Molecular & Biochemical Parasitology 232 (2019) 111199

Contents lists available at ScienceDirect

Molecular & Biochemical Parasitology

journal homepage: www.elsevier.com/locate/molbiopara

An enigmatic catalase of *Blastocrithidia*

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ARTICLE INFO

Keywords:
Oxygen peroxide
Catalase
Trypanosomatidae

ABSTRACT

Here we report that trypanosomatid flagellates of the genus *Blastocrithidia* possess catalase. This enzyme is not phylogenetically related to the previously characterized catalases in other monoxenous trypanosomatids, suggesting that their genes have been acquired independently. Surprisingly, *Blastocrithidia* catalase is less enzymatically active, compared to its counterpart from *Leptomonas pyrrhocoris*, posing an intriguing biological question why this gene has been retained in the evolution of trypanosomatids.

Catalase (EC 1.11.1.6) is a ubiquitous enzyme, usually involved in oxidative stress protection. It contains a heme cofactor in its active site and converts hydrogen peroxide (H_2O_2) to water and oxygen [1]. The hydrogen peroxide is typically produced in the mitochondria by a superoxide dismutase using extremely harmful anion superoxide as a substrate [2]. H_2O_2 is a small molecule that can penetrate the cell membrane, often playing a role of a secondary messenger in many biochemical reactions [3]. However, in the presence of iron, H_2O_2 can be converted to a highly reactive and toxic hydroxyl radical by the Fenton reaction.

Most species use catalase to control intracellular level of hydrogen peroxide. Nevertheless, several prominent examples of organisms, lacking this enzyme, have been recently reported. It is presumed that these species rely on other biochemical pathways to detoxify intracellular H_2O_2 (reviewed in [4]).

Kinetoplastid flagellates of the family Trypanosomatidae [5] is one of such peculiar examples. These parasites infect either exclusively invertebrates (monoxenous species) or invertebrates and vertebrates or plants (dixenous species) [6]. Most of the analyzed trypanosomatids do not possess catalase-encoding gene in their genomes. The only known exception to this rule so far was a group of monoxenous relatives of *Leishmania* (representatives of the insect-infecting genera *Crithidia*, *Leptomonas*, and *Novymonas*) [7]. Conspicuously, dixenous *Leishmania* have secondarily lost catalase. It has been proposed that hydrogen peroxide plays a role in promastigote-to-amastigote differentiation of these parasites [8]. Thus, presence of a catalase appears to be incompatible with dixenous life cycle of *Leishmania* [4].

One of the trypanosomatid groups insufficiently investigated in this regard is the genus *Blastocrithidia*. These parasites recently came into prominence because of their confounding genetic code with all three stop codons reassigned to encode amino acids. One of these codons (UAA) also serves the genuine translation terminator [9].

Genomic analysis of two species belonging to this genus (*Blastocrithidia* sp. p57 and *B. triatomae*) reveals that both possess a catalase gene (GenBank accession numbers MK934828 and MK934829, respectively). A phylogenetically-related flagellate with standard genetic code of the so-called "*jaculum*" group [7] (hereafter called Trypanosomatidae sp. Fi-14) also encodes this enzyme in its genome (GenBank accession number MK934827). The sequences of trypanosomatid catalases display high level of conservation (Fig. 1). All amino acids involved in the heme binding (marked by black squares in Fig. 1) are invariant in all trypanosomatid species analyzed, while sequence motifs of the NADPH binding site (green boxes in Fig. 1) and tetramer interface (blue boxes in Fig. 1) differ between Leishmaniinae (*Crithidia* and *Leptomonas* spp.) and members of the *Blastocrithidia* / "*jaculum*" clade. The catalase sequences of *Blastocrithidia* spp. (but not Trypanosomatidae sp. Fi-14) contain amino acid-coding in-frame stop codons UAG (Glu), UAA (Glu), and UGA (Trp) (Fig. 1). NCBI-CDD analysis

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https://doi.org/10.1016/j.molbiopara.2019.111199
Received 24 May 2019; Received in revised form 1 July 2019; Accepted 1 July 2019
Available online 02 July 2019
0166-6851/

