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Catalase in Leishmaniinae: With me or against me?

Natalya Kraeva [a,1], Eva Horáková [b,1], Alexei Y. Kostygov [a,c], Luděk Kořený [b,d], Anzhelika Butenko [a], Vyacheslav Yurchenko [a,b,e,*], Julius Lukeš [b,f,g,**]

[a] Life Science Research Centre, Faculty of Science, University of Ostrava, 710 00 Ostrava, Czech Republic
[b] Biology Centre, Institute of Parasitology, Czech Academy of Sciences, 370 05 České Budějovice (Budweis), Czech Republic
[c] Zoological Institute of the Russian Academy of Sciences, St. Petersburg 199034, Russia
[d] Department of Biochemistry, University of Cambridge, Cambridge CB2 1GA, United Kingdom
[e] Institute of Environmental Technologies, Faculty of Science, University of Ostrava, 710 00 Ostrava, Czech Republic
[f] Faculty of Science, University of South Bohemia, 370 05 České Budějovice (Budweis), Czech Republic
[g] Canadian Institute for Advanced Research, Toronto, Ontario, M5G 1Z8, Canada

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ABSTRACT

The catalase gene is a virtually ubiquitous component of the eukaryotic genomes. It is also present in the monoxenous (i.e. parasitizing solely insects) trypanosomatids of the subfamily Leishmaniinae, which have acquired the enzyme by horizontal gene transfer from a bacterium. However, as shown here, the catalase gene was secondarily lost from the genomes of all *Leishmania* sequenced so far. Due to the potentially key regulatory role of hydrogen peroxide in the inter-stagial transformation of *Leishmania* spp., this loss seems to be a necessary prerequisite for the emergence of a complex life cycle of these important human pathogens. Hence, in this group of protists, the advantages of keeping catalase were uniquely outweighed by its disadvantages.

1. Evolutionary history of catalase in eukaryotes

The chemical reaction of hydrogen peroxide (H_2O_2) decomposition mediated by various vegetable and animal extracts and leading to production of molecular oxygen was first described by a German-Swiss chemist Christian Friedrich Schönbein (Schönbein, 1863). This power of H_2O_2 decomposition was considered a general property of all enzymes until Loew assigned the activity to a special enzyme to which he gave the name catalase (Loew, 1901). Ever since, this powerful enzyme was encountered in so many taxa that it might, in fact, be one of the most widely distributed enzymes on Earth. Still, as will be shown below, catalase is not omnipresent and has been lost in several eukaryotic lineages for reasons that are tightly associated with its function.

Catalase plays an important role in removing intracellular H_2O_2, thereby protecting the cells from reactive oxygen species (ROS). These are constantly formed as a by-product of aerobic metabolism (Mates, 2000). Although not a free radical and generally poorly reactive, H_2O_2 is still ranked among the ROS due to its ability to react with iron. As a consequence, the reactive hydroxyl radical (OH•) formed through the Fenton reaction can be very harmful to cells (Halliwell, 1999). Importantly, H_2O_2 can also act as a second messenger in signaling pathways in multicellular eukaryotes, while in protists it primarily stimulates the production of antioxidants and ROS removing enzymes (Zamocky et al., 2010). The activity of catalase, which is composed of four polypeptide chains, is intertwined with the synthesis or acquisition of heme, an iron-carrying cofactor that allows the enzyme to react with H_2O_2 (Kirkman and Gaetani, 1984). Catalase is not the only enzyme responsible for fine tuning the intracellular levels of H_2O_2, as proteins such as several glutathione peroxidases (GPX) and other peroxiredoxins work in synergy with catalase (Molavian et al., 2015).

