Molecular Microbiology (2010) 76(3), 706–718 ■
doi:10.1111/j.1365-2958.2010.07128.x
First published online 31 March 2010

Mitochondrial translation in absence of local tRNA aminoacylation and methionyl tRNA^{Met} formylation in *Apicomplexa*

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Summary

Apicomplexans possess three translationally active compartments: the cytosol, a single tubular mitochondrion, and a vestigial plastid organelle called apicoplast. Mitochondrion and apicoplast are of bacterial evolutionary origin and therefore depend on a bacterial-like translation machinery. The minimal mitochondrial genome contains only three ORFs, and in *Toxoplasma gondii* the absence of mitochondrial tRNA genes is compensated for by the import of cytosolic eukaryotic tRNAs. Although all compartments require a complete set of charged tRNAs, the apicomplexan nuclear genomes do not hold sufficient aminoacyl-tRNA synthetase (aaRSs) genes to be targeted individually to each compartment. This study reveals that aaRSs are either cytosolic, apicoplastic or shared between the two compartments by dual targeting but are absent from the mitochondrion. Consequently, tRNAs are very likely imported in their aminoacylated form. Furthermore, the unexpected absence of tRNA^{Met} formyltransferase and peptide deformylase implies that the requirement for a specialized formylmethionyl-tRNA^{Met} for translation initiation is bypassed in the mitochondrion of *Apicomplexa*.

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Introduction

The phylum *Apicomplexa* consists in a large group of obligate intracellular protozoan parasites, several of which have a considerable impact on human and animal health. *Plasmodium falciparum* is responsible for the most deadly form of malaria while *Toxoplasma gondii* can cause spontaneous abortion, congenital birth defects, severe illness and death in warm-blooded animals. Most apicomplexans possess the relic of a plastid organelle called the apicoplast, which results from the ancient secondary endosymbiotic acquisition of a red alga. This non-photosynthetic organelle fulfills a number of metabolic functions critical for parasite survival and possesses a 35 kb circular genome that codes for genes implicated in transcription and translation, including a set of 30 *tRNA* genes sufficient for the translation of the ORFs (Wilson *et al.*, 1996). Most apicomplexans also possess a single tubular mitochondrion that contributes to haem and iron–sulphur cluster biosynthesis in addition to hosting the Krebs cycle and oxidative respiration (Seeber *et al.*, 2008). With a reduced size of 6 kb, the mitochondrial genome of these parasites exclusively codes for the subunits I and III of cytochrome *c* oxidase and cytochrome *b* plus short fragments representing the small and large rRNA subunits (Feagin, 2000; Mcfadden *et al.*, 2000).

Absence of some essential mitochondrial *tRNA* genes was reported in plants, some fungi and protozoa, and is compensated for by the import of the corresponding nuclear-encoded tRNAs. The number and identity of the imported tRNAs vary greatly from one organism to the other (Duchene *et al.*, 2009). In this respect, *Trypanosoma brucei* is an extreme case with all tRNAs imported into the mitochondrion except the eukaryotic-type tRNA^{Met-i} and tRNA^{Sec} (Tan *et al.*, 2002; Bouzaidi-Tiali *et al.*, 2007). In *T. gondii*, the full set of tRNAs is predicted to be imported into the mitochondrion and import has been demonstrated experimentally for several of them with the exception of the initiator tRNA (tRNA^{Met-i}) (Esseiva *et al.*, 2004).

Several drugs that target organellar transcription and translation machinery are effective against apicomplexans acting likely both on mitochondrial enzymes and their

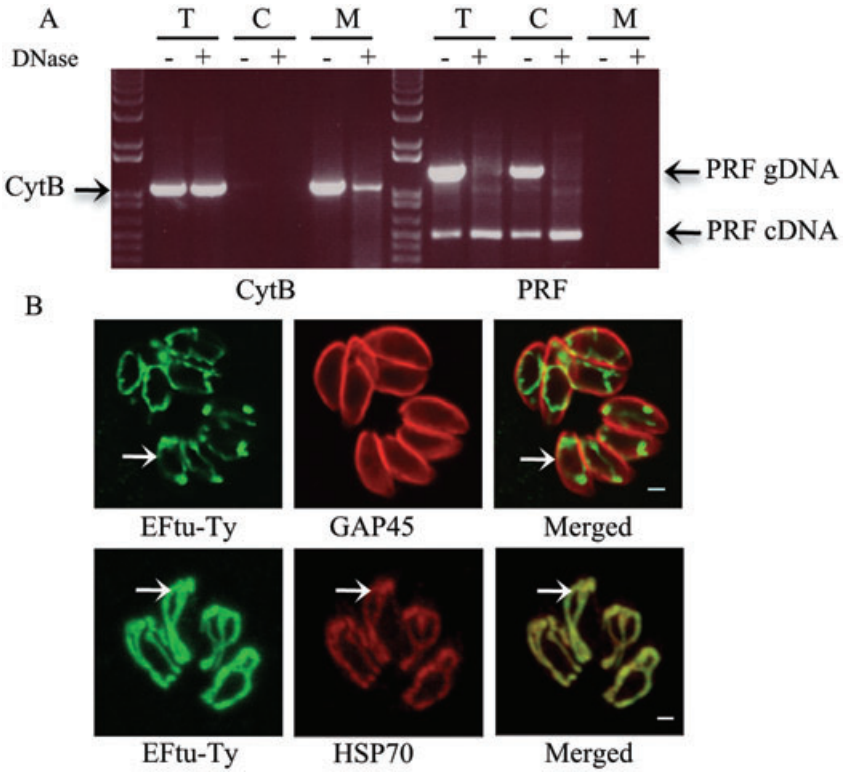

Fig. 1. Evidence for mitochondrial transcription.
A. Detection of *TgCytb* mRNAs by RT-PCR in the membrane faction. CytB mRNA is detected in the total and the membrane fractions but not in the cytosolic fraction. The profilin gene (*TgPRF*) that contains introns was used as control generating cytosolic transcripts. *TgPRF* mRNAs are detected in the total and cytosolic fractions, but not in the membrane fraction. The successful amplification of CytB after DNase treatment confirms that the mitochondrial encoded *TgCytB* is efficiently transcribed. T: Total fraction; C: Cytosolic fraction; and M: 'Mitoplast' fraction (Mitochondrion, Apicoplast and membranes).
B. IFA on stable transgenic parasites expressing full-length mEF-Tu Ty tagged protein under the control of a Tub8 promoter. GAP45 is used to stain the periphery of the parasites. mEF-Tu-Ty colocalizes to the mitochondrion with the mitochondrial marker HSP70. Open-head arrows show the mitochondria. Scale bars represent 1 μm.

counterparts in the apicoplast (Fleige and Soldati-Favre, 2008). In consequence, direct evidence for mitochondrial translation in *Apicomplexa* has been elusive. However, atovaquone, a specific inhibitor of the cytochrome bc1 complex of the respiratory chain, exhibits potent parasiticidal effects. Moreover, resistance to atovaquone has been mapped to mutations in the mitochondrially encoded cytochrome b gene, indicating that the protein it encodes must be translated (Siregar *et al*., 2008). In *T. gondii*, some copies of the cytb gene are also found in the nuclear genome; however, the fact that the cytB mRNA is exclusively found in the mitochondria shows that they are pseudogenes (Mcfadden *et al*., 2000 and Fig. 1).