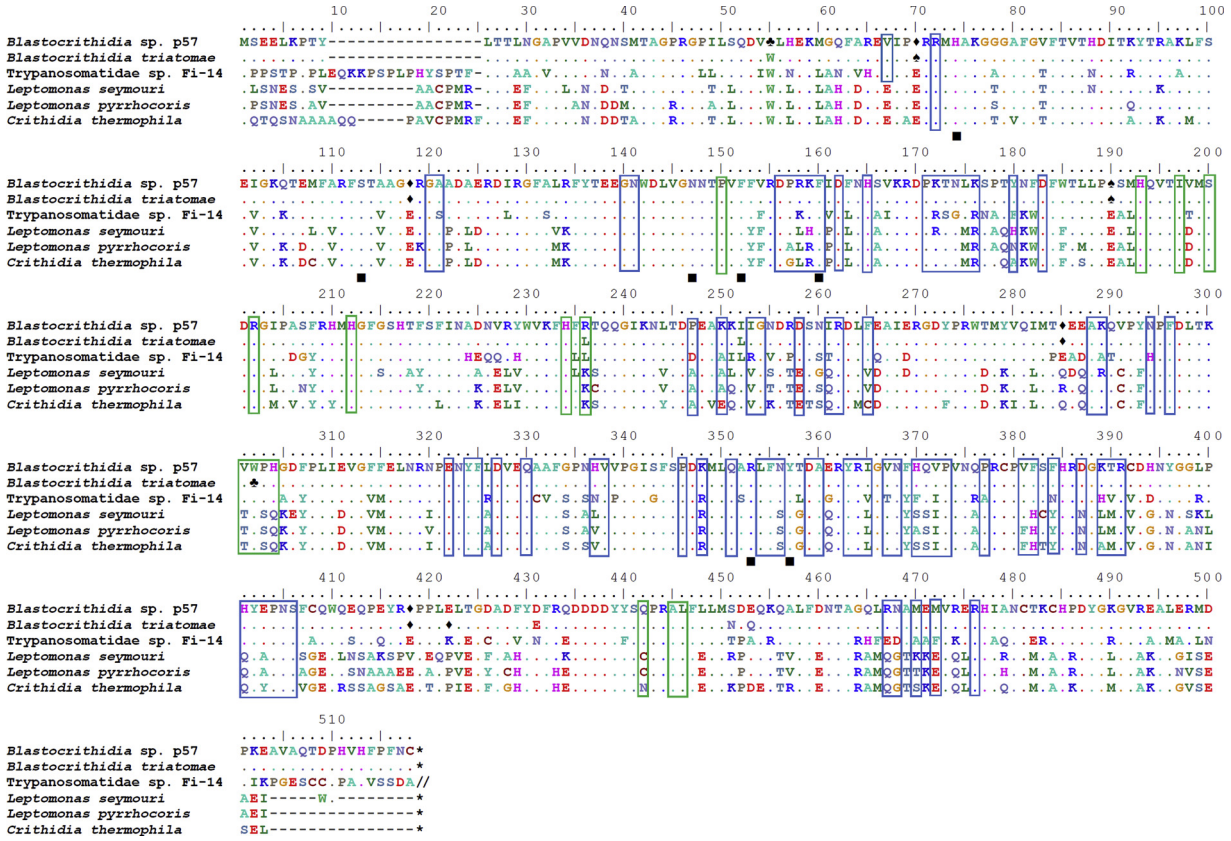

Fig. 1. Alignment of amino acid sequences of trypanosomatid catalases. Predicted functional domains and sites: ■ –heme binding site, green box –NADPH binding site, blue box –tetramer interface. Two slashes mark the trimmed sequence. Amino acids coded by in-frame stop codons in *Blastocrithidia* spp.: ♦ UAG (Glu), ♠ UAA (Glu), ♣ UGA (Trp). The catalase sequences of different trypanosomatids (*Blastocrithidia* sp. p57, *Blastocrithidia triatomae*, *Crithidia thermophila*, *Leptomonas pyrrhocoris*, *L. seymouri*, Trypanosomatidae sp. Fi-14) were aligned with MAFFT (v. 7) using G-INS-i iterative refinement method. NCBI-CDD was used to search for the conserved domains and sites.

suggests that trypanosomatid catalases belong to the catalase clade 3. Clade 3 catalases are found in all three kingdoms of life. Their tetramer-forming subunits are relatively small (43–75 kDa) and have been shown to bind a protoheme IX (heme b) and NADPH as a second redox-active cofactor [10].

