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Iron-Sulfur cluster assembly in *Monocercomonoides exilis*

Syntéza železo-sirných center v *Monocercomonoides exilis*

Summary of Ph.D. Thesis
Autoreferát dizertační práce

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Abstract:

In the search for the mitochondrion of oxymonads, DNA of *Monocercomonoides exilis* – an oxymonad isolated from the gut of *Chinchilla,* was isolated and its genome was sequenced. Sequencing resulted in a fairly complete genome which was extensively searched or genes for mitochondrion related proteins, but no reliable candidate for such gene was identified. Even genes for the ISC pathway, which is responsible for Fe-S cluster assembly and considered to be the only essential function of reduced mitochondrion-like organelles (MROs), were absent. Instead, we were able to detect the presence of a SUF pathway which functionally replaced the ISC pathway.

Closer examination of the SUF pathway based on heterologous localisation revealed that this pathway localised in the cytosol. *In silico* analysis showed that SUF genes are highly conserved at the level of secondary and tertiary structure and most catalytic residues and motifs are present in their sequences. The functionality of these proteins was further indirectly confirmed by complementation experiments in *Escherichia coli* where SUF proteins of *M. exilis* were able to restore at least partially Fe-S cluster assembly of strains deficient in the SUF and ISC pathways. We also proved by bacterial adenylate cyclase two-hybrid system that SufB and SufC can form complex.

SUF genes were also found in transcriptomes and genomes of nine other Preaxostyla species (a group of anaerobic protists that belong to Metamonada), while the ISC pathway was consistently absent. Interestingly, in most Preaxostyla, we were able to identify at least partial fusions of SufD, S, and U, which is a unique situation. In a phylogenetic analysis of concatenated genes SufB, C, D, and S, Preaxostyla formed a well-supported clade nested inside of bacteria. The resulting clade is clearly distinct from that of the SUF pathways known from plastids and previously described SufBC systems of *Blastocystis hominis, Pygsuia biforma*, and related protists.

These results strongly suggest that the SUF pathway was acquired from bacteria by the last common ancestor of Preaxostyla, where it functionally replaced the mitochondrial ISC pathway. Based on these results it was proposed that *M. exilis* has completely lost its MRO and therefore it is the first described amitochondriate organism. The loss of MRO in the case of *M. exilis* was probably allowed by a combination of an anaerobic and endobiotic lifestyle of this organism, together with the replacement of the mitochondrial ISC pathway by cytosolically - localised SUF pathway which rendered the MRO expendable.

Abstrakt:

Při hledání mitochondrií u oxymonád jsme osekvenovali genom *Monocercomonoides exilis* — oxymonády izolované ze střeva činčily. Sekvenování poskytlo relativně kompletní genom, který byl důkladně prohledán na geny proteinů asociovaých s mitochondriální organelou, ale ani po intenzivním hledání se nám nepodařilo odhalit žádné věrohodné mitochondriální geny. Nepodařilo se nalézt ani geny pro ISC dráhu, která je zodpovědná za syntézu Fe-S center a je považována za esenciální funkci organel mitochondriálního původu (MRO). Namísto ní se nám podařilo najít geny pro SUF dráhu, která ji zřejmě funkčně nahradila.

Bližší zkoumání SUF dráhy pomocí heterologních lokalizací naznačilo, že tato dráha je pravděpodobně lokalizována v cytosolu. *In silico* analýza prokázala, že proteiny SUF dráhy jsou konzervovány na úrovni sekundární a terciální struktury a že většina katalyticky významných aminokyselin a motivů je v nich přítomna. Funkčnost těchto proteinů byla nepřímo prokázána pomocí komplementací v *Escherichia coli* s mutovanou SUF a ISC drahou.

SUF dráhu se podařilo nalézt také v transkriptomech a genomech dalších devíti zástupců skupiny Preaxostyla (anaerobních protist patřících do skupiny Metamonada). U většiny preaxostyl se nám podařilo nalézt alespoň částečnou fúzi genů pro SufD, SufS a SufU. Tato fúze je unikátní pro tuto skupinu protist. V konkatenované fylogenetické analýze SUF proteinů utvořila Preaxostyla dobře podpořený klád umístěný mezi bakteriálními sekvencemi. Pozice tohoto kládu zřetelně ukazuje, že SUF systém preaxostyl je fylogeneticky odlišného původu od SUF dráhy známé z plastidů a SUF dráhy popsané u *Blastocystis hominis*, *Pygsuia biforma* a dalších protist.