The evolutionary history of catalase is rather complicated due to the promiscuous nature of the corresponding gene. Indeed, the topology of catalase-based trees suggests an unusually high number of horizontal gene transfer (HGT) events, especially among bacteria (Faguy and Doolittle, 2000). Eukaryotes acquired the catalase gene from various sources (Fig. 1), as they form several unrelated groups in the respective phylogenetic tree (Suppl. Fig. 1). One of these groups brings together representatives of most of the eukaryotic supergroups, suggesting that catalase could be already present in the last eukaryotic common ancestor (LECA) (Fig. 1 and Suppl. Fig. 1, highlighted in blue). However, the branching within this clade does not reflect the current view of eukaryotic evolution: metazoans group with stramenopiles, bryophytes with fungi and *Acanthamoeba*, and Heterolobosea form a sister lineage to

* Correspondence to: V. Yurchenko, Life Science Research Centre, University of Ostrava, Chittussiho 10, 710 00 Ostrava, Czech Republic.
** Correspondence to: J. Lukeš, Institute of Parasitology, Branišovská 31, 370 05 České Budějovice (Budweis), Czech Republic.
E-mail addresses: vyacheslav.yurchenko@osu.cz (V. Yurchenko), jula@paru.cas.cz (J. Lukeš).
[1] Shared first authorship.

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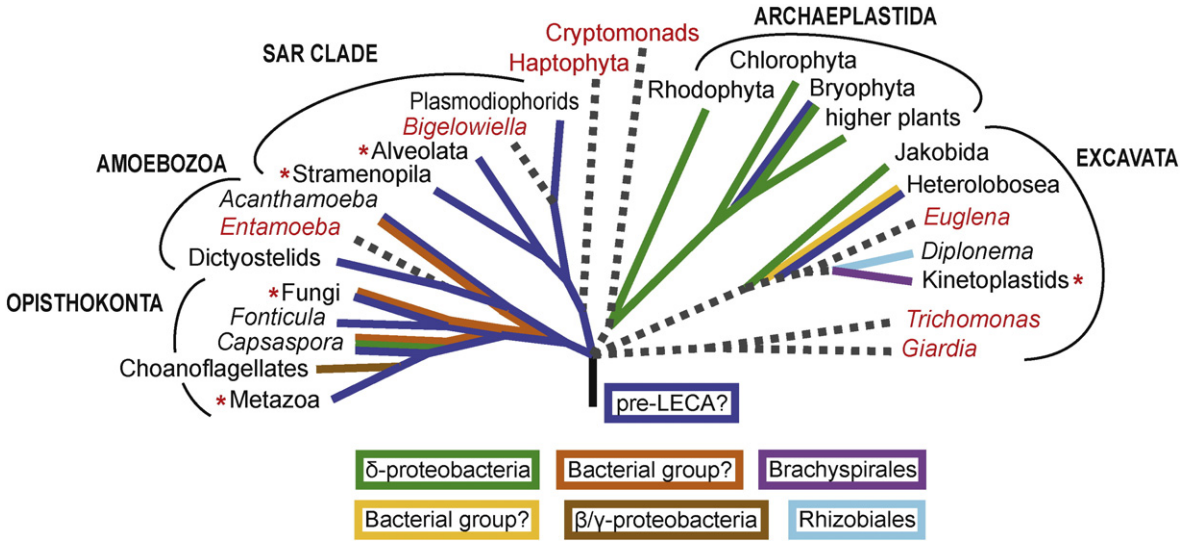

Fig. 1. Evolutionary history of eukaryotic catalases. The schematic tree depicts the current view of eukaryotic phylogeny. Taxa lacking the catalase gene in their genomes are in red, while the larger eukaryotic groups where only subsets of species lack catalase are marked with red asterisks. The colored lines highlight the presence of catalase and different colors stand for different gene origins, which are explained in the boxes below the tree. The origins were estimated based on the phylogenetic affiliations in Suppl. Fig. 1.

Amoebozoa. It is therefore plausible that the gene encoding catalase has been acquired later in the evolution of eukaryotes, likely from a bacterium, and passed onto several groups via multiple intra-eukaryotic HGTs. Other eukaryotic catalases are more limited in taxonomic sense and their origin via HGT from various bacterial lineages is obvious from their phylogenetic affiliations. However, due to high frequency of HGTs between bacterial lineages, it is often impossible to uncover which specific bacterial taxon served as a donor of a given eukaryotic catalase. One of these genes was likely transferred from δ-proteobacteria onto the ancestor of Archaeplastida and consequently onto jakobids and *Capsaspora* (Fig. 1, highlighted in green). Some eukaryotes, namely fungi, *Acanthamoeba*, *Capsaspora*, *Naegleria* spp. and bryophytes, have two or more catalase genes of different bacterial origins, further complicating the picture. Others seem to have acquired the catalase gene relatively recently and its distribution is therefore limited to a rather narrow taxonomic range. Trypanosomatid flagellates represent such a case and will be described in detail below.

