toh et al. 2009). Motif A binds two magnesium ions required for catalysis according to the general two-metal-ion mechanism of polymerases described by Steitz (1998). Motif D is involved in nucleotide selection and forms hydrogen bonds to the incoming base, acting as an amino acid-based template (Li et al. 2002). Additional elements are required to switch the
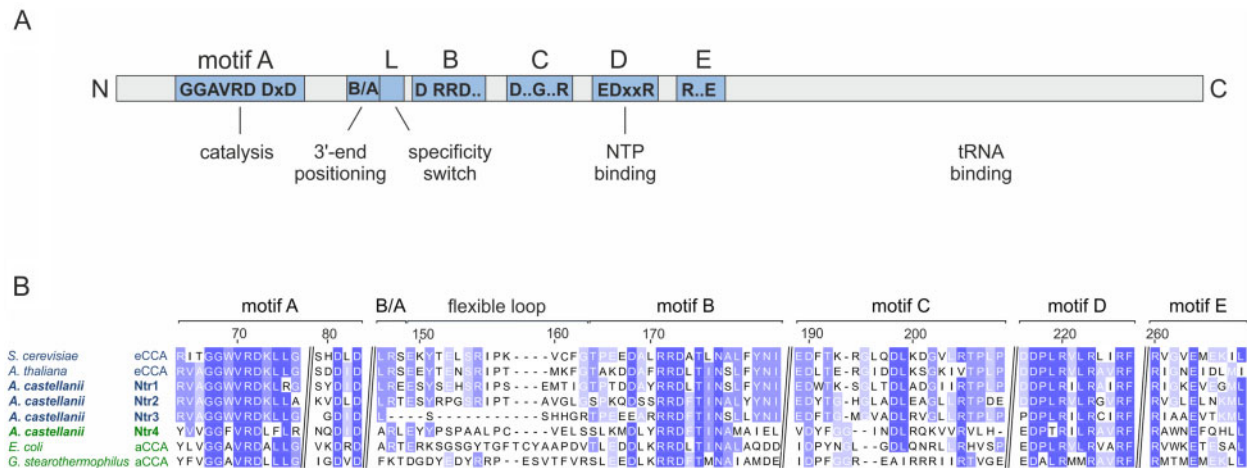
FIG. 1. Catalytic core motifs in *Acanthamoeba castellanii* tRNA nucleotidyltransferases. (*A*) Bar diagram depicting the composition of the catalytic core located in the N-terminal region of eukaryotic and bacterial tRNA nucleotidyltransferases. Highly conserved residues and the function of individual motifs are indicated. The considerably less conserved C-terminus is involved in tRNA binding. (*B*) The four protein sequences of the *A. castellanii* tRNA nucleotidyltransferases (Ntr) were aligned to sequences of well characterized CCA-adding enzymes of eukaryotic (*Saccharomyces cerevisiae* and *Arabidopsis thaliana*; blue) and bacterial origin (*Escherichia coli* and *Geobacillus stearothermophilus*, green). Ntr1, Ntr2, and Ntr3 (blue) share a high sequence similarity with the eukaryotic CCA-adding enzymes, indicating a close evolutionary relation. Although Ntr1 and Ntr2 carry highly conserved catalytic core motifs, Ntr3 shows deviations in the B/A motif and the flexible loop region characteristic for CC-adding enzymes. The sequence of Ntr4 (green) is highly similar to bacterial CCA-adding enzymes, including all conserved motifs.

enzymes nucleotide specificity from CTP toward ATP during synthesis. In this process, motif C and a flexible loop between motifs A and B contribute to the structural rearrangement of the enzyme (Neuenfeldt et al. 2008; Toh et al. 2009; Hoffmeier et al. 2010; Ernst et al. 2015; Erber, Franz et al. 2020). Lastly, a basic and an acidic residue (B/A motif) downstream of motif A positions the growing 3′-end of the tRNA for addition of the terminal A residue (Tomita et al. 2004; Neuenfeldt et al. 2008; Toh et al. 2009; Betat et al. 2010).

Interestingly, in a considerable number of bacterial species, the incorporation of C and A residues is split between two tRNA nucleotidyltransferases with partial and complementing activities. A CC-adding enzyme adds two C residues to the tRNA 3′-end but fails to incorporate the terminal A. The second enzyme cannot add C residues but incorporates the terminal A, representing an A-adding enzyme. This collaboration results in the synthesis of a complete CCA-end (Tomita and Weiner 2001, 2002; Bralley et al. 2005; Neuenfeldt et al. 2008; Bralley et al. 2009). In the CC-adding enzymes, deletions of the B/A motif and the flexible loop are responsible for the restricted activity, whereas in A-adding enzymes, the tRNA-binding C-terminus acts as a guide rail allowing only a tRNA ending with CC to enter the catalytic core (Neuenfeldt et al. 2008; Tretbar et al. 2011).

A similar split activity for CCA-addition was recently described in Eukaryotes, where several groups independently demonstrated that *Schizosaccharomyces* spp. and several choanoflagellates also possess CC- and A-adding enzymes (Preston et al. 2019; Reid et al. 2019; Erber, Franz et al. 2020). This situation comes to an extreme in *A. castellanii*, where we identified the bizarre constellation of four putative

tRNA nucleotidyltransferases carrying the catalytic core motifs typical for class II.

Here, we investigate the in vitro activity of all four putative tRNA nucleotidyltransferases (Ntrs) of *A. castellanii* as recombinant proteins. Furthermore, we present a phylogenetic characterization of the enzymes as well as the conservation of these tRNA nucleotidyltransferases among several *Acanthamoeba* species.