Tyto výsledky silně poukazují na to, že Preaxotyla získala SUF dráhu od blíže neurčené skupiny bakterií a že k tomu došlo už u posledního společného předka preaxostyl. Na základě těchto výsledků jsme formulovali hypotézu, že minimálně v případě *M. exilis*, byla ztráta mitochondrie umožněna nahrazením mitochondriální ISC dráhy SUF dráhou lokalizovanou v cytosolu. Tato změna umožnila převést tvorbu Fe-S center do cytosolu a mitochondrie tak ztratila svou jedinou esenciální funkci, což umožnilo její postupnou ztrátu.

Introduction:

Iron-Sulfur (Fe-S) clusters are ancient and essential inorganic cofactors of enzymes and are virtually ubiquitous in all living organisms. Fe-S clusters exist in many forms but most common are 2Fe-2S (so-called rhombic) and 4Fe-4S (cubane) clusters (Beinert 1997; Beinert 2000). Thanks to their structure they have an ability to delocalise electrons over Fe and S atoms (Noodleman and Case 1992; Glaser et al. 2000), which makes them ideal candidates for their primary role in the cell, mediators of electron transport.

As electron transporters, they are involved in many important cellular processes involve electron transport such as mitochondrial respiration, photosynthesis, and citric acid cycle. However, Fe-S clusters are involved in many other processes like gene regulation, sensory function, substrate activation, DNA metabolism, protein synthesis, RNA modifications, iron homeostasis, etc.

Although Fe-S clusters can form spontaneously *in vitro*, if apoprotein is exposed to inorganic Fe and S sources under anaerobic conditions (Malkin and Rabinowitz 1966), concentrations of Fe and S necessary for this reaction to occur reach toxic levels. Therefore in living organisms, several different pathways have evolved – in order of their discovery those are NIF (**NI**trogen **F**ixation) (Zheng et al. 1993), ISC (**I**ron-**S**ulfur **C**luster assembly) (Zheng et al. 1998), SUF (**S**ulfur **U**tilisation **F**actor) (Takahashi and Tokumoto 2002), and CSD pathway(**C**ysteine **S**ulfinate **D**esulfinase) (Loiseau et al. 2005).

In eukaryotic organisms, Fe-S clusters are synthesised usually by mitochondrial ISC pathway of α-proteobacterial origin (Richards and Van Der Giezen 2006) and CIA pathway which is considered to be a eukaryotic novelty and which maturates cytosolic and nuclear Fe-S proteins. However, CIA pathway is unable to synthesise Fe-S clusters *de novo* and needs unknown sulfur or iron-sulfur intermediate (termed X-S) (Kispal et al. 1999; Pandey et al. 2019), synthesised by mitochondrial ISC pathway and transported via Atm1 to the cytosol (Biederbick et al. 2006; Pondarré et al. 2006).

Core proteins of ISC pathway were found in almost all mitochondria and mitochondrion-related organelles(Tachezy et al. 2001; Williams et al. 2002; Tovar et al. 2003) with exceptions of few anaerobic protists (Ali et al. 2004; Nyvltova et al. 2013; Stairs et al. 2014) and is considered to be an only essential function of the mitochondrion (Williams et al. 2002).

Preaxostyla are a group of anaerobic flagellates protists which are part of Metamonada (Hampl et al. 2009; Adl et al. 2019). They are named after preaxostyle - lattice-like cytoskeleton structure connecting two pairs of basal bodies. Preaxostyla are a sister group to Parabasalia and Fornicata and consists of three monophyletic groups — free-living Trimastigidae and Paratrimastigidae and endobiotic Oxymonadida (Zhang et al. 2015).