Despite its extremely wide distribution, several eukaryotes lack an identifiable homolog of catalase in their genomes. The absence of this enzyme in species that inhabit anoxic environments with little exposure to oxidative stress, such as parasitic protists *Giardia*, *Trichomonas*, *Entamoeba* spp. and *Cryptosporidium* spp., is not surprising (Mehlotra, 1996). Unexpectedly, catalase is also lacking from numerous photosynthetic eukaryotes that possess secondary plastids, including euglenids, chlorarachniophytes, haptophytes, cryptophytes and some stramenopiles. Another striking pattern that emerged from our analysis of the catalase distribution in eukaryotes is that it was specifically lost in some parasitic lineages, in particular in those that dwell in vertebrate blood. The apicomplexan parasites represent a conspicuous example. The two gregarine and seven coccidian species that have their genomes sequenced possess the catalase gene, while it is missing from the genomes of hematozoans *Theileria, Babesia*, and *Plasmodium* spp. Similarly, catalase is present in five species of free-living flatworms but absent from the related blood-dwelling flukes such as *Schistosoma* spp. (Suppl. Fig. 1).

Such a seemingly mutual exclusiveness of catalase and vertebrate blood led us to consider a scenario, in which the parasites are prone to the loss of catalase, as they occupy an environment with very low levels of H_2O_2. There seems to be little consensus on this issue in the literature, as some authors have measured substantially high levels of H_2O_2 (up to 35 uM) in vertebrate blood (Halliwell et al., 2000), while others have claimed close-to-zero levels (Mueller et al., 1997). In support of the latter notion is the fact that human red blood cells efficiently scavenge extracellular H_2O_2 and inhibit formation of hypochlorous acid and hydroxyl radicals (Winterbourn and Stern, 1987). Moreover, it was shown that when added to human plasma, H_2O_2 disappears rapidly (Halliwell et al., 2000). In any case, our finding that blood-dwelling unicellular and multicellular parasites tend to lose the catalase gene is of particular interest and the reason for that should be investigated further.

2. Catalase in trypanosomatid flagellates

As mentioned above, kinetoplastid protists of the family Trypanosomatidae deserve special attention when the distribution of catalase is considered. These flagellates are obligatory parasites of arthropods, leeches, vertebrates, and plants (Maslov et al., 2013) that apparently evolved from free-living biflagellated relatives (Lukeš et al., 2014). Trypanosomatids of the well-studied genera *Phytomonas*, *Trypanosoma*, and *Leishmania* are dixenous, i.e. shuttling between two hosts in their life cycle, while members of the other genera (*Crithidia*, *Leptomonas*, *Herpetomonas*, and others) are monoxenous, which means they are confined to a single insect host (Maslov et al., 2013; Votýpka et al., 2015).

Since several species of Trypanosomatidae, in particular the human parasites *Trypanosoma brucei* and *Leishmania* spp., are among the best studied model protists, a lot of information is available on their protection against oxidative stress. It seems to be mediated mainly by the bis-glutathionyl derivative of spermidine-trypanothione, Fe-superoxide dismutase (SOD), NADP-isocitrate dehydrogenase, and several peroxidases (Fairlamb et al., 1985; Tomás and Castro, 2013). In addition to these, several trypanosomatids have acquired a bacterial-type catalase. The presence of this enzymatic activity has been first reported over 50 years ago in several monoxenous representatives of the subfamily Leishmaniinae (*Crithidia fasciculata* and *Crithidia luciliae*) and Strigomonadinae (*Strigomonas oncopelti* and *S. culicis*) and even proposed as a taxonomical trait (Wertlieb and Guttman, 1963). In contrast to other eukaryotes (where the enzyme is mostly peroxisomal), catalase in trypanosomatids was localized to the cytoplasm (Edwards and Lloyd, 1977; Souto-Padron and de Souza, 1982). Surprisingly, all the dixenous species investigated in this respect (i.e. *Trypanosoma* and *Leishmania* spp.), lack catalase (Mehlotra, 1996; Wertlieb and Guttman, 1963; Harvey, 1949), but these early findings did not raise much attention at

that time. Glycosomes of trypanosomatids do not contain typical peroxisomal marker enzymes, such as D-amino acid oxidase, acyl-CoA oxidase, and 2-hydroxy-acid oxidase (Blast cut-off E-10) (Opperdoes et al., 2016) and, therefore, these species may be less reliant on the presence of catalase.