Aminoacyl-tRNA synthetases (aaRSs) are key enzymes that attach amino acids to their corresponding tRNAs. Despite the existence of three compartments where protein synthesis occurs (Chaubey *et al*., 2005), the apicomplexans do not possess three complete sets of 20 aaRSs that would be theoretically required. In the flowering plant *A. thaliana*, examination of the aaRSs revealed that instead of the full set of 60 genes, this organism only possesses 45, many of which are shared between two different compartments, with the alanyl-RS being localized to all three subcellular compartments (Duchene *et al*., 2005). Similarly, dual targeting of aaRSs has also been reported in many organisms including humans (Tolkunova *et al*., 2000), yeast (Huang *et al*., 2006) and trypanosomes (Rinehart *et al*., 2004). In apicomplexans, evidence for dual targeting of anti-oxidant and metabolic enzymes to the apicoplast and the mitochondrion was previously reported (Gunther *et al*., 2007; Pino *et al*., 2007; Saito *et al*., 2008).

The present study uncovers two exceptional features regarding mitochondrial translation in *Apicomplexa*. The mitochondrion of *T. gondii* does not contain any aaRS and initiation of mitochondrial translation occurs in the absence of formylated $tRNA^{Met}$. These characteristics potentially reflect an intermediate state in the evolution of these organisms towards the loss of their already extremely reduced organellar genome.

Results

The T. gondii *mitochondrial genome is transcribed*

To provide a more direct evidence of mitochondrial transcription in *T. gondii*, we applied a fractionation protocol involving partial permeabilization with digitonin to separate the organelles (intact apicoplast and mitochondrion) from the cytosolic fraction (Esseiva *et al*., 2004). *T. gondii cytochrome b* (*TgCytB*) mRNAs were detected in the total and organellar fractions but not in the cytosolic fraction (Fig. 1A). *T. gondii* profilin gene (*TgPRF*) contains introns and was used as a control that generates cytosolic spliced transcripts of a distinct size compared

with a genomic amplification (Plattner *et al*., 2008). DNAse treatment resulted in the disappearance of the upper band (gDNA) in the profilin amplification. The successful amplification of CytB (that does not contain introns) after DNAse treatment confirmed the presence of *TgCytB* transcripts in the mitochondrion (Fig. 1A).

The genes coding for mitochondrial translation factors are easily identifiable in the nuclear genome (Table S1). Annotation of the nuclear-encoded mitochondrial elongation factor Tu (*mEF-Tu*) sequence clearly indicated the presence of a mitochondrial targeting signal whereas the apicoplast genome code for its own EF-Tu gene. The mitochondrial targeting of mEF-Tu was confirmed by the expression of a carboxy-terminus epitope tagged transgene under the control of the tubulin promoter (TgmEF-Tu-Ty) (Fig. 1B). The targeting of a nuclear-encoded translation factor to the mitochondrion further supports that translation occurs in that organelle.

Apicomplexan parasites contain a reduced set of aminoacyl-tRNA synthetases

A bioinformatics survey of the available apicomplexan genomes revealed a very limited set of 34–35 putative *aaRSs* encoding genes in the phylum (Table 1). In *T. gondii*, the aaRSs of 14 amino acids are encoded by two genes instead of three, whereas the remaining six are represented each by a single *aaRS* gene. Interestingly, the repertoire of single-gene and two-gene encoded enzymes is not identical across the different apicomplexan genomes, suggesting that most gene losses occurred after divergence of the major apicomplexan lineages (Table S2). *Cryptosporidium* does not possess an apicoplast and harbours the relic of a mitochondrion (mitome), lacking a genome (Abrahamsen *et al*., 2004; Putignani *et al*., 2004). Accordingly, this parasite only possesses the 20 *aaRSs* genes that ensure cytosolic translation (Table 1). Phylogenetic trees based on all the predicted amino acid sequences of aaRSs revealed a very complex pattern. Nonetheless, these phylogenetic associations are fully consistent with the idea that *Cryptosporidium* kept the most representative cytosolic enzymes found in eukaryotes (Table 1). Frequently, the cytosolic enzymes are more similar to homologues from Archaea, whereas the typical organellar enzymes show a closer phylogenetic affiliation to homologues from eubacteria, including cyanobacteria and alpha-proteobacteria (Table 1 and Fig. S1). The MetRSs, ArgRSs and AspRSs are exceptional in that the two apicomplexan enzymes responsible for a given amino acid share the same phylogenetic affiliation, suggesting that the second copy originated from a relatively late gene duplication event (Table S1, Fig. S2).

Absence of aaRS in the mitochondrion of Toxoplasma gondii

The apparently insufficient repertoire of *aaRSs* genes to sustain translation in the three compartments could be accommodated by dual and triple targeting of the gene products. To test this hypothesis a series of aaRSs were localized by epitope tagging in *T. gondii* (constructs Fig. S3). *T. gondii* possesses two putative *TrpRS* genes, one from archeal origin *TgTrpRS1*, and a eubacterial type *TgTrpRS2* (Table 1). Unlike TgTrpRS1, TgTrpRS2 exhibits an N-terminal extension that consists of a putative signal peptide followed by a hydrophilic section with a net positive charge, characteristic of the bipartite signal that commonly directs proteins to the apicoplast (Waller *et al*., 2000; Foth *et al*., 2003). TgTrpRS2-Ty localized to the apicoplast whereas TgTrpRS1-Ty was cytosolic but, unexpectedly, both proteins were absent from the mitochondrion (Fig. 2A). To avoid any targeting artefact due to a strong or temporally inappropriate promoter, transgenic parasites expressing TgTrpRS2-Ty under the control of *TgTrpRS2* promoter were generated to confirm the localization (Fig. 2A). Finally, to exclude any mitochondrial localization of the apparently cytosolic TgTrpRS1, subcellular fractionation experiments were performed. TgTrpRS2-Ty was found only in digitonin-extracted pellets, co-fractionating with the apicoplast marker TgPDH-E2 (Fleige *et al*., 2007), whereas TgTrpRS1-Ty was solely present in the cytosolic fraction together with the cytosolic marker TgPRF (Plattner *et al*., 2008) (Fig. 2B).

Two additional sets of *aaRS* genes were analysed and confirmed the results observed for TgTrpRSs. TgLeuRS1 and TgSerRS1 lack a targeting signal and are expected to be cytosolic, whereas TgLeuRS2 and TgSerRS2 possess an N-terminal bipartite extension and were shown by epitope tagging (TgLeuRS2-Ty, TgSerRS2-Ty) to localize exclusively to the apicoplast (Fig. 2A).