Oxymonads are endobiotic, typically inhabiting the gut of insects, with the largest diversity found in lower termites and wood-eating cockroaches of the genus *Cryptocercus* (Hampl 2017). Some species of oxymonads also live in the intestines of vertebrates. Currently are the oxymonads are only large group of protists which no MRO was identified, although they were studied quite intensively by TEM in the past. Trimastigidae and Paratrimastigidae, on the other hand, contain MRO resembling hydrogenosome (Simpson 2003; Hampl et al. 2008; Zhang et al. 2015)

Aims of thesis:

- Search for mitochondrion related genes in the genomic and transcriptomic data of *Monocercomonoides exilis*.
- Localize SUF pathway of *Monocercomonoides exilis* in heterologous systems.
- Find and characterize Fe-S cluster assembly proteins in available genomes and transcriptomes of Preaxostyla and resolve their evolutionary history.
- Predict and annotate Fe-S cluster proteins in *M. exilis* and compare them to other closely related protists to elucidate if change of Fe-S cluster pathway influenced inventory of Fe-S cluster proteins.
- Confirm functionality of SUF proteins of *M. exilis* by complementation experiments in *E. coli.*

Materials and methods:

Materials and methods are described in detail in individual published publications. Here is a brief list of used methods: DNA and RNA isolation, mRNA selection, PCR, restriction cloning, bacterial two-hybrid adenylate cyclase systém, B-galactosidase assay, genome annotation and phylogenetic analyses, immunofluorescence microscopy

Summary:

In the search for MRO of oxymonads, we sequenced the genome of *Monocercomonoides exilis* by 454 whole-genome shotgun method (Karnkowska et al. 2016). Sequencing resulted in the fairly complete genome with an approximate size of 75 Mb and average coverage 35x. Completeness of the genome was estimated by CEGMA (Core Eukaryotic Genes Mapping Approach) (Parra et al. 2007) which identified presence 63.3% of core eukaryotic genes and after removing of mitochondrial genes and using manually curated *M. exilis* gene model estimated completeness increased to 90%.

|Genome was extensively searched for the mitochondrion related genes by BLAST and HMMER based approaches. However, we were not able to identify any specifically mitochondrial genes in the genome including mitochondrial translocases, which are otherwise present in all known MROs (Dolezal et al. 2010; Zarsky et al. 2012). Also, we were not able to find any components of mitochondrial ISC pathway, which is considered to be the only essential function of MROs and is present in virtually all known MROs, with few previously described exceptions — *Entamoeba histolytica* (Ali et al. 2004; Mi-ichi et al. 2009), *Mastigamoeba balamuthi* (Nyvltova et al. 2013) and Pygsuia biforma (Stairs et al. 2014).

Instead of the ISC pathway, we were able to identify genes of SUF pathway for Fe-S cluster assembly. In other eukaryotes, the SUF pathway is limited only to plastids and few protists like *P. biforma, S. incarcerata* (Stairs et al. 2014) or *B. hominis* (Tsaousis et al. 2012). The SUF pathway of *M. exilis* consists of SufB, SufC, and fusion gene SufDSU. Currently, such fusion is known only from Preaxostyla. Fluorescence *in situ* hybridisation with specific probes showed that SUF genes are specific for *M. exilis* and not the bacterial contamination. None of the SUF genes has any recognisable N-terminal targeting sequence and heterologous localisation of SufC in *S. cerevisiae* and SufB and SufC in *T. vaginalis* showed cytosolic localisation indicating that SUF pathway is most likely localised in the cytosol of *M. exilis.*

In the genome, we were able to identify four genes of CIA pathway- Nbp35, Cia1, Cia2b (two copies) and Nar1. This minimalistic set of proteins is comparable with other members of Metamonada (Pyrih et al. 2016). We were not able to identify any genes involved in the transport of X-S from MRO like Atm1 or Erv1 and also genes encoding electron transport chain proteins (Tah18 and Dre2) are missing. From this we can conclude that the cytosolic and nuclear Fe-S proteins are most likely maturated by the unique combination of SUF and CIA pathway and that whole system is localised in the cytosol.