To shed more light on this complex pattern of distribution of catalase in Trypanosomatidae, we have analyzed all the available genomic datasets for the presence/absence of the corresponding gene (Table 1, Fig. 2). This was complemented by the analysis of the recently sequenced closest free-living relative of the obligatory parasitic trypanosomatids, *Bodo saltans* (Opperdoes et al., 2016; Jackson et al., 2016). No catalase gene was identified in any species outside of the Leishmaniinae clade, suggesting that its acquisition was a relatively recent evolutionary event. Moreover, within this clade, catalase was restricted to the monoxenous genera *Crithidia*, *Leptomonas*, and *Lotmaria*, and was apparently secondarily lost from the dixenous *Leishmania* spp. The previously reported catalase activity in *Strigomonas* spp. (Wertlieb and Guttman, 1963) is probably a technical mistake. The original culture of *S. oncopelti* was shown to contain two different species: a real *Strigomonas* with cytoplasmic bacterial endosymbionts and a symbiont-free trypanosomatid of unknown phylogenetic affinity, which differed in morphology (Krylov et al., 1985). Hence, the catalase activity could be explained by the presence of this second species.

To track the origin of catalase in the monoxenous Leishmaniinae, we performed an extensive phylogenetic analysis of the corresponding gene (Fig. 3). Besides the trypanosomatid sequences from publicly available genomes and those obtained in frame of our study (Table 1), we included first 100 BlastP hits obtained from the NCBI non-redundant protein database with the catalase of *Novymonas esmeraldas* as a query. Our results strongly suggest that this gene was acquired from a bacterium and that it was a singular event occurred in the common ancestor of all Leishmaniinae. The catalase of spirochaetes of the genus *Brachyspira* is phylogenetically most closely related to that of trypanosomatids. However, this bacterial genus was recently demonstrated to acquire

Table 1
Presence/absence of the catalase-encoding gene in sequenced genomes of Trypanosomatidae.