Results

Bioinformatic Analysis of the Putative tRNA Nucleotidyltransferase Candidates Revealed Unannotated Features

Multiple genes for putative tRNA nucleotidyltransferases were identified in the genome of *A. castellanii* in a detailed bioinformatic analysis based on the available provisional refseq data. As the published genomes of this organism provided by the NCBI database (Benson et al. 2013) are incomplete shotgun assemblies, we verified the correctness and completeness of the published gene sequences by cDNA analysis. In order to search for correct 5′-upstream and 3′-downstream sequences and to avoid the analysis of possibly incorrectly annotated genes, we performed 5′- and 3′-RACE of the mRNA sequences for the tRNA nucleotidyltransferase candidates. By this, we discovered several unannotated features of the candidate sequences. For tRNA nucleotidyltransferase gene 1 (*ntr*1), the sequence was identical to that available on NCBI (accession number XM_004334772) and no additional upstream or downstream regions could be detected. For *ntr*2 (XM_004334624), a 5′-upstream region

was identified as well as a small insertion in a nonconserved part of motif A (see Supplementary Material online). The identified 5′-sequence could be assigned as mitochondrial import sequence by MitoProt II analysis (Claros and Vincens 1996), which was corroborated by the detection of the Ntr2 protein sequence in the mitochondrial proteome (Gawryluk et al. 2014). Analysis of the third gene *ntr*3 (XM_004340480) showed some deviations from the NCBI prediction with several single base deviations, small insertions, and deletions not interfering with the open reading frame (ORF). Interestingly, the majority of the detected deviations is silent or located outside the catalytic core and its conserved motifs. Analysis of the fourth gene, *ntr*4 (XM_004334993) revealed an elongated 5′-region that was annotated in the NCBI database as an independent protein with a DHH1A binding domain (XM_004334994). However, amplification with specific primers showed that this and the *ntr*4 ORF are transcribed as a single mRNA unit consisting of one continuous ORF, suggesting that both proteins are expressed as a single polypeptide chain. Similar N-terminal extensions are reported for bacterial A-adding enzymes—tRNA nucleotidyltransferases with restricted activity that add only the terminal A residue. The actual function of these extensions, however, is not clarified yet (Tomita and Weiner 2001; Bralley et al. 2005). In addition, we detected several single base deviations, insertion, and deletions that were not present in the NCBI mRNA sequence prediction (Supplementary Material online).

Most tRNA Nucleotidyltransferase Genes Belong to the Eukaryotic e-Type

According to their evolutionary origin, eukaryotic tRNA nucleotidyltransferases are divided into two groups. The a-type enzymes (aCCA) descend from alpha-proteobacteria in a horizontal gene transfer (HGT) event, whereas the e-type enzymes (eCCA) represent the original ancient eukaryotic version (Betat et al. 2015). To clarify the evolutionary origin of the *Acanthamoeba* enzymes, the mRNA sequences verified by Sanger sequencing were translated into the corresponding protein sequences and compared with enzymes with confirmed activities and evolution. The CCA-adding enzymes of *Arabidopsis thaliana* and *Saccharomyces cerevisiae* were chosen as representatives of e-type enzymes, whereas the enzymes from *Escherichia coli* and *Geobacillus stearothermophilus* correspond to the a-type in our sequence alignment (fig. 1B). Focusing on the catalytic core region where most evolutionary information is present, the sequence alignment shows that three of the four *A. castellanii* enzymes are closely related to the ancestral eukaryotic (eCCA) type (Betat et al. 2015). Here, Ntr1 and Ntr2 share the highest sequence similarity, whereas Ntr3 shows some deviations in the B/A motif, the flexible loop region and motif B. In addition, Ntr3 has a slight variation in the amino acid template region of motif D (PDxxR instead of the conserved sequence EDxxR or DDxxR, x representing any residue). In several enzymes restricted to C-addition (CC-adding enzymes), however, this first acidic position of this signature is not conserved (Tomita and Weiner 2002; Jones 2019). Together with the lack of the basic/acidic motif (B/A) and the deletion of the flexible loop—hallmarks

for CC-adding enzymes (Neuenfeldt et al. 2008; Erber, Franz et al. 2020)—this sequence deviation indicates that Ntr3 likely represents a CC-adding activity. In contrast, the sequence of Ntr4 exhibits a high similarity to bacterial A-adding enzymes, leading to the assumption that this gene sequence derived via HGT from a bacterial species.

All Candidates Are Active tRNA Nucleotidyltransferases

Based on the alignment shown in figure 1B, Ntr1 and Ntr2 were predicted as bona fide CCA-adding enzymes, whereas Ntr3 and Ntr4, although of different evolutionary origin, likely represent a pair of complementing CC- and A-adding enzymes. To verify this prediction, the ORFs of the four *A. castellanii* nucleotidyltransferase variants were cloned and recombinantly expressed in *E. coli*. The corresponding purified enzymes were tested in vitro for their activity. To this end, two different ^{32}P-labeled transcripts of yeast tRNA^{Phe} were offered as substrates. This tRNA represents a widely used tRNA model for in vitro investigations, as the transcript folds into a structure almost identical to the modified in vivo tRNA (Shi and Moore 2000; Byrne et al. 2010). The first substrate represents a tRNA without CCA-terminus, ending at the discriminator position 73. This tRNA allows for monitoring the complete CCA-addition. The second substrate carries the two C positions 74 and 75 of the CCA-end, representing a reaction intermediate where only A76 has to be added (numbering according to Sprinzl and Vassilenko [2005]). Both substrates were incubated with recombinant enzymes in the presence of all four NTPs, ATP only, and CTP only. The reaction products were separated by denaturing polyacrylamide gel electrophoresis and visualized by autoradiography (fig. 2).

Ntr1 and Ntr2 show identical activities and add a complete CCA to tRNA^{Phe} and the lacking A residue to tRNA^{Phe}-CC (fig. 2A and B), indicating that both enzymes represent fully active CCA-adding enzymes. When only ATP is offered, Ntr1 and Ntr2 catalyze the misincorporation of an A residue on tRNA^{Phe}, leading to tRNA^{Phe}-A. This type of nucleotide misincorporation represents an in vitro artifact that was already described for the *E. coli* CCA-adding enzyme (Hou 2000; Just et al. 2008; Betat et al. 2010).