In *T. gondii*, the six aaRSs corresponding to Gln, Asp, Asn, Cys, Pro and Ala are encoded by single copy genes. With the exception of GlnRS, each protein is preceded by a predicted bipartite N-terminal extension for targeting to the apicoplast; however, both IFA and organellar fractionation analyses performed on parasites expressing TgGlnRS-Ty, TgProRS-Ty and Nterm-TgCysRS-Ty revealed cytosolic localization (Fig. 3A and B). In contrast, when the full-length *TgCysRS* was expressed under the control of its own promoter rather than under the tubulin promoter (Fig. 3B, third panel and Fig. S3), TgCysRS-Ty was detectable both in the cytosol and in the membrane fraction. These results suggest that the bipartite targeting signal present on TgCysRS is appropriately used only in the context of the wild-type 5′UTR sequence. While it is unclear how the cytosolic form of TgCysRS is generated, it might involve either alternative initiation of transcription

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Table 1. Repertoires of aminoacyl-tRNA synthetases (aaRSs) in apicomplexan genomes.

	Toxoplasma gondi[a]		*Theileria annulata*[a]	*Babesia bovis*[a]	*Plasmodium falciparum*[a]	*Plasmodium yoelii*[a]	*Cryptosporidium hominis*[a]	Closest phylogenetic affiliation of apicomplexan sequences to aaRSs from[b]
	ToxoDB 4.2	ToxoDB 5.3						
Ala	38.m01067	TGME49_019540	TA16780	XP_001612285	PF13_0354	PY03081	EAL35675	Eukaryotes
Cys	145.m00322	ADC80543	TA03175	XP_001608890	PF10_0149	PY04618	EAL37860	Fungi, animals
Gln	37.m00743	ADC80544	TA12530	XP_001611769	PF13_0170	PY02178	EAL35592	Eubacteria
Leu-1	80.m00095	TGME49_092080	TA10995	XP_001611815	PFF1095w	PY02181	EEA05061 (*C. muris*)	Cytosol; archaea
Leu-2	57.m01806	TGME49_066730	TA04430	XP_001609402	PF08_0011	PY05778	not found	Organelles (plants); eubacteria
Tyr-1	50.m00021	TGME49_051880	TA08075	XP_001609749	MAL8P1.125	PY04194	EAL37613	Cytosol; archaea
Tyr-2	59.m06092	TGME49_073410	TA06885	XP_001611016	PF11_0181	XP_678804 (*P. berghei*)	not found	Organelles; eubacteria
Trp-1	80.m00063	TGME49_088360	TA20460	XP_001611386	PF13_0205	PY06252	EAL38376	Cytosol; archaea
Trp-2	52.m01634	ADC80549	TA10040	XP_001612001	PFL2485c	XP_744571 (*P. chabaudi*)	not found	Organelles; eubacteria
Ile-1	25.m01749	TGME49_007640	TA12460	XP_001611793	PF13_0179	PY01849	EAL35035	Cytosol
Ile-2	65.m01098	TGME49_077030	TA13750	XP_001610095	PFL1210w	XP_745094 (*P. chabaudi*)	not found	Organelles; eubacteria
Pro-1	38.m00021	ADC80545	TA19220	XP_001609304	PFL0670c	PY02018	CAD98257 (*C. parvum*)	Cytosol (including metazoan bifunctional Glu/Pro-RS)
Pro-2	not found	not found	TA12695	XP_001612221	PFI1240c	PY00927	not found	Mitochondria (fungi, animals); eubacteria
Glu-1	55.m05093	TGME49_063870	TA17035	XP_001612304	PF13_0257	PY02891	EAL35652	Cytosol (including metazoan bifunctional Glu/Pro-RS)
Glu-2	49.m03265	TGME49_041870	TA06725	XP_001610985	MAL13P1.281	PY00363	not found	Organelles; eubacteria
Phe-1alpha	46.m01611	TGME49_034500	TA10840	XP_001611853	PFA0480w	PY00417	EAL37950	Cytosol
Phe-1beta	542.m00221	TGME49_106960	TA11150	XP_001612083	PF11_0051	XP_678724 (*P. berghei*)	EAL38303	Cytosol
Phe-2	27.m00821	TGME49_010750	TA11685	XP_001610235	PFF0180w	PY04422	not found	Organelles
Ser-1	50.m00020	TGME49_051690	TA03465	XP_001610648	PF07_0073	PY03295	EAL36390	Cytosol
Ser-2	TgGLEAN_4192 & 59.m00043	TGME49_058720 & TGME49_071730	TA19195	XP_001609299	PFL0770w	XP_673568 (*P. berghei*)	not found	Diatoms; eubacteria
Thr-1	145.m00604	TGME49_100260	TA06605	XP_001610740	PF11_0270	PY06957	EAL36495	Cytosol & mitochondria
Thr-2	38.m01112	TGME49_019430	not found	not found	not found	not found	not found	Eubacteria; archaea; organelles (plants)
Asn-1	59.m03518	TGME49_070510	TA14880	XP_001612247	PFB0525w	PY05639 (PY02504)	EAL36932	Organelles (plants); eubacteria
Asn-2	not found	not found	TA05825	XP_001610875	PFE0475w	PY03253	not found	Mitochondria (fungi, animals)

Table 1. *cont.*

	Toxoplasma gondii[a]		*Theileria annulata*[a]	*Babesia bovis*[a]	*Plasmodium falciparum*[a]	*Plasmodium yoelii*[a]	*Cryptosporidium hominis*[a]	Closest phylogenetic affiliation of apicomplexan sequences to aaRSs from[b]
	ToxoDB 4.2	ToxoDB 5.3						
Lys-1	20.m03931	TGME49_005710	TA04275	XP_001609428	PF13_0262	PY00115	EAL37975	[Cytosol & mitochondria: single gene in fungi/animals]; Cytosol (plants)
Lys-2	39.m00356	TGME49_020350	TA08620	XP_001609643	PF14_0166	PY05658	not found	Eubacteria; plant organelles; archaea
His-1	72.m00395	TGME49_080600	XP_765672 (*T. parva*)	XP_001609284	PF14_0428	PY03706	EAL36292	[Cytosol & mitochondria: single gene in fungi/animals]; Cytosol (plants)
His-2	83.m01308	TGME49_095050	TA13155	XP_001611649	PFI1645c	PY00739	not found	Organelles (plants); trypanosomes
Val-1	52.m00009	TGME49_053290	TA10235	XP_001611967	PF14_0589	PY00514	EAL36810	[Cytosol & mitochondria: single gene in fungi/animals]
Val-2	35.m00886	TGME49_016500	XP_764207 (*T. parva*)	XP_001611804	PFC0470w	PY07509	not found	Eubacteria; archaea; organelles (plants)
Gly-1	55.m04665	TGME49_056990	TA20715	XP_001609027	PF14_0198	PY01198	EAL35110	[Cytosol & mitochondria: single gene in fungi/animals]
Gly-2	52.m01640	TGME49_054200	not found	not found	not found	not found	not found	Oomycetes & diatoms; eubacteria; archaea
Met-1	80.m02206	TGME49_089300	TA05325	XP_001608849	PF10_0340	PY03894	EAL38284	Organelles; eubacteria
Met-2	76.m01644	TGME49_086180	TA09735	XP_001610587	PF10_0053	PY06890	not found	Organelles; eubacteria
Arg-1	27.m00832	TGME49_010840	TA08180	XP_001609801	PFL0900c	PY01800	EAL35040	Cytosol (animals); plants; eubacteria
Arg-2	44.m02819	TGME49_033860	TA16890	XP_001609088	PFI0680c	PY02481	not found	Cytosol (animals); plants; eubacteria
Asp-1	20.m03726	TGME49_002530	TA04845	XP_001609334	PFA0145c (MAL1P1.20)	PY01511	EAZ51570 (*C. parvum*)	Cytosol
Asp-2	not found	not found	not found	not found	PFE0715w	PY01996	not found	Cytosol
Similar to multisynthetase complex-auxiliary component p43 (AIMP1 = aminoacyl tRNA synthetase complexinteracting multifunctional protein 1)	41.m00027	TGME49_023140	TA11475	XP_001612148	PF14_0401	PY02994	EAL34834	