Species	Isolate	Catalase presence/absence	Source of data
Angomonas deanei	TCC036E	−	GenBank
Angomonas desouzai	TCC079E	−	GenBank
Blechomonas ayalai	B08-376	−	Our unpublished data
Bodo saltans	Konstanz	−	Wellcome Trust
Crithidia acanthocephali	TCC037E	+	GenBank
Crithidia fasciculata	CF-C1	+	TriTrypDB v.9.0
Crithidia luciliae thermophila	ATCC 30817	+	Our unpublished data
"*Endotrypanum*" *monterogeii*	LV88	−	TriTrypDB v.9.0
Herpetomonas muscarum	TCC001E	−	GenBank
Kentomonas sorsogonicus	MF-08	−	Our unpublished data
Leishmania aethiopica	L147	−	TriTrypDB v.9.0
Leishmania amazonensis	MHOM/BR/71973/M2269	−	TriTrypDB v.9.0
Leishmania arabica	LEM1108	−	TriTrypDB v.9.0
Leishmania braziliensis	MHOM/BR/75/M2903	−	TriTrypDB v.9.0
Leishmania braziliensis	MHOM/BR/75/M2904	−	TriTrypDB v.9.0
Leishmania donovani	BPK282A1	−	TriTrypDB v.9.0
Leishmania enriettii	LEM3045	−	TriTrypDB v.9.0
Leishmania gerbilli	LEM452	−	TriTrypDB v.9.0
Leishmania infantum	JPCM5	−	TriTrypDB v.9.0
Leishmania major	Friedlin	−	TriTrypDB v.9.0
Leishmania mexicana	MHOM/GT/2001/U1103	−	TriTrypDB v.9.0
Leishmania panamensis	MHOM/COL/81/L13	−	TriTrypDB v.9.0
Leishmania peruviana	PAB-4377	−	TriTrypDB v.9.0
Leishmania sp.	MARLEM 2494	−	TriTrypDB v.9.0
Leishmania tarentolae	ParrotTarII	−	TriTrypDB v.9.0
Leishmania tropica	L590	−	TrTrypDB v.9.0
Leishmania turanica	LEM423	−	TrTrypDB v.9.0
Leptomonas pyrrhocoris	H10	+	TriTrypDB v.9.0
Leptomonas seymouri	ATCC 30220	+	TriTrypDB v.9.0
Lotmaria passim	SF	+	GenBank
Novymonas esmeraldas	E262AT.01	+	Our unpublished data
Paratrypanosoma confusum	Cul-13	−	Our unpublished data
Phytomonas serpens	9T	−	GenBank
Phytomonas sp.	EM1	−	GenBank
Phytomonas sp.	HART1	−	GenBank
Strigomonas culicis	TCC012E	−	GenBank
Strigomonas galati	TCC219	−	GenBank
Strigomonas oncopelti	TCC290E	−	GenBank
Trypanosoma brucei gambiense	DAL972	−	TriTrypDB v.9.0
Trypanosoma brucei	Lister 427	−	TriTrypDB v.9.0
Trypanosoma brucei	TREU927	−	TriTrypDB v.9.0
Trypanosoma congolense	IL3000	−	TriTrypDB v.9.0
Trypanosoma cruzi marinkellei	B7	−	TriTrypDB v.9.0
Trypanosoma cruzi	CL Brener Esmeraldo-like	−	TriTrypDB v.9.0
Trypanosoma cruzi	CL Brener non-Esmeraldo-like	−	TriTrypDB v.9.0
Trypanosoma cruzi	Dm28c	−	TriTrypDB v.9.0
Trypanosoma cruzi	JRcl4	−	TriTrypDB v.9.0
Trypanosoma cruzi	Sylvio ×10	−	TriTrypDB v.9.0
Trypanosoma cruzi	Tulacl2	−	TriTrypDB v.9.0
Trypanosoma evansi	STIB805	−	TriTrypDB v.9.0
Trypanosoma grayi	ANR4	−	TriTrypDB v.9.0
Trypanosoma rangeli	SC58	−	TriTrypDB v.9.0
Trypanosoma vivax	Y486	−	TriTrypDB v.9.0

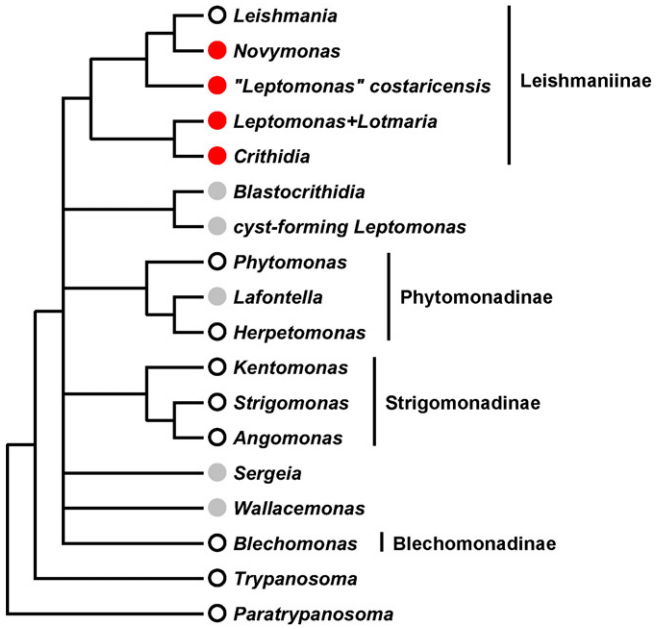

Fig. 2. Schematic phylogenetic tree of trypanosomatids showing the distribution of the catalase within this group (summarized from (Gerasimov et al., 2012; Votýpka et al., 2014; Yurchenko et al., 2016; Kostygov et al., 2016)). Red circles — catalase present, open circles — catalase absent; grey circles — not assessed.

many genes by phage-mediated HGT from other microorganisms (Håfström et al., 2011). The results of our phylogenetic analysis suggest that the catalase of *Brachyspira* is not related to the enzyme present in other spirochaetes (which were not even found within the first 1000 BlastP hits). Therefore, we conclude that both Leishmaniinae and *Brachyspira* spp. obtained this gene from a so far unidentified prokaryote. The enzyme appears to be so "popular" that it is being very frequently transferred between different unrelated lineages of microorganisms (Fig. 1).