The recombinant Ntr3 enzyme readily added a C residue at position 74 of tRNA^{Phe}, whereas incorporation of the second C was rather inefficient (fig. 2C). In contrast, no A addition on tRNA^{Phe}-CC was observed. Hence, Ntr3 represents a CC-adding enzyme, although—at least in vitro—the second C residue is added at low efficiency. For the putative HGT-derived enzyme Ntr4, no activity was detectable when tRNA^{Phe} was offered (fig. 2D). However, the enzyme readily accepts tRNA^{Phe}-CC as a substrate and adds a terminal A residue when incubated with NTPs or ATP, clearly identifying this enzyme variant as a true A-adding enzyme. Hence, Ntr3 and Ntr4 represent a pair of tRNA nucleotidyltransferases with separated C- and A-adding activities.
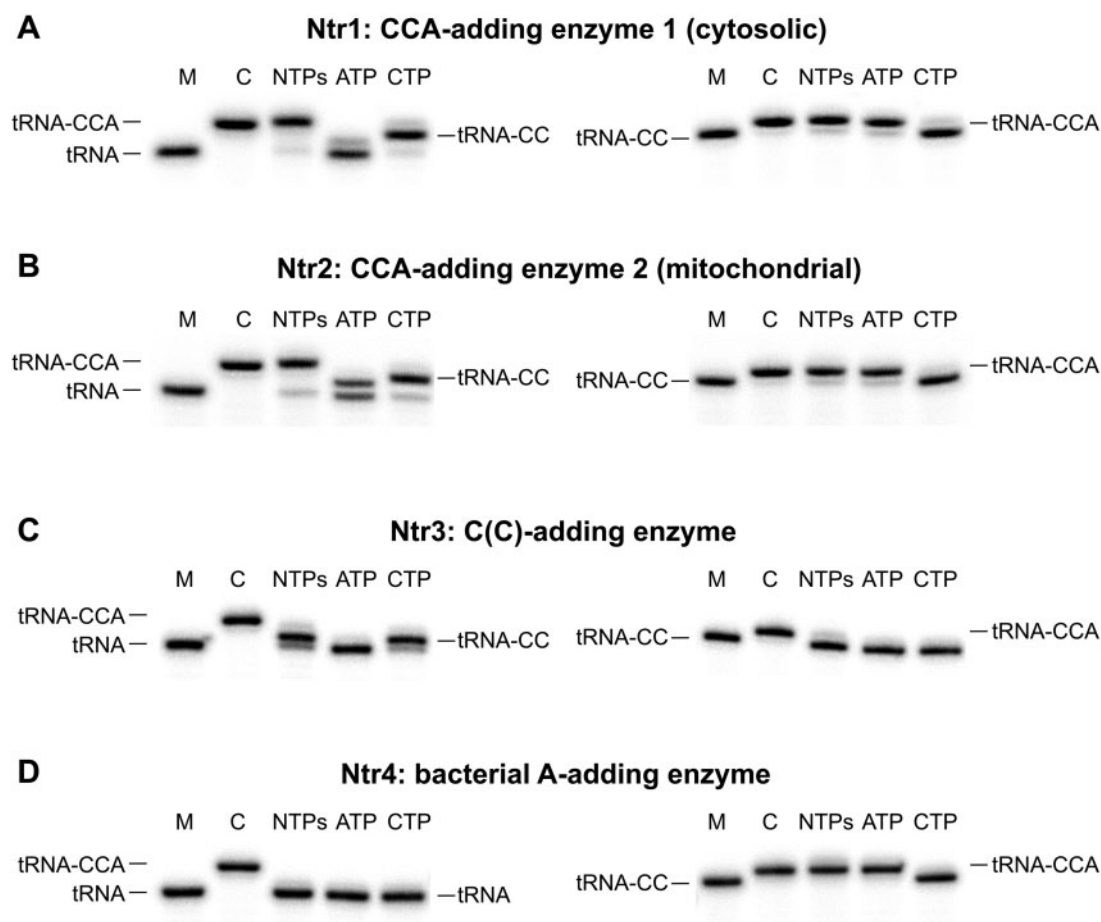
Fig. 2. In vitro activity of the tRNA nucleotidyltransferases of *Acanthamoeba castellanii*. The recombinant enzymes were incubated with α^{32}P-ATP-labeled yeast tRNA[Phe] without and with CC-end (tRNA and tRNA-CC) in the presence of NTPs, ATP only, or CTP only. (*A*, *B*) Ntr1 and Ntr2 accept a tRNA lacking the CCA-terminus and incorporate a complete CCA-end when NTPs are present. When only CTP is offered, both enzymes add two C residues, resulting in tRNA-CC. In the presence of ATP, a misincorporation of a single A residue is observed, corresponding to a well described in vitro artifact of this type of tRNA nucleotidyltransferases. On tRNA-CC, Ntr1 and Ntr2 add the terminal A residue, completing the CCA-end. According to these activities, both nucleotidyltransferases represent true CCA-adding enzymes. (*C*) In contrast, Ntr3 adds only C residues to tRNA lacking the CCA-end. Although the first residue is readily incorporated, the enzyme is very inefficient in adding the second C, indicating a loss of activity due to mutational inactivation. (*D*) Ntr4 only accepts tRNA-CC as substrate and adds the terminal A residue in the presence of NTPs or ATP, representing an A-adding enzyme.

Phylogenetic Analysis Supports an HGT Event from *Desulfovibrio* spp. to *A. castellanii*