a. Listed are gene model identifiers or accession numbers (see http://www.toxodb.org, http://www.genedb.org, http://www.plasmodb.org, or http://www.ncbi.nlm.nih.gov/).
b. These results are based on phylogenetic trees that included sequences from plants, fungi and animals as eukaryotic representatives, and from Archaea and Eubacteria (with an emphasis on cyanobacteria and alpha-proteobacteria); '[cytosol & mitochondria]' refers to enzymes that have been annotated as being dually targeted or that exist as only a single gene in fungi and animals, strongly suggesting a dual localization in these two compartments.

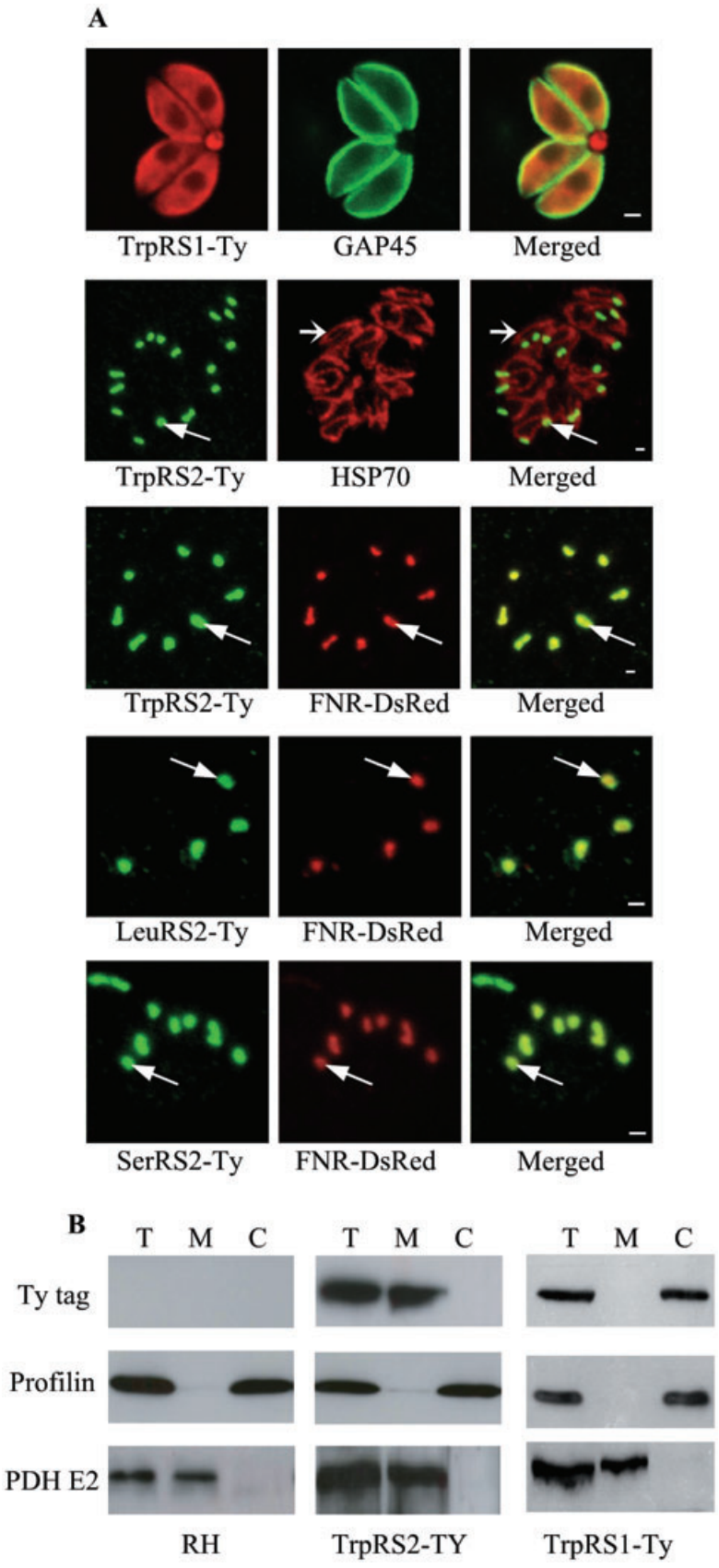


Fig. 2. Absence of aaRS from *T. gondii* mitochondrion (two-genes encoded enzymes).
A. IFA on stable transgenic parasites expressing full-length TrpRS1-, TrpRS2-LeuRS2- and SerRS2-Ty tagged proteins. Anti-GAP45 stains the pellicle and anti-TgHSP70 stains the mitochondrion. Parasites were transfected with the known apicoplast marker FNR-DsRed for colocalization with the apicoplast. Closed-head arrows show the apicoplast, open-head arrows show the mitochondrion. Scale bars represent 1 μm.
B. Western blot analysis on transgenic parasites fractions; Profilin is a marker for cytosolic protein and PDH-E2 is mitoplast maker. T: Total fraction; C: Cytosolic fraction; and M: 'Mitoplast' fraction (Mitochondrion, Apicoplast and membranes).