With all the available sequence information and considering the promiscuous character of the catalase gene, it is clearly not a good phylogenetic marker as was proposed earlier (Wertlieb and Guttman, 1963). Moreover, the topology of the tree reconstructed using its sequences differs from the previously published ones (Kostygov et al., 2014; Kostygov et al., 2016; Votýpka et al., 2014; Schwarz et al., 2015). This incongruence may be related to the fact that some of the species are thermotolerant (namely *Crithidia luciliae thermophila* and *Leptomonas seymouri*) (Roitman et al., 1977; Kraeva et al., 2015) and that as a consequence of this feature, their catalase may be subject to faster adaptive evolution, resulting in phylogenetic reconstruction artefacts.

In trypanosomatids, there are several additional enzymes responsible for H_2O_2 utilization. Some of them are nearly as efficient as catalases (catalytic rate ~10^7/s), as exemplified by the tryparedoxin peroxidase first characterized from *Trypanosoma cruzi*. Later this enzyme was identified in other Trypanosomatidae, including Leishmaniinae, but its turnover number in these flagellates is lower by 1 to 2 orders of magnitude (Piñeyro et al., 2011; Machado-Silva et al., 2016). Thus, catalase in Leishmaniinae seems to be the most powerful enzyme responsible for H_2O_2 decomposition. We propose that it was indispensable for the monoxenous ancestor of this group but has been subsequently lost when the life cycle became dixenous.

3. Why is catalase incompatible with dixenous life cycle of *Leishmania*?

The well-known life cycle of *Leishmania* spp. includes two hosts: female sandflies (of the genera *Phlebotomus* and *Lutzomyia*) and vertebrates (mammals or reptiles). In the vector, the parasites colonize intestinal tract and develop from procyclic promastigotes into highly infective metacyclic promastigotes. The latter are transmitted to vertebrates, in which they multiply in the phagolysosomes of macrophages (da Silva and Sacks, 1987; Banuls et al., 2007).

Insects are generally resistant to microorganisms (Hamilton et al., 2015; Sant'Anna et al., 2012). Production of ROS is one of their innate immunity defense mechanism that likely evolved in order to regulate homeostasis of their gut microbes (Pal et al., 2010). In contrast to bacterial pathogens, *Leishmania* infection does not result in elevated ROS production by *Lutzomyia* sandflies, thus allowing the parasites to develop (Diaz-Albiter et al., 2012). Absence of the ROS response to *Leishmania* infection in the insect hosts indicates that catalase is not required at this stage of the parasite's life cycle. Moreover, high activity of such an efficient ROS scavenger like catalase could lead to a disturbance of the gut microbial homeostasis and uncontrolled proliferation of both pathogenic and commensal microbiota. Similar disturbance of the insect gut's homeostasis was demonstrated following the treatment of sand flies with an exogenous antioxidant uric acid (Diaz-Albiter et al., 2012). Being sensitive to a prolonged exposure of H_2O_2, *Leishmania* flagellates react by upregulating expression of their peroxiredoxins (Barr and Gedamu, 2003). Indeed, transcription of these genes is significantly higher in procyclic promastigotes compared to the lesion-derived or axenically-differentiated amastigotes (Holzer et al., 2006).

In mammalian host, *Leishmania* resides within macrophages and must be able to resist their antimicrobial defense. Upon phagocytosis of the parasite, macrophages produce ROS (e.g. superoxide anion O_2^-, H_2O_2, hydroxyl anion OH^-) and reactive nitrogen species (RNS) including nitric oxide (NO). To bypass the deleterious effect of free radicals, *Leishmania* is equipped with antioxidant enzymes such as SOD, peroxiredoxins, and heme-containing ascorbate peroxidase (APX) (Van Assche et al., 2011). Although facing such high levels of free radicals, *Leishmania* spp. lack the most effective and powerful enzyme in decomposing H_2O_2 — the catalase.