Interestingly, the *A. castellanii* A-adding enzyme Ntr4 shares a high sequence similarity to A-adding enzymes of *Desulfovibrio* species. To elucidate its evolutionary origin, we analyzed the phylogenetic distribution of this enzyme compared with eukaryotic and bacterial tRNA nucleotidyltransferase sequences. In the phylogenetic tree reconstruction, we included the sequences of all *A. castellanii* nucleotidyltransferases (Ntr1, Ntr2, Ntr3, and Ntr4), Ntr sequences of Amoebozoa (Dictyostelia, Entamoeba), and several Metazoa with bacterial CCA-, CC-, and A-adding enzyme sequences. The underlying sequence alignment was fed into the program SplitsTree 4 for phylogenetic network construction (fig. 3). The sequences of *A. castellanii* Ntr1 (CCA1), Ntr2 (CCA2) branch along with eukaryotic sequences of Dictyostelia and Entamoeba, supporting the observation that these enzymes belong to the subgroup of ancestral eukaryotic enzyme types (e-type; see also fig. 4). The metazoan sequences form an evolutionary unit together with the bacterial CCA-adding enzymes and represent the subgroup of alpha-proteobacterial-like enzyme types (a-type) (Betat et al. 2015). Ntr3 (CC) branches between eukaryotic and bacterial enzymes and possibly represents a basal enzyme form. Ntr4, representing the *A. castellanii* A-adding enzyme, however, is located very close to the bacterial A-adding enzymes. This fact and the high sequence similarity of Ntr4 to *Desulfovibrio* spp. A-adding enzymes (supplementary fig. S1, Supplementary Material online) led us to the assumption that the *A. castellanii* Ntr4 gene was derived via HGT event. However, it is not possible to identify a specific bacterial ancestor that transmitted this gene. Given the high sequence similarity to *Desulfovibrio* spp., a member of the Proteobacteria phylum seems highly likely.
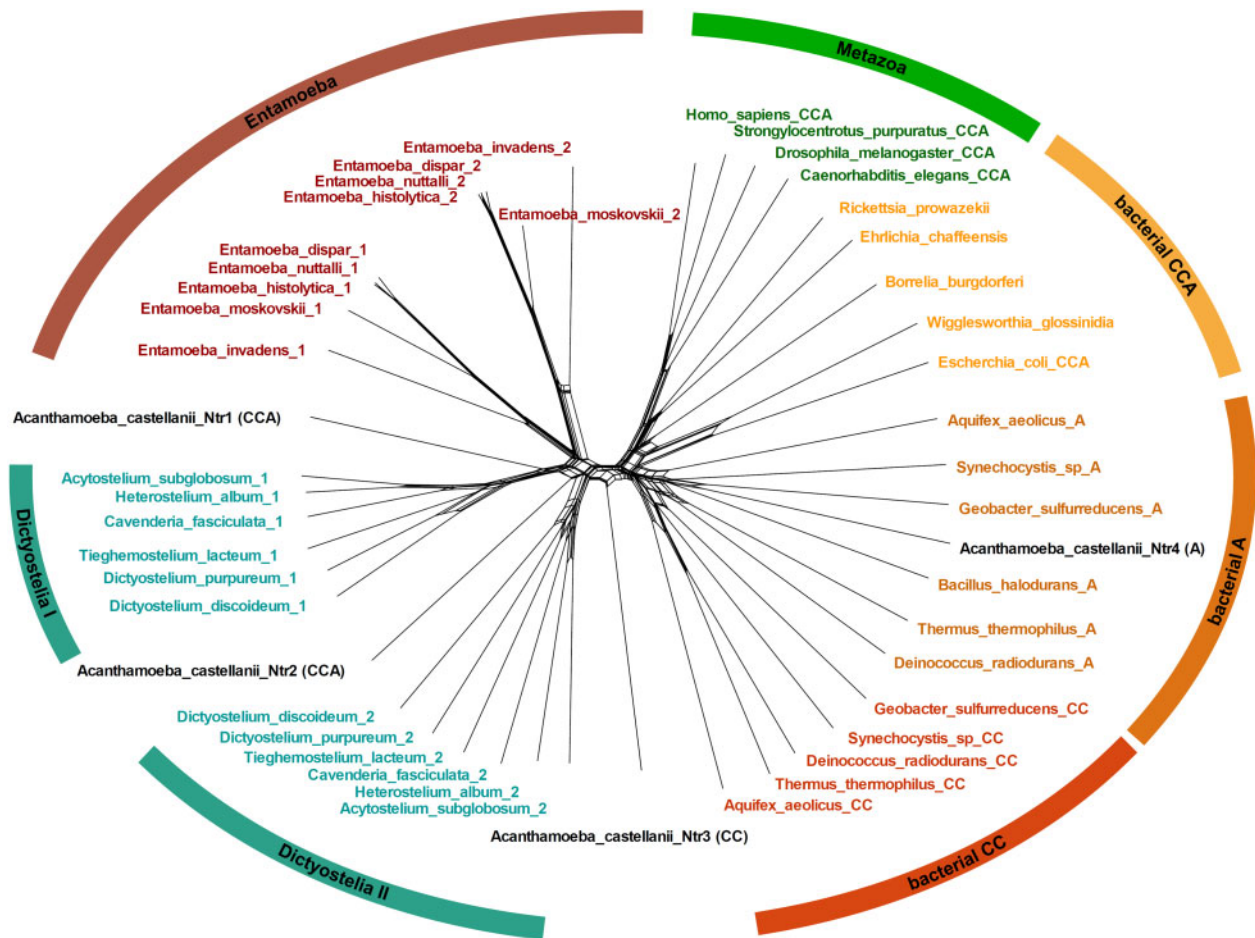
Fig. 3. Phylogenetic analysis of the *Acanthamoeba castellanii* tRNA nucleotidyltransferases. In the phylogenetic network, Ntr1 and Ntr2 branch closely to the Dictyostelia enzymes (dark cyan), whereas the Ntr3 sequence branches between the eukaryotic (Dictyostelia II, dark cyan) and the bacterial CC-adding enzymes (bacterial CC, dark orange). The A-adding enzyme Ntr4 shares a common split with the bacterial A-adding enzymes (bacterial A, orange).

Evolution of Multiple tRNA Nucleotidyltransferase Genes in the *Acanthamoeba* Genus

In order to analyze whether the unusual occurrence of multiple gene sequences for tRNA nucleotidyltransferases is limited to *A. castellanii* or is a typical evolutionary feature of the *Acanthamoeba* genus, we analyzed the available genomes of four different *Acanthamoeba* species (*A. mauritaniensis*, *A. pearcei*, *A. polyphaga*, and *A. quina*). To this end, the genomes were manually searched for tRNA nucleotidyltransferase genes and then spliced in silico based on sequence similarity to the known sequences of *A. castellanii*. Our results demonstrate that all analyzed genomes contain the four tRNA nucleotidyltransferase sequences as found in *A. castellanii*. We further performed a phylogenetic analysis of the found putative tRNA nucleotidyltransferase genes of the *Acanthamoeba* spp. (fig. 4). Interestingly, the sequences of all *Acanthamoeba* spp. branch very closely together, only splitting at the top end of the branch. As described for the A-adding enzyme Ntr4, the corresponding enzymes of other *Acanthamoeba* spp. also branch closely together with the bacterial A-adding enzymes, indicating that other *Acanthamoeba* species also acquired this type of gene via

HGT. Ntr2 and Ntr3 seem to split more distantly to other phylogenetic groups and rather form separate branches. These results demonstrate that the unusual occurrence of multiple tRNA-nucleotidyltransferase genes is widely distributed among *Acanthamoeba* species and represents an ancient evolutionary trait, indicating that the common ancestor already carried four tRNA nucleotidyltransferases.