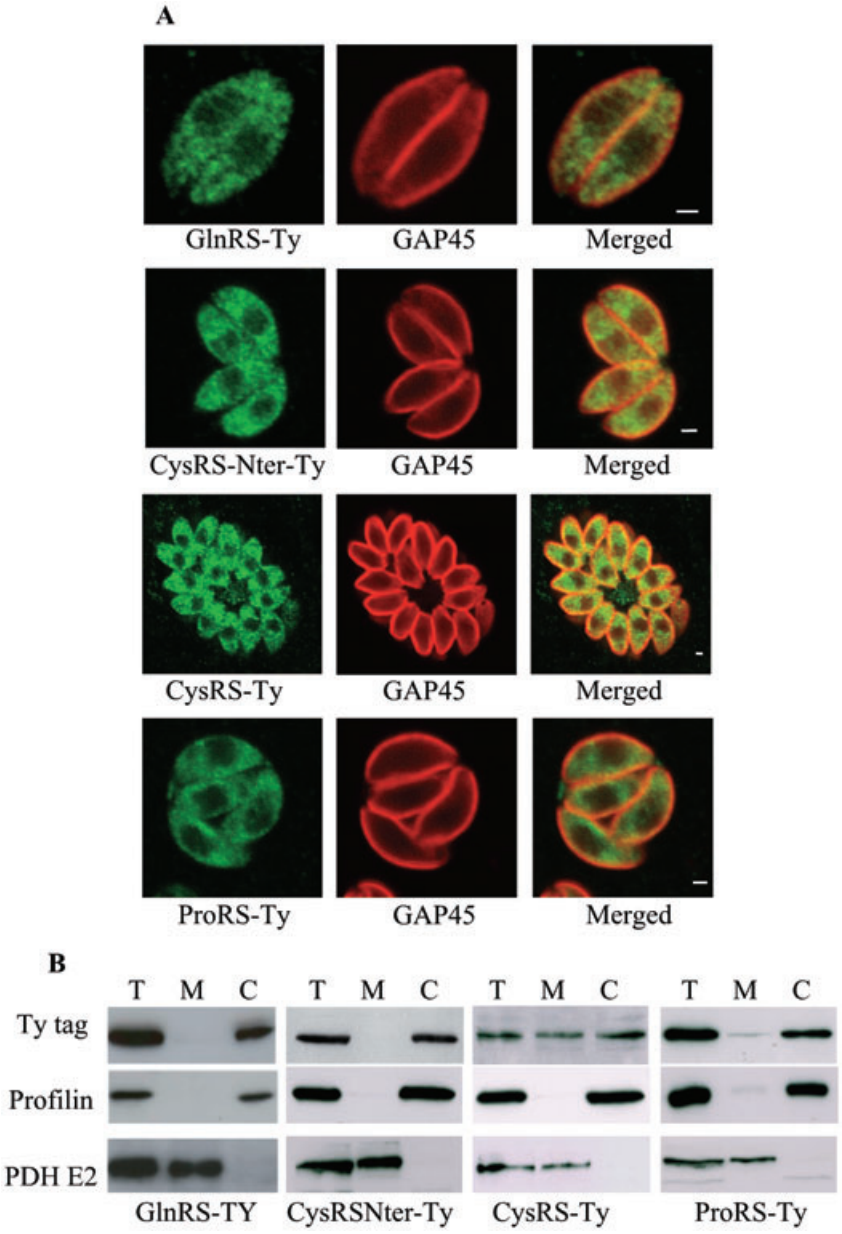
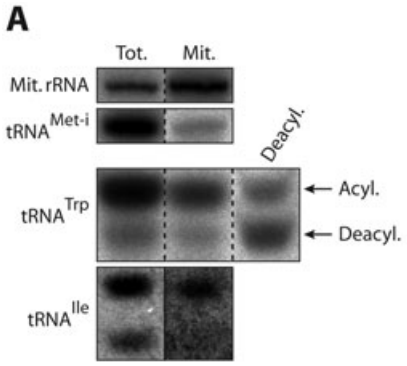


Fig. 3. Absence of aaRS from *T. gondii* mitochondrion (one-gene encoded enzymes).
A. IFA on stable transgenic parasites expressing full-length GlnRS, CysRS and ProRS TrpRS1-Ty and CysRS-Nterm-Ty tagged proteins. Anti-GAP45 stains the pellicle. Scale bars represent 1 μm.
B. Western blot analysis on transgenic parasites fractions; Profilin is used as cytosolic maker and PDH-E2 is used as a mitoplast maker. GlnRS-Ty and ProRS-Ty appear to be cytosolic. CysRS-Nterm-Ty driven by the Tub8 promoter appears to be cytosolic whereas the full-length CysRS-Ty driven by its endogenous promoter is dually targeted to the cytosol and the apicoplast. T: Total fraction; C: Cytosolic fraction; and M: 'Mitoplast' fraction (Mitochondrion, Apicoplast and membranes).

or translation, or alternative splicing or alternative processing of the protein. Given that the two organelles cannot be physically separated, the detection of TgCysRS in the membrane fraction does not allow discriminating whether targeting occurred exclusively to the apicoplast or to both organelles. However, in the light of the results obtained here, we suspect that TgCysRS exclusively resides in the apicoplast.

Presence of aminoacylated nuclear-encoded tRNA in the mitochondrion of T. gondii

It has previously been shown that nuclear-encoded tRNAs are imported into the mitochondrion of *T. gondii* (Esseiva *et al.*, 2004). However, the aminoacylation status of these imported tRNAs has not been analysed. In order to address this question we prepared RNA fractions from total cells and from digitonin-extracted pellets. Northern blot analysis of the mitochondrial rRNA and the cytosol-specific $tRNA^{Met\text{-}i}$ (Esseiva *et al.*, 2004) showed that the pellet fraction is highly enriched for mitochondrial RNA (Fig. 4A). Subsequently, the mitochondrial RNA fraction was resolved on a high-resolution acid urea polyacrylamide gel, which allows separation of the charged and uncharged tRNAs (Varshney *et al.*, 1991). Northern blot analysis in Fig. 4 showed that 6.9% and 2.2% of the total cellular content of nuclear-encoded $tRNA^{Trp}$ and $tRNA^{Ile}$ are localized in the mitochondria. Moreover, approxi-



B

RNA in Mit.	
Mit. rRNA	100 %
tRNA Met-i	0.6 %
tRNA Trp	6.9 %
tRNA Ile	2.2 %

Fig. 4. Mitochondrial tRNAs of *T. gondii* are aminoacylated.
A. Total *(Tot.)* and mitochondrially enriched RNA fractions (Mit.) isolated under acidic conditions were separated on a 8 M Urea-10% polyacrylamide gel, blotted and analysed by specific oligonucleotide hybridization for the presence of the mitochondrial LSU rRNA fragment and the cytosol-specific $tRNA^{Met\text{-}i}$. The bottom two panels show Northern blots of the same RNA fractions hybridized with probes specifically recognizing either the $tRNA^{Trp}$ or the $tRNA^{Ile}$ respectively. In these cases the RNA fractions were resolved on a long acid urea gel, which allows separation of aminoacylated from deacylated tRNAs. The lane on the right in the third panel shows total RNA that has been partially deacylated *in vitro*. It serves as a control to indicate the position of acylated and deacylated $tRNA^{Trp}$.
B. Quantification of the Northern blots in (A) depicting the fraction of the total amounts of $tRNA^{Trp}$ and $tRNA^{Ile}$, including both aminoacylated and deacylated molecules, that are found in mitochondria. Mitochondrial rRNA was set to 100%; the $tRNA^{Met\text{-}i}$ represents cytosolic contamination.

mately 80% of the two imported tRNAs are recovered in their aminoacylated forms irrespectively of whether they were isolated from total cells or from mitochondrial pellets. Thus, even though no aaRSs could be found in the mitochondrion of *T. gondii*, mitochondrially localized tRNAs are essentially only recovered in their aminoacylated form. This suggests that imported tRNAs are charged in the cytosol, imported in their aminoacylated form and directly used for mitochondrial protein synthesis. Interestingly, a similar situation has been described in yeast where the $tRNA^{Lys}$ cannot be charged inside the mitochondria and is imported in its charged state. However, unlike in *T. gondii*, yeast mitochondria encode their own $tRNA^{Lys}$ and the imported cytosolic $tRNA^{Lys}$ is only required for mitochondrial protein synthesis at elevated temperature (Kamenski *et al*., 2007).