In silico analysis of SUF pathway of *M. exilis* showed that these proteins show a high level of conservation on the level of secondary and tertiary structure and also contain all catalytically important residues. SufB and SufC seem to be best conserved when compared to their bacterial homologues. Also, their predicted protein models show remarkable similarity protein crystal structures of their counterparts from bacteria. Parts of fusion gene SufDSU contain several insertions when compared to sequences of their bacterial homologues. However, these insertions do not disturb the overall tertiary structure of predicted protein models.

In complementation experiments with *M. exilis* genes in single-gene mutants of *E. coli,* where the deficient gene was replaced by the gene from *M. exilis, i*n conditions of oxidative stress and iron-starvation, only SufB showed weak rescue effect. Complementation in *E. coli* strains deficient in ISC pathway and the examined gene — genes for SufB, SufS and SufSU — showed significantly higher production of Fe-S clusters than the negative control, when measured by β-galactosidase assay. Proving that SUF genes of *M. exilis* are capable of a partial restoration of Fe-S assembly in *E. coli*. However, the effect is not strong enough to restore the viability of *E. coli* cells. Another piece of evidence for the functionality of the SUF pathway in *M. exilis* was provided by the BACTH assay (Bacterial Adenylate Cyclase Two-Hybrid system), that confirmed the ability of *M. exilis* SufB and SufC to interact with each other.

Prediction of Fe-S cluster protein based on the MetalPredator (Valasatava et al. 2016) showed that *M. exilis* genome contains ~ 70 potential Fe-S proteins, this number is comparable to other anaerobically living eukaryotes (Karnkowska et al. 2019). These results suggest that the switch from the ISC to SUF pathway did not affect number Fe-S cluster proteins in *M. exilis.*

We were also able to identify genes of SUF pathway protein in available genomes and transcriptomes of nine members of Preaxostyla, at the same time we were not able to identify any genes of ISC pathway in them. Although, there is a possibility that some genes might be missing due to the incompleteness of the transcriptomes. We were able to detect fusion gene SufDSU in all Preaxostyla with sequenced genomes and at least partial fusion of SufS and SufU in the transcriptome of *T. marina*. Phylogenetic analysis of concatenated SufB, SufC, SufD and SufS genes showed that origin of SUF pathway in Preaxostyla is distinct to SUF pathway known from plastids and SufBC genes reported from *Blastocystis hominis*, *Pygsuia biforma*, *Stygiela incarcerata* and other protists, which was acquired from methanogenic archaea (Tsaousis et al. 2012; Stairs et al. 2014; Tsaousis 2019). These results suggest that the last common ancestor of Preaxostyla acquired SUF pathway by lateral gene transfer from unknown bacteria before a loss of mitochondrion (Vacek et al. 2018).

Conclusions:

Our results proved that *M. exilis* has completely lost mitochondrion and is therefore first described truly amitochondriate organism. Together with mitochondrion it also lost the ISC pathway which was replaced by the SUF pathway. Heterologous immunofluorescence localisation showed that SUF system of *M. exilis* is probably localised in the cytosol, where it is involved in Fe-S cluster synthesis together with CIA pathway.

By combining results of *in silico* analysis, complementation experiments in *E. coli* and bacterial adenylate cyclase two-hybrid experiments we brought first although indirect evidence, that SUF system of *M. exilis* is capable of Fe-S cluster synthesis.

Phylogenetic analysis showed, that SUF pathway was acquired by last common ancestor of Preaxostyla *via* horizontal gene transfer from an unidentified group of bacteria. Fossil records of oxymonads from Burmese amber indicate that, Preaxostyla acquired SUF pathway before Early Cretaceous period (~145 - 105.5 million years ago).

Based on our results we proposed hypothesis that replacement of ISC pathway by SUF pathway allowed to transport Fe-S cluster assembly from MRO to the cytosol and rendered ISC pathway obsolete as it lost its indispensability for cell. Loss of only essential function – Fe-S cluster assembly – allowed for complete loss of MRO in *M. exilis* and most likely in majority of oxymonads. Acquisition of SUF pathway was therefore key event for loss of mitochondria.

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- *In silico* analyses and protein modelling, complementation experiments, Bacterial adenylate cyclase two-hybrid experiments

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2014 - 2017 – Project lead researcher GAUK No. 1584314, funded by Charles University

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Publications:

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