Discussion

Acanthamoeba castellanii Has Four Active tRNA Nucleotidyltransferases

In the last years, the diversity of eukaryotic tRNA nucleotidyltransferases has been more and more unveiled. In these investigations, enzyme pairs with restricted but complementing activities as CC- and A-adding enzymes were observed, similar to the bacterial situation (Betat et al. 2015; Preston et al. 2019; Reid et al. 2019; Erber, Franz et al. 2020). Here, we describe an extreme situation in *A. castellanii*, where four independent genes for tRNA nucleotidyltransferases could be identified. This organism is described as a "genetic melting pot" with increased HGT (Boyer et al. 2009; Maumus and Blanc 2016; Wang and Wu 2017) due to its feeding behavior on bacteria
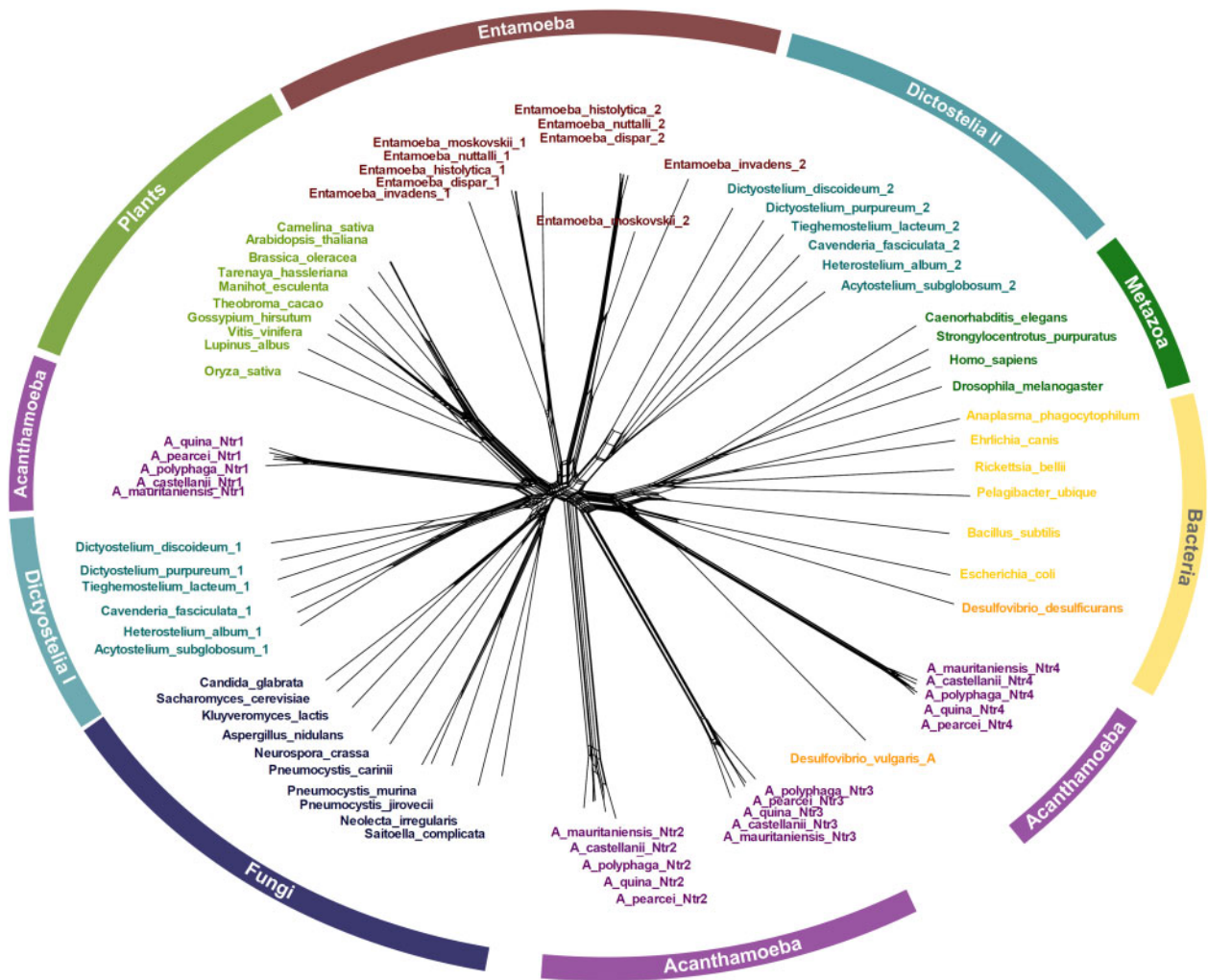
FIG. 4. Phylogenetic network of tRNA nucleotidyltransferases from *Acanthamoeba* species. tRNA nucleotidyltransferase sequences of *Acanthamoeba mauritaniensis, A. polyphaga, A. quina,* and *A. pearcei* (Acanthamoeba, purple) were aligned to corresponding sequences of Fungi, Dictyostelia, Plants, Entamoeba, Metazoa, and Bacteria. All *Acanthamoeba* enzymes follow the same distribution. Ntr1 enzymes branch between Dictyostelia (Dictyostelia I, cyan) and plant enzymes (Plants, green), whereas Ntr2, Ntr3, and Ntr4 are located between eukaryotic (Fungi, blue) and bacterial CCA-adding enzymes (yellow). Ntr4 enzymes share a common split with the A-adding enzyme of *Desulfovibrio vulgaris* (yellow), underlining their origin by HGT.

(Weekers et al. 1993), the frequent infection by nucleocyto-plasmic large DNA viruses (NCLDV) (Aherfi et al. 2016) or virophages (La Scola et al. 2008), and its function as host for a broad variety of microorganisms (Sandström et al. 2011; Guimaraes et al. 2016). In 2013, Clarke et al. published the complete genome sequence of *A. castellanii* and stated that about 450 genes (representing 2.9% of the proteome) are derived by HGT from other organisms such as Bacteria and Archaea (Clarke et al. 2013). Hence, it is not a surprise that such an unusual combination of tRNA nucleotidyltransferase genes is detected in *Acanthamoeba*. As a genetic manipulation of *A. castellanii* is far from being trivial and was successfully performed only in very few cases (Swart et al. 2018), we focused on a biochemical characterization of the recombinantly expressed enzymes.