Formylmethionine-independent initiation of translation in apicomplexans

In eukaryotes, protein synthesis is initiated by Met-$tRNA^{Met\text{-}i}$, which is distinct from the elongator tRNA, Met-$tRNA^{Met\text{-}e}$. A similar situation is found in eubacteria, plastids and mitochondria, except that the initiator tRNA is further distinguished from its elongation counterpart by the formylation of the methionine on the charged tRNA. The formylation reaction is catalysed by methionyl-tRNA formyltransferase (FMT). Ultimately, the formylated methionine used during initiation is deformylated on the nascent peptide by a peptide deformylase (PDF). *T. gondii* possesses a single gene coding for each FMT and PDF respectively, and both gene products are predicted to carry a bipartite targeting signal. When expressed stably, both TgFMT-Ty and TgPDF-Ty localized solely to the apicoplast (Fig. 5). Since the enzymes implicated in formylation and deformylation are absent this in-turn suggests that the requirement for formylated $tRNA^{Met}$ in mitochondrial translation initiation has been bypassed in *Apicomplexa*.

Discussion

Transcription and translation in the endosymbiotic organelles of *Apicomplexa* constitute attractive targets for the development of effective drugs (Fidock *et al*., 2008, Fleige and Soldati-Favre, 2008). The number of genes encoded by the organellar genomes is reduced to a strict minimum, and our understanding of importance of these genes for the complete life cycle of these parasite and their evolutionarily preservation is fragmentary. By loosing the apicoplast and a fully functional mitochondrion, *Cryptosporidium* has evolved alternative solutions to acquire host lipids and isoprenoids and to produce energy (Putignani *et al*., 2004; Zhu, 2004). In contrast, *Toxoplasma* and *Plasmodium* have maintained a minimal mitochondrial genome to ensure respiration. Although mitochondrial translation has not been formally demonstrated in *T. gondii*, the presence of *TgCytb* mRNAs in the organellar fraction and the targeting of EF-Tu to the mitochondrion provide compelling evidence that it indeed occurs. In addition, a large set of nuclear-encoded specific translation initiation and elongation factors are predicted to be mitochondrial (Table S1).

The complete absence of *tRNA* genes in the mitochondrial genome of *Apicomplexa* is a striking feature otherwise observed only in *Kinetoplastida*. In contrast, the absence of *aaRS* genes in the mitochondrial genome is a universal phenomenon, compensated for by the import of nuclear-encoded enzymes (Duchene *et al*., 2009). Results reported here uncover an extreme situation in *T. gondii*, where none of the 34 *aaRS* genes is predicted to be targeted to the mitochondrion. A bioinformatics analysis of the *P. falciparum* aaRSs repertoire was performed, and based on targeting predictions the different aaRSs enzymes have been described as mitochondrial or apicoplastic (Bhatt *et al*., 2009). However, no experimental data were produced to support these targeting predictions. Here we have experimentally localized several aaRSs and under no circumstances could any of these aaRSs be found in the mitochondrion (Figs 2 and 3). When two

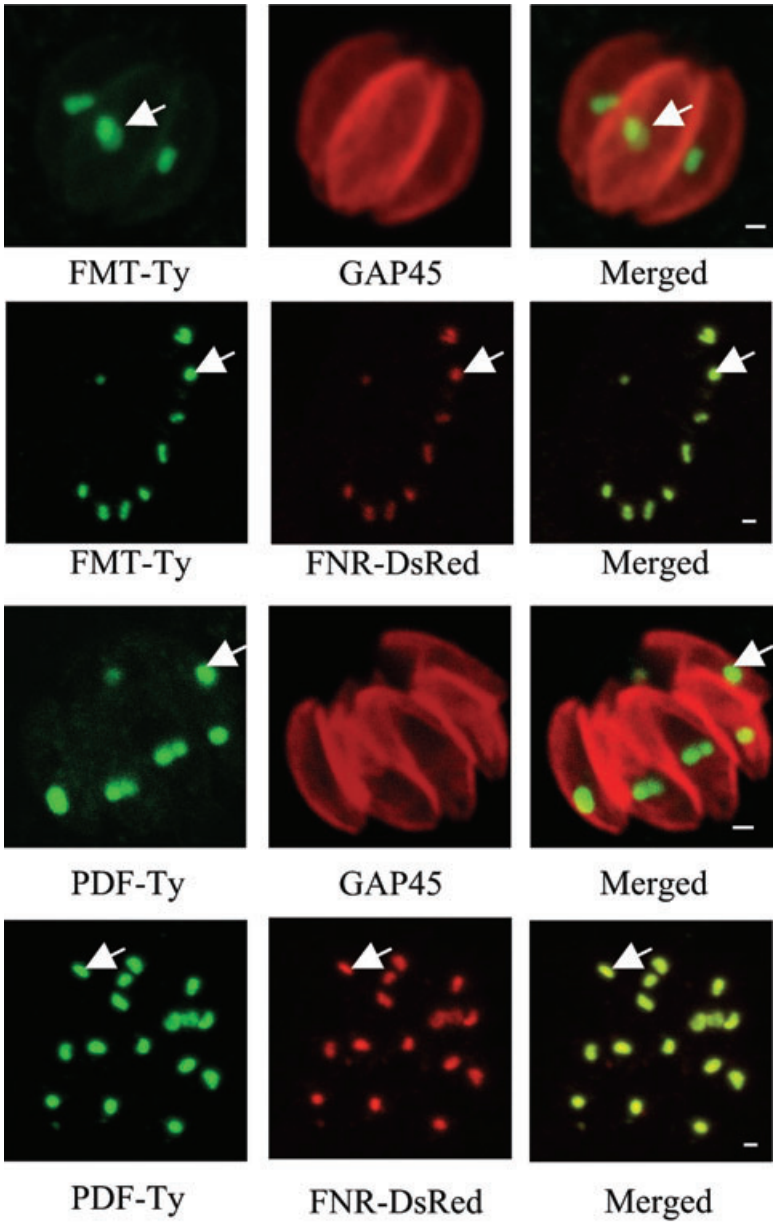


Fig. 5. Absence of methionine formylation and deformylation in *T. gondii* mitochondrion. IFA on stable transgenic parasites expressing full-length FMT- and PDF-Ty tagged proteins. Anti-GAP45 stains the pellicle, Anti-HSP70 the mitochondrion and FNR-DsRed the apicoplast. Closed-head arrows show the apicoplast. Scale bars represent 1 μm.