Recent studies shed some light on why such a potent enzyme, which is clearly advantageous in the ROS/RNS-rich environment, has been lost by *Leishmania*. Growth of *Leishmania amazonensis* on iron-depleted medium mimics the conditions within phagolysosomes. Iron deficiency induces expression of the ferrous *Leishmania* iron transporter 1 (LIT1), which is followed by increase of the iron content and Fe-SOD activity, accumulation of H_2O_2, growth arrest, and the onset of differentiation from promastigotes into amastigotes (Mittra et al., 2013). Similarly, treating the log-phase wild-type *Leishmania* promastigotes with either superoxide-generating drug menadione or H_2O_2 also resulted in the promastigotes to amastigotes differentiation. Correspondingly, LIT1 ablation led to the accumulation of superoxide radicals because of the low level of Fe-SOD activity, and appearance of the rounded amastigotes after exposure to H_2O_2 but not to menadione (Mittra et al., 2013). These data point to the direct role H_2O_2 may play as a signaling molecule in regulating *Leishmania* differentiation. Hyperpolarization of the parasite's mitochondrion, a concomitant increase in superoxide radical production, and an enhanced level of SOD activity also take place in response to the ROS surge, following the exposure of promastigotes to elevated temperature and low pH (Alzate et al., 2007), as in the "classical" in vitro axenic amastigote differentiation protocol (Bates, 1994). Importantly, a signaling role of H_2O_2 in differentiation and virulence has been recently demonstrated for another trypanosomatid, *Trypanosoma cruzi* (Nogueira et al., 2011; Goes et al., 2016).

Accumulation of intermediate O_2^- or higher level of H_2O_2 in promastigotes was shown to increase the virulence of *Leishmania*. When compared with the wild type, the null APX mutant of *Leishmania major* induced more severe infection in mice, while APX-overexpressing cells were not capable of inducing lesions development (Pal et al., 2010). Moreover, overexpression of SOD (which catalyzes dismutation of the superoxide radical O_2^- into H_2O_2) in promastigotes increases their ability to infect macrophages in vitro (Plewes et al., 2003).