Recently, the first transcriptomic data of *A. castellanii* were published (Shabardina et al. 2018), which show mRNA expression for all four *ntr* genes with RPKM values (reads per kilo base per million mapped reads) ranging from 5.1 (*ntr4*) up to 48.2 (*ntr2*). These observations convincingly demonstrate that all four *ntr* genes are expressed, indicating the relevance of these genes for *A. castellanii*.

Ntr1 and Ntr2: CCA-Addition in Cytosol and Mitochondria

As shown in figure 2, Ntr1 and Ntr2 represent true CCA-adding enzymes and catalyze the addition of the complete nucleotide triplet. Ntr2, and to a reduced extent also Ntr1, shows misincorporation of a single A residue into a tRNA at position 74 (corresponding to the first position of the CCA-end). Such extra nucleotide additions are described for several class 1 and class 2 enzymes, where—depending on the incubation conditions—additional C or A residues are incorporated (Hou 2000; Seth et al. 2002; Betat et al. 2004; Wilusz et al. 2011; Ernst et al. 2018). Although a regulatory function of

these extra nucleotide additions in vivo cannot be excluded (in terms of tRNA stability or availability), the fact that a tRNA-A is not further elongated by the enzyme represents a proof-reading mechanism executed by the basic/acidic motif (B/A) during CCA-addition. In the catalytic core, these amino acid residues are involved in the correct positioning of the growing tRNA 3′-end for nucleophilic attack on the incoming next NTP to be incorporated (Tomita et al. 2004; Neuenfeldt et al. 2008; Toh et al. 2009). Only a primer ending in a C residue is recognized, whereas other 3′-ends are not properly oriented for CCA completion, avoiding the production of nonfunctional tRNAs that might interfere with aminoacylation and protein synthesis (Lizano et al. 2008).

As Ntr2 carries a putative mitochondrial import sequence, it is likely that at least a part of this enzyme pool is translocated into this organelle. Ntr1, on the other hand, is lacking such a signal sequence. Hence, it seems that these enzymes act on two different tRNA pools in the cell, the cytosolic (Ntr1, and to a certain extent also Ntr2) and the mitochondrial one (exclusively Ntr2). This situation resembles the distribution of aminoacyl-tRNA-synthetases in eukaryotes, where several enzymes specific for mitochondrial tRNAs are imported into the organelle, whereas others are dually targeted to cytosol and mitochondria or reside exclusively in the cytosol (Tyynismaa 2013; Hällberg and Larsson 2014). Although structural deviations of mitochondrial tRNAs in many species require such extra sets of synthetases, it seems highly unlikely that this is also the case for the CCA-adding enzymes, as the substrate requirements for CCA-addition are not as stringent as for aminoacylation and the mitochondrial tRNAs of A. castellanii are of canonical shape. One difference between cytosolic and mitochondrial tRNAs in A. castellanii is the extensive editing in the mitochondrial tRNAs (Lonergan and Gray 1993; Lohan and Gray 2007). However, as these editing reactions convert the tRNA structures into the consensus form, it is highly unlikely that the mature mitochondrial tRNAs require a specialized CCA-adding enzyme.

Hence, it seems that there exist two different solutions to deliver a CCA-adding activity to cytosol and mitochondria. In Acanthamoeba spp., this is achieved by two separate genes, whereas in most other eukaryotes, a single gene provides the activity for both cell compartments (Chen et al. 1992; Wolfe et al. 1996; Nagaike et al. 2001; Reichert et al. 2001).

Ntr3 and Ntr4 with Partial Activities: Collaboration or Independent Function?

Although the existence of eukaryotic CC- and A-adding enzymes is described for Salpingoeca rosetta and Schizosaccharomyces pombe (Preston et al. 2019; Reid et al. 2019; Erber, Franz et al. 2020), the existence of enzymes with split activity in A. castellanii is surprising considering that this organism already possesses two CCA-adding enzymes. In contrast to Sal. rosetta and Sc. pombe, the pair of A. castellanii CC- (Ntr3) and A-adding (Ntr4) enzymes obviously evolved independently from each other, as Ntr4 is presumably derived by HGT as a bacterial A-adding enzyme. In addition to that, the structure and conservation of the A. castellanii CC-adding enzyme differs from CC-adding enzymes in Sal. rosetta and

Sc. pombe, where the restricted activity is linked to a loss of conserved amino acids in the flexible loop region and the B/A motif (Erber, Franz et al. 2020). However, in the A. castellanii CC-adding enzyme Ntr3, the flexible loop is deleted (fig. 1), similar to bacterial CC-adding enzymes (Neuenfeldt et al. 2008). Ntr3 catalyzes only a rather inefficient incorporation of the second C residue (C75; fig. 2C). As Ntr1 and Ntr2 synthesize complete CCA-ends, it seems that there is no selective pressure on Ntr3 activity, resulting the accumulation of detrimental mutations and, consequently, progressive loss of enzymatic function. Alternatively, it is possible that the in vitro reaction conditions are not suitable for Ntr3. However, we exclude this possibility, as all other nucleotidyltransferases tested in our lab, ranging from CCA- to CC- and A-adding enzymes of a whole range of organisms, are highly active under these conditions.

The A-adding enzyme Ntr4 exhibits a high fidelity for the terminal A-incorporation, as no misincorporations could be observed. Alignments and phylogenetic analyses of the A. castellanii A-adding enzyme and bacterial enzymes support a HGT from a species closely related to the Desulfovibrio genus from the class of Deltaproteobacteria. Desulfovibrio spp. carry two genes that encode for putative CC- and A-adding enzymes, as the corresponding amino acid sequences carry several characteristic features. As an example, one tRNA nucleotidyltransferase in Desulfovibrio vulgaris shows typical elements of CC-adding enzymes, like sequence deviations in the flexible loop, a QRD sequence instead of RRD in motif B and an xDxxR amino acid template in motif D instead of EDxxR (Tomita and Weiner 2002; Bralley et al. 2005; Neuenfeldt et al. 2008; Jones 2019; Erber, Franz et al. 2020). The second D. vulgaris enzyme carries the sequence PAALP in its flexible loop, a motif characteristic for A-adding enzymes that we identified in published sequence alignments (Jones 2019). With a sequence identity of about 40%, the A. castellanii Ntr4 enzyme is closely related to these putative Desulfovibrio A-adding enzymes. This relation is corroborated by the clear A-adding activity of Ntr4 and its N-terminal extension, representing a frequent feature of bacterial A-adding enzymes, although its function is still unclear (Tomita and Weiner 2001; Bralley et al. 2005).