genes are present in the genome, one of the enzymes is cytosolic whereas the other is exclusively targeted to the apicoplast via a bipartite N-terminal extension. In the cases where a single gene codes for the aaRS, a bipartite N-terminal extension can be found, except for GlnRS. Although we have not assessed the localization of each of these gene products experimentally, we anticipate that, as for *CysRS*, expression of *AspRS*, *AsnRS* and *ProRS* genes leads to dual targeting of the enzymes between the cytosol and the apicoplast. The mechanism by which such dual targeting is achieved is not understood; however, in the case of CysRS, our data indicate that the full protein under the control of its own promoter is required (Fig. 3). This highlights the importance of using full-length proteins and preferably endogenous promoters in the context of epitope tagging approaches (Pino *et al*., 2007). GlnRS is an exception with the apparent absence of a targeting signal and an exclusively cytosolic localization (Fig. 3). Although classically, 20 aaRSs specifically load one amino acid onto the corresponding tRNA, numerous exceptions to this one-to-one correspondence have been identified. Most bacterial and all known archeal genomes do not encode a GlnRS and use instead the tRNA-dependent amino acid transformation pathway to generate Gln-tRNA^{Gln} (Sheppard *et al*., 2008). This two-step process involves the misacylation of a Glu residue on a tRNA^{Gln} and the subsequent conversion of Glu to Gln by a tRNA-dependent amidotransferases (AdT). AdT's can be

either heterotrimeric enzymes (GatCAB) as found in both bacteria and Archaea or heterodimeric (GatDE) as found only in Archaea. A putative GatA and B subunits but no GatC can be found in *P. falciparum*. The very small size of GatC probably explains the lack of hits in BLAST searches. Interestingly, we identified only a putative GatB in the *T. gondii* genome but no GatA (Table S2). More work will be needed to unravel how apicomplexans resolve this issue. Recently, the generation of a ku80-ko in *T. gondii* has offered an option to increase the efficiency of homologous recombination (Fox *et al*., 2009; Huynh and Carruthers, 2009). In consequence, it is now possible to quickly expand the analysis performed here to a larger pool of *aaRS* gene.

Taken together, these results highlight the absence of aaRS in the mitochondrion of *T. gondii*. Nevertheless, mitochondrial protein synthesis is expected to be operational and the six previously tested nuclear-encoded elongator-type tRNAs (Ala, Ile, Ser, Trp, Gln and Met) must be imported into the organelle in their aminoacylated form. In *S. cerevisiae*, the mitochondrial import of cytosolic $tRNA^{Lys}$ in its aminoacylated state testifies to this plausible scenario (Kamenski *et al*., 2007). However, in contrast to yeast, *T. gondii* needs to import all of its mitochondrial tRNAs and the import of each of them may be essential for mitochondrial gene expression. The absence of aaRS should lead to an accumulation of uncharged tRNAs inside the mitochondrion. Our results, however, show that this is not the case since the extent of aminoacylation is identical for cytosolic and mitochondrial tRNAs respectively (Fig. 4). Two models emerge to explain how uncharged tRNAs do not accumulate: (i) uncharged tRNAs are immediately degraded after each translation elongation cycle; (ii) uncharged tRNAs are efficiently exported to the cytosol and recycled. While both models make predictions that in principle should be testable, this is technically hampered by the difficulty in isolating functional mitochondria in *Apicomplexa*. Various systems for mitochondrial tRNA import have been described, relying or not on known protein import pathways, but they remain controversial (Alfonzo and Soll, 2009). Protein synthesis in eubacteria, mitochondria and chloroplasts is selectively initiated with a formylmethionine, whereas in Archea and the eukaryotic cytoplasm the translation starts with a methionine. We have shown here that the product of the single *TgFMT* gene is exclusively imported into the apicoplast (Fig. 5). This is compatible with the fact that in *T. gondii* mitochondrion, bacterial-type initiator tRNA is absent and replaced by a eukaryotic $tRNA^{Met\text{-}e}$ (Esseiva *et al*., 2004). Nevertheless, confronted with a similar situation, *T. brucei* appears to have chosen to use its FMT to formylate a $tRNA^{Met\text{-}e}$ of eukaryotic origin as substrate (Esseiva *et al*., 2004). Such an adaptation is hard to envisage in *Apicomplexa*, where the single FMT would have to recognize both the bacterial-type tRNA initiator synthesized in the apicoplast and the eukaryotic one imported into the mitochondrion.

It has been shown previously that yeast lacking mitochondrial FMT can still synthesize mitochondrial proteins (Li *et al*., 2000). Thus, the suggestion that the requirement for a formylated $tRNA^{Met\text{-}i}$ has been bypassed in mitochondria of apicomplexan appears more likely than the postulated import of formylated-Met-$tRNA^{Met\text{-}i}$ from apicoplast to mitochondrion (Barbrook *et al*., 2006; Howe and Purton, 2007). Indeed, it is hard to imagine only one specific charged-tRNA crossing the four membranes of the apicoplast and the two membranes of the mitochondrion. In addition, in most organisms a PDF is present to remove the formyl group from nascent polypeptides and the latter is also absent in the mitochondrion of *T. gondii* (Figs 5 and 6) and *P. falciparum* (Tonkin *et al*., 2004). An ultimate argument that *Apicomplexa* have bypassed the requirement of a formylated $tRNA^{Met}$ for mitochondrial translation initiation resides in the fact that both *Babesia* and *Theileria* species naturally lack the *FMT* and *PDF* coding genes.

In summary, while the apicoplast translation is more 'self-sufficient', with its own set of tRNAs, the import of the nuclear-encoded aaRSs as well as the FMT and PDF involved in the pathway of methionine formylation/deformylation, the mitochondrial machinery is exclusively relying on cytosolic components (Fig. 6). This reflects an extraordinary situation suggesting that we might be watching evolution at work, by studying organisms on their way to loose their mitochondrial genome. Such a minimal mitochondrion might constitute an intermediate state in the process of its conversion into a mitome that would be ultimately maintain to fulfill strict minimal functions to ensure survival.

Experimental procedures

Reagents and parasite culture

RH strain *T. gondii* tachyzoites were grown in human foreskin fibroblasts. The cDNAs described here were cloned by RT-PCR with Superscript II (Invitrogen, http://www.invitrogen.com) followed by PCR amplification using ExTaq (Takara). Total RNAs was isolated by Triazol Kit (Invitrogen, http://www.invitrogen.com). Oligonucleotide primers used for PCRs are listed in Table S3. Transgenic parasites generated as described earlier (Pino *et al*., 2007). Rabbit anti-HSP70 antibodies were used as mitochondrial marker, and an FNR-DsRed construct was used for apicoplast localization. Rabbit anti–PRF and anti-GAP45 were previously described (Plattner *et al*., 2008). The antiHSP70 antibodies were validated as staining the mitochondrion by colocalization with the previously published mitochondrial marker TgPRX3 (Pino *et al*., 2007) (Fig. S4).