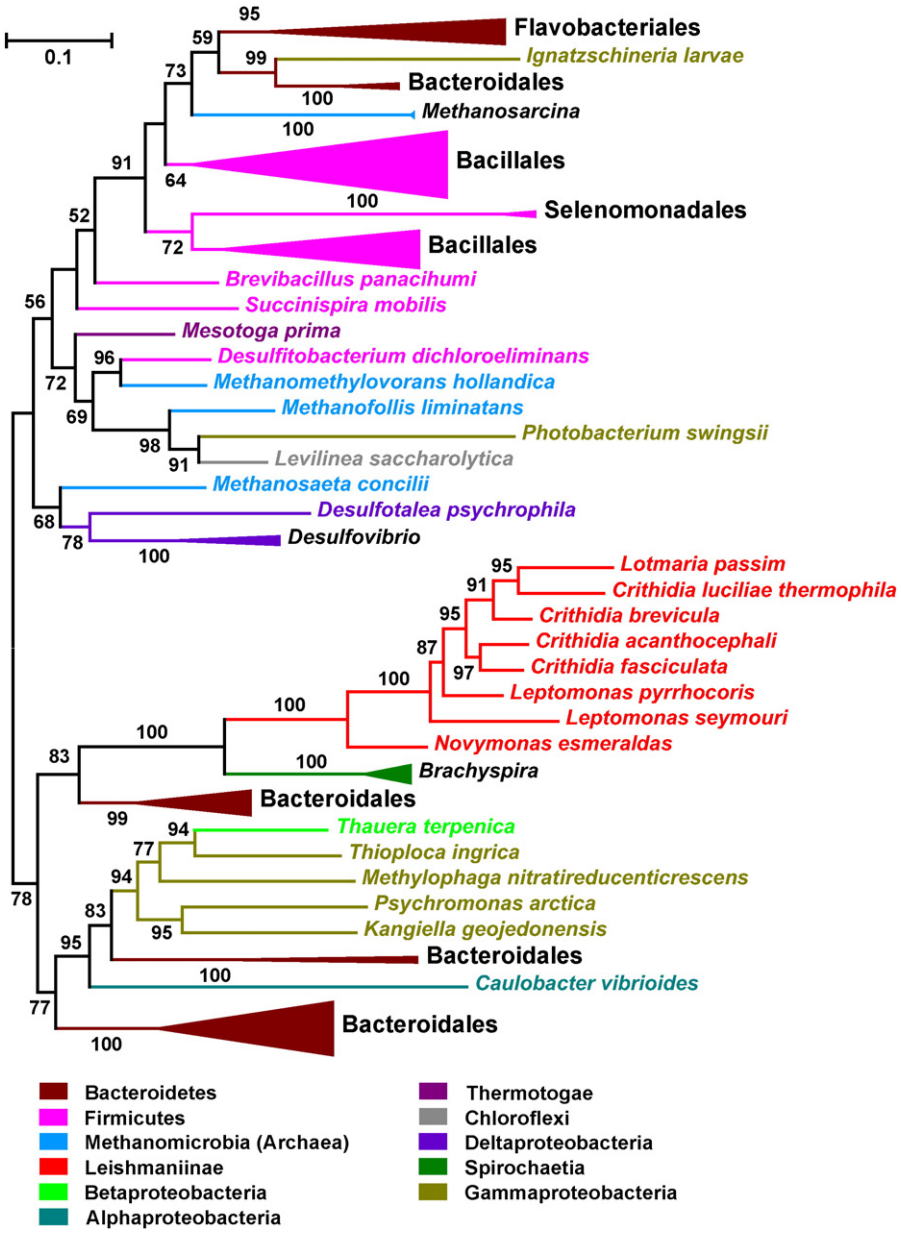

Fig. 3. Unrooted phylogenetic tree demonstrating the origin of catalase in trypanosomatids. The sequences of the catalase gene of Leishmaniinae were retrieved from publicly available genomic data and combined with those obtained in frame of our study. First 100 BlastP hits obtained with the catalase of *Novymonas esmeraldas* as a query were added. The sequences were aligned using MAFFT (Katoh and Standley, 2013), then non-conserved and ambiguously aligned positions were removed with the use of Gblocks (Castresana, 2000). Phylogeny was inferred using maximum likelihood method in IQ-TREE (Nguyen et al., 2015) under LG + I + G model as selected by the program. The statistical support (numbers at branches) was assessed using ultrafast bootstrap method for 1000 bootstrap replicates (Minh et al., 2013).

An interesting situation is observed in the gut of the hematophagous vectors, where trypanosomatids face another type of harsh environment rich in H_2O_2 (Graca-Souza et al., 2006). Expression analysis has shown that transcription of the tse-tse fly catalase gene is up-regulated in the posterior midgut, where digestion of the huge blood meal occurs. At the same time, in the engorged fly the antioxidant gene expression was shown to promote the establishment of trypanosome infection (Munks et al., 2005). Interestingly, the monoxenous *C. fasciculata*, which encodes its own catalase, proliferated in the tse-tse fly upon injection into its haemocoel, whereas the dixenous catalase-lacking *Leishmania* and *Trypanosoma* spp. failed to survive in the same environment (Ibrahim and Molyneux, 1987). Moreover, unlike absolute majority of examined dipteran insects, the tse-tse flies were never found to host monoxenous catalase-carrying trypanosomatids in the wild (Týč et al., 2013). We propose that it is the activity of catalase, which allows *C. fasciculata* to multiply and prosper in the tse-tse fly, eventually killing it. However, in the case of *Trypanosoma* spp., proliferation that would cause the death of the insect host would clearly be incompatible with its dixenous life style. The same likely applies to *Leishmania* spp. and their insect vectors. In such a case, the loss of catalase from the genome of some flagellates would be the key and necessary step preceding the emergence of their eventually highly successful dixenous life style.

4. Conclusions

We propose that the presence of catalase in trypanosomatids is incompatible with a dixenous life cycle. Such an enzyme would prevent

sensing minute changes in H_2O_2 concentrations which serve as a differentiation signal in *Leishmania*. It was indispensable for the monoxenous ancestor of Leishmaniinae but has been subsequently lost when the life cycle became dixenous.

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