Since A. castellanii feeds on bacteria, one might assume that the ntr4 gene is a bacterial gene that was falsely annotated to A. castellanii. However, regarding the exon/intron structure (supplementary fig. S2, Supplementary Material online) and the codon usage (supplementary fig. S3, Supplementary Material online), a contamination with genetic material of bacterial origin can be excluded. The encoded amino acid sequence, however, clearly shows a close evolutionary relation between the Ntr4 enzyme and the nucleotidyltransferase gene of D. vulgaris (NCBI entry WP_010938942.1). The described HGT of ntr4 is not a unique scenario, as similar integration events of other bacterial genes into the genome of A. castellanii were frequently described (Moliner et al. 2009; Clarke et al. 2013; Dröge et al. 2014; Chelkha et al. 2018; Shabardina et al. 2018). An explanation for this extensive HGT might be the proximity of A. castellanii to other organisms such as Bacteria, Fungi, and viruses

(Doolittle 1998). The majority of such horizontally derived genes degenerate after time and are expulsed from the genome (van Passel et al. 2008). In *ntr*4, however, the high amount of accumulated introns indicates a successful adaptation of the bacterial A-adding enzyme to the gene expression machinery of *A. castellanii*, suggesting a relevant function of Ntr4 in this organism. The terminal A residue of the CCA-end underlies a frequent turnover due to nuclease activity or hydrolysis, resulting in tRNA molecules ending with CC. Several of the involved nucleases were identified in *E. coli* (RNase T) (Deutscher et al. 1985), *S. cerevisiae* (Rex1p) (Copela et al. 2008), and humans (angiogenin) (Czech et al. 2013), and it is very likely that other organisms, including *A. castellanii*, have similar nuclease activities. Although it was shown that CCA-adding enzymes can restore the missing position (Deutscher et al. 1977; Lizano et al. 2008; Wellner, Betat, Mörl 2018; Wellner, Czech, et al. 2018), it is conceivable that in *A. castellanii*, the A-adding activity of Ntr4 fulfills (or at least supports) this important repair function in the cytosol and/or nucleus.

Evolution of Four Different Enzymes: Adaptation to the Environment?

Although enzyme paralogs and isozymes are rather common, the occurrence of four different *ntr* genes raises the question as to why *A. castellanii* requires such a number of tRNA nucleotidyltransferases, whereas in the majority of organisms a single gene is sufficient. The fact that further *Acanthamoeba* species carry the same number of tRNA nucleotidyltransferase genes indicates that this is not a single exceptional event of randomly acquired genes in *A. castellanii*. Especially, the acquisition of introns in the *ntr*4 gene, which we found in *A. mauritaniensis*, *A. pearcei*, *A. polyphaga*, and *A. quina* as well, highlights the extensive adaptations of this gene and indicates an important function in *Acanthamoeba*.

Acanthamoeba castellanii is able to adapt its metabolism and gene expression to many different environmental conditions such as shortage of resources (Byers et al. 1991; Lloyd 2014), temperature (Jones et al. 1993), and anaerobiosis (Lloyd et al. 1983), resistance to bacterial toxins (Shabardina et al. 2018) or harsh environmental conditions (Aksozek et al. 2002). It was speculated that the acquisition of foreign genes promotes an enhanced adaptation to environmental conditions (Clarke et al. 2013; Shabardina et al. 2018). Thus, the accumulation of several tRNA nucleotidyltransferase genes might be beneficial for the adaptation to stress situations. Further, it was shown that the expression of tRNAs is altered during the encystment of *A. castellanii* (McMillen et al. 1974; McMahon et al. 1980), which might require the activity of certain subspecialized tRNA nucleotidyltransferases. It is also conceivable that one of the CCA-adding enzymes in *A. castellanii* (Ntr1 or Ntr2) has an additional function in tRNA quality control. Wilusz and Sharp demonstrated that tRNA nucleotidyltransferases can add a second CCA-end to certain tRNA transcripts that are structurally destabilized (due to mutations or lacking modifications), and that the resulting CCACCA-end represents a tag that labels these transcripts for degradation (Wilusz et al. 2011). A situation where both Ntr1 and Ntr2 are involved in tRNA maturation by CCA-addition, whereas only one enzyme monitors tRNA quality would represent a scenario where both genes are essential for *A. castellanii*.

The existence of multiple enzyme versions in *A. castellanii* is not limited to tRNA nucleotidyltransferases, as also multiple gene copies for tRNA-synthetases, for antibacterial resistance, genes important for adaptation to external conditions and ribosomal proteins were found in the transcriptome data (Shabardina et al. 2018). Some of the aminoacyl-tRNA synthetase genes were described to be derived by HGT as well (Clarke et al. 2013). The occurrence of multiple gene copies for this type of enzymes was already shown for some bacteria, however, their function in the organism is still unclear (Chaliotis et al. 2017). One explanation here might be the involvement in pathways besides the translation machinery. This acquisition of new functions might also be the case for the multiple gene copies of ribosomal protein paralogs that have been discovered in *A. thaliana* and *S. cerevisiae* (Komili et al. 2007; Carroll et al. 2008).

Conclusion

The tRNA nucleotidyltransferase genes discovered in *A. castellanii* demonstrate the huge gene repertoire of this amoeba. The acquisition of a bacterial A-adding enzyme in a eukaryotic organism was never observed before and highlights the gene traffic that is occurring between the *Acanthamoeba* genus and bacterial organisms. Although the selective advantage for the evolution of such a multitude of tRNA nucleotidyltransferase genes has to be elucidated, it is possible that these genes are involved in additional processes beyond CCA-addition and thus might enhance the adaptation of *A. castellanii* to environmental conditions.

Materials and Methods

Isolation of *A. castellanii* Total RNA and mRNA

Cell material stored in Trizol was treated according to the manufacturer's protocol (Thermo Scientific). mRNA was isolated with the Dynabeads mRNA Purification Kit (Thermo Scientific).