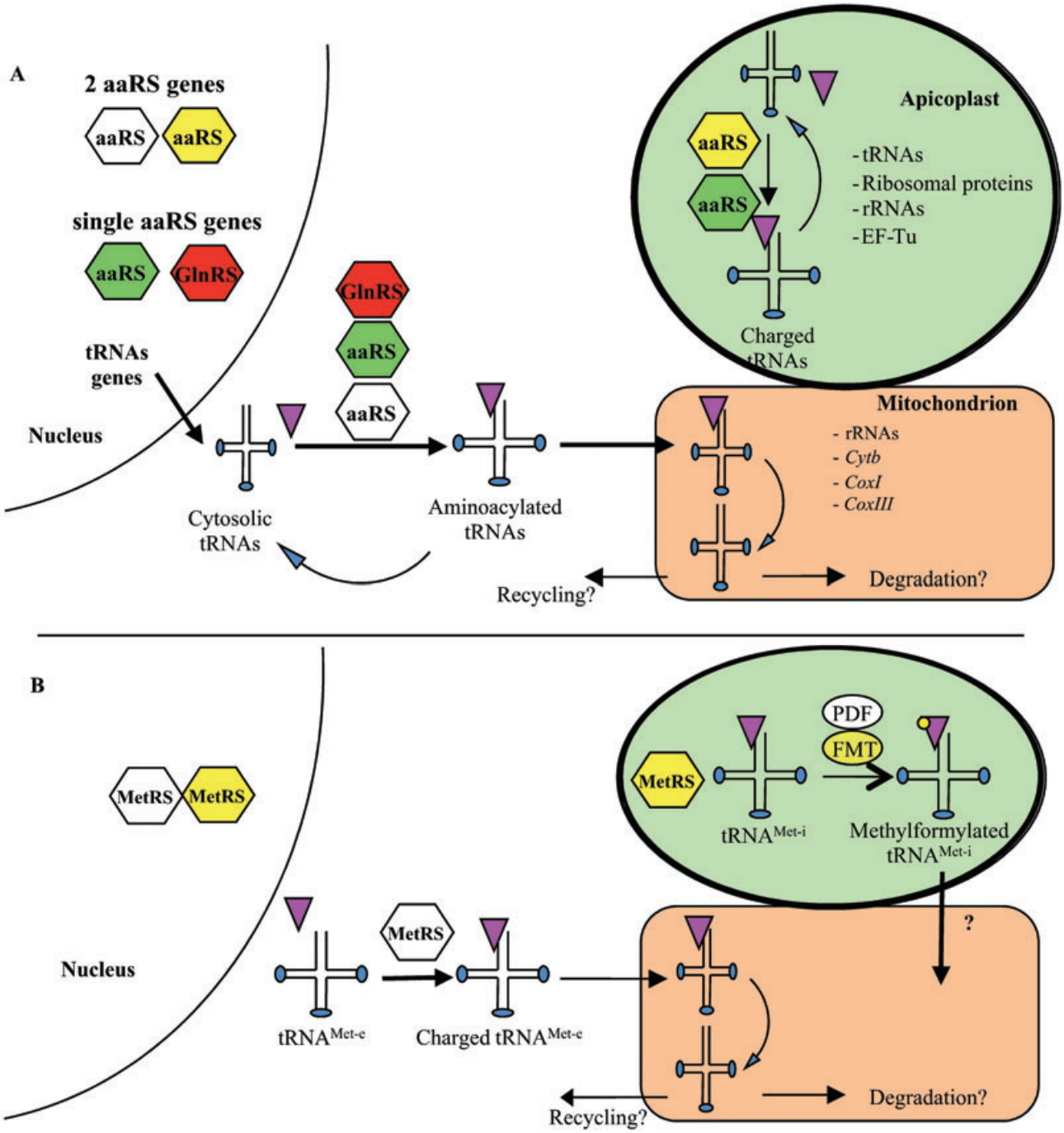


Fig. 6. The aaRSs are absent in the mitochondrion (A). The absence of tRNA genes in the mitochondrial genome of *Apicomplexa* is compensated for by the import of the nuclear-encoded cytosolic tRNAs. The aaRSs are either uniquely cytosolic or apicoplastic or shared between the two compartments by dual targeting but absent in the mitochondrion. Failure to detect any aaRS into the mitochondrion suggests that the imported aminoacylated tRNAs are degraded or recycled. Absence of formylation of methioninyl-tRNA (B). Both the methionyl-tRNA formyltransferase (FMT) and peptide deformylase (PDF) are localized to the apicoplast only. This implies that the requirement for an initiation by a specialized tRNA charged with formylmethionine has been bypassed in the mitochondrion of these parasites.

Subcellular fractionation of T. gondii

PBS-washed *T. gondii* cells were resuspended in SoTE (0.6 M sorbitol, 20 mM Tris-HCl, pH 7.5, and 2 mM EDTA). Five per cent of the sample was removed to isolate the total RNA. After the addition of SoTE containing either 0.1% (0.05% final) or 0.2% (0.1% final) of digitonin, the samples were mixed by pipetting and incubated on ice for 5 min. The suspensions were centrifuged (800 *g* for 5 min at 4 °C), and the supernatants were discarded. The resulting pellets were

treated with RNase A and incubated on ice for 15 min to digest the contaminating cytosolic RNAs. The organellar fractions used to isolate RNA were recovered in the pellets after a final centrifugation step (Esseiva *et al*., 2004).

RNA Isolation and northern analysis

RNA from total cells or digitonin-treated fractions was purified by using the acidic guanidinium isothiocyanate method as previously described (Esseiva *et al*., 2004). Blotting and hybridizations using 5′-end-labelled oligonucleotides were done as described (Esseiva *et al*., 2004).

IFA and confocal microscopy

Intracellular parasites grown in HFF on glass slides were fixed with 4% paraformaldehyde as previously described (Pino *et al*., 2007). Confocal images were collected with a Leica (http://www.leica.com/) laser scanning confocal microscope (TCS-NT DM/IRB and SP2) using a 1003 Plan-Apo objective with NA 1.4. All other micrographs were obtained with a Zeiss Axiophot (http://www.zeiss.com/) with a camera (Photometrics Type CH-250; http://www.photomet.com/). Adobe Photoshop (Adobe Systems, http://www.adobe.com/) was used for image processing.

Bioinformatics

Putative SPs were predicted using SignalP and iPSORT [63–65], and putative mitochondrial transit peptides using TargetP [66] and MitoProt II [67] (http://ihg.gsf.de/ihg/mitoprot.html). Publicly available protein sequences were taken from NCBI, PlasmoDB.org, ToxoDB.org, the DOE Joint Genome Institute (http://www.jgi.doe.gov/) and the *Cyanidioschyzon merolae* Genome Project (http://merolae.biol.s.u-tokyo.ac.jp/). Sequence alignments were generated with ClustalX (v.1.83) (Chenna *et al*., 2003) using both the 'Slow-Accurate' and the 'Fast-Approximate' pairwise alignment modes. The alignments were arrived at in an iterative fashion whereby obvious insertions present in only a few or individual sequences were deleted before recalculating the alignment. The final alignments were generated using the default alignment parameters in 'Slow-Accurate' pairwise alignment mode. Just prior to the phylogenetic analysis hypervariable and difficult-to-align regions of the alignments were manually removed, ensuring that only those blocks of the alignments were used that contained well-aligned sequence positions especially in regard to the apicomplexan sequences. Phylogenetic Maximum Likelihood and Neighbor-Joining analyses were carried out using the PHYLIP programs *seqboot*, *proml*, *protdist*, *neighbor* and *consense* (version 3.69) (Felsenstein, 1989). The Jones-Taylor-Thornton model was selected in *proml* and *protdist*, global rearrangements were employed in *proml*, and sequence input order was randomized in *proml* and *neighbor*. The resulting trees were visualized with TreeView.

Acknowledgements

We gratefully acknowledge Drs J. Curran and D. Garcin for their critical reading of the manuscript. We thank Dr W. Bohne for kindly providing anti-PDH-E2 antibodies. This work is part of the activities of the BioMalPar European Network of Excellence supported by a European grant (LSHP-CT-2004-503578) from the Priority 1 'Life Sciences, Genomics and Biotechnology for Health' in the 6th Framework Program. P.P. was supported by The Wellcome Trust. D.S. is supported by the Swiss National Foundation and is an International Scholar of the Howard Hughes Medical Institutes.

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