Rapid Amplification of 5′- and 3′-cDNA Ends

For 3′-RACE, mRNA of *A. castellanii* was reverse transcribed by Avian Myeloblastosis Virus Reverse Transcriptase (AMV RT). To this end, 80 ng purified mRNA, 1 µM oligo-dT-anchor primer (5′-GACCACGCGTATCGATGTCGAC(T) 16V-3′; V = A, C, G) and 2 mM dNTP mix (equimolar amounts) were incubated for 3 min at 65 °C. RT reaction was carried out in 1× AMV buffer with 10 U AMV reverse transcriptase for 1 h at 42 °C. Reaction was stopped by heat inactivation for 5 min at 90 °C. Varying amounts of template cDNA were amplified with 0.4 U Phusion DNA polymerase HF (Thermo Scientific) in 1× HF buffer with 200 µM dNTPs (equimolar amounts) and 0.5 µM primer (forward: gene specific primer; reverse: 5′-GACCACGCGTATCGATGTCGAC-3′). Polymerase chain reaction (PCR) cycles were as follows: 1 min at 98 °C for initial denaturation, 30 cycles of

98 °C (10 s; denaturation), 57 °C (10 s; annealing), and 72 °C (1 min; elongation), followed by a final extension of 5 min at 72 °C.

For the analysis of mRNA 5′-ends, the 5′-RACE system for rapid amplification of cDNA ends of Thermo Scientific was used (version 2.0). The procedure was performed according to the manufacturers' protocol except that AMV RT (NEB) was used for reverse transcription and Phusion DNA polymerase HF (Thermo Scientific) for PCR amplification as described above. In total, 140 ng purified mRNA was used for first strand synthesis. Reaction products of 3′- and 5′-RACE were cloned into pJET vector using the CloneJet PCR cloning kit (Thermo Scientific). Individual clones were analyzed by Sanger sequencing.

Cloning of tRNA Nucleotidyltransferases ORFs

For cloning of the putative tRNA nucleotidyltransferase ORFs, specific primers and Phusion DNA polymerase HF (GC-rich protocol) were used in a touchdown PCR (Korbie and Mattick 2008). Reaction products were cloned into pET28b(+) expression vector. Correct inserts were verified by Sanger sequencing.

Enzyme Expression and Purification

Expression vectors containing the ORFs of the putative *A. castellanii* tRNA nucleotidyltransferases fused to a C-terminal His-tag were used to transform *E. coli* BL21 DE3 (*cca::-cam*, Δ*cca*) cells lacking the endogenous CCA-adding enzyme in order to exclude contamination with the corresponding enzymatic activity of *E. coli*. Cells were grown in TB-medium in the presence of kanamycin and chloramphenicol as described (de Wijn et al. 2018). For purification of expressed recombinant enzymes, cell pellets were solved in 20 mM Tris/HCl, 500 mM NaCl, 5 mM MgCl$_2$, and 5 mM imidazole and lysed in a FastPrep-24^{TM} homogenizer (MP Biomedicals) and 0.1 mm zirconia beats. The lysate was used for affinity chromatography purification in an Äkta purifier system (GE Healthcare) using a 1 ml HisTrap FF column (GE Healthcare). Fractions containing the desired protein were dialyzed overnight (25 mM Tris/HCl, 150 mM NaCl, pH 7.4). Purity was analyzed by SDS-PAGE and Coomassie Brilliant blue staining. The protein-containing fractions were stored in 10–40% glycerol at −80 °C until use.

Enzyme Activity Assays

For in vitro activity tests of the *A. castellanii* tRNA nucleotidyltransferases, in vitro transcribed *S. cerevisiae* tRNA^{Phe} or tRNA^{Phe}-CC (α-^{32}P-ATP-labeld) was produced as described (Mörl and Hartmann 2014; Schürer et al. 2002). To ensure proper three-dimensional folding of the tRNAs, transcripts were heated up to 65 °C for 5 min and cooled down prior to reaction. Reaction conditions were as following: 5 pmol tRNA and varying amounts of enzyme (1–50 ng) were incubated with 1 mM NTP-mix (equimolar amounts) or 0.1 mM ATP or 0.1 mM CTP in 30 mM HEPES/KOH (pH 7.6), 30 mM KCl, 2 mM DTT, and 6 mM MgCl$_2$ for 30 min at 30 °C. Reaction was stopped by ethanol precipitation, products were dissolved in loading dye, and analyzed by denaturing

PAGE and autoradiography using a Typhoon 9410 scanner (GE Healthcare). For each enzyme, at least three independent experiments were performed.

Sequence Assembly of Putative tRNA Nucleotidyltransferases in *Acanthamoeba* spp.

Although several genomes of *Acanthamoeba* spp. are available, they are mostly only partially annotated. In order to manually search for tRNA nucleotidyltransferase sequences, genomes of *A. mauritaniensis*, *A. pearcei*, *A. polyphaga*, and *A. quina* were obtained from the NCBI genome database (Benson et al. 2013) and blasted against the sequences found in *A. castellanii*. Putative gene sequences were aligned with the corresponding mRNA sequence of *A. castellanii* using Clustal Omega 2.1 (Sievers et al. 2011) and manually spliced. Finally, sequences were translated using the ExPASy translate tool (Artimo et al. 2012) to verify the correctness by ORF identification.

Phylogenetic Analysis of the *Acanthamoeba* spp. tRNA Nucleotidyltransferases

Sequences of *A. mauritaniensis*, *A. pearcei*, *A. polyphaga*, and *A. quina* were aligned with experimentally verified Ntr sequences of *A. castellanii* and corresponding sequences of Bacteria, Metazoa, Fungi, Plants, and other Amoebozoa using the web-tool Clustal Omega (Sievers et al. 2011). The sequence alignment was used for construction of a phylogenetic network using the program SplitsTree (version 4.14.8, built 15 Nov 2018 [Huson and Bryant 2006]).

Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

Author Contributions

M.M. and H.B. initiated the study and designed the project. L.E. performed all experiments and phylogenetic analyses. All data were analyzed by L.E., H.B., and M.M. L.E., H.B., and M.M. wrote the manuscript. All authors read and approved the final manuscript.

Data Availability

Accession numbers of *ntr* genes of *A. castelanii* are indicated in the Result section.

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