Next, we investigated phylogenetic relationships of *Blastocrithidia* spp. and Trypanosomatidae sp. Fi-14 catalase sequences. Blast search and subsequent phylogenetic analyses revealed that all trypanosomatid catalases have bacterial origin (Fig. 2). To our surprise, enzymes of the Leishmaniinae (magenta in Fig. 2A) and those of the *Blastocrithidia* / "*jaculum*" clade (red in Fig. 2A) are not phylogenetically related and originated from different groups of bacteria. While *Brachyspira*-related spirochetes have likely donated the catalase gene to Leishmaniinae, the closest known relative of its homolog in *Blastocrithidia* and Trypanosomatidae sp. Fi-14 encodes the enzyme of the gut symbiotic β-proteobacterium infecting honey bees and bumble bees, *Snodgrassella alvi* (Fig. 2B,C). Co-inhabiting the same environmental niche (insect's gut) may greatly facilitate gene transfer between bacteria and flagellates.

To investigate biochemical properties of trypanosomatid catalases, we employed a high-resolution respirometry technique to measure the amount of molecular oxygen produced upon addition of H_2O_2. A catalase is the only cellular enzyme facilitating this biochemical reaction, so the level of the produced oxygen is a direct indicator of the catalase's activity *in vivo*. *Trypanosoma brucei* strain Lister 427 (a species lacking catalase) was used to estimate background level of oxygen in the experiment. We documented that *Leptomonas pyrrhocoris* H10 (representative of the Leishmaniinae clade) converts H_2O_2 into molecular oxygen significantly better than *Blastocrithidia* sp. p57 (c.a 9 and 2 fmol O_2/cell/min, respectively, Fig. 3A). This also correlates with ˜5-fold difference in levels of heme b (measured as in [11]) between these flagellates (Fig. 3A). Whether the lower peroxide-utilization enzymatic activity of catalase in *Blastocrithidia* can be explained by the scarcity of the available cofactor remains to be investigated further, but this appears to be a plausible explanation. An alternative hypothesis is that trypanosomatids of the *Blastocrithidia* / "*jaculum*" clade are overall less efficient is using hemoproteins (including catalase). The high frequency of "stop" codons in the sequence of *Blastocrithidia* spp. catalases, suggesting that these genes are translated with a lowered efficiency [9] and substantially reduced levels of both heme a and b, support this hypothesis.

Finally, we measured whether the lower catalase activity makes *Blastocrithidia* cells more sensitive to hydrogen peroxide in comparison to their Leishmaniinae counterparts. The ability of parasites to survive in different concentrations of H_2O_2 was determined by the fluorimetric-based assay employing utilization of resazurin (Alamar Blue). *Blastocrithidia* sp. p57 demonstrated lower sensitivity ($p < 0.0001$) to the hydrogen peroxide than L. *pyrrhocoris* with IC_{50} of 0.35 and 0.15 mM, respectively (Fig. 3B). We concluded that *Blastocrithidia* spp. mainly rely on other (catalase-independent) mechanisms of oxidative stress protection. While catalase might be one of the most efficient enzyme to reduce hydrogen peroxide [12], cells can also employ other biochemical pathways (for example, based on glutathione peroxidase and peroxiredoxin) to remove this harmful molecule [13].Genes encoding enzymes of these alternative pathways are present in the *Blastocrithidia* genome.

Our data document the presence of an enzymatically active catalase in *Blastocrithidia* / "*jaculum*" clade. This catalase is not phylogenetically related to its counterpart in Leishmaniinae and is less active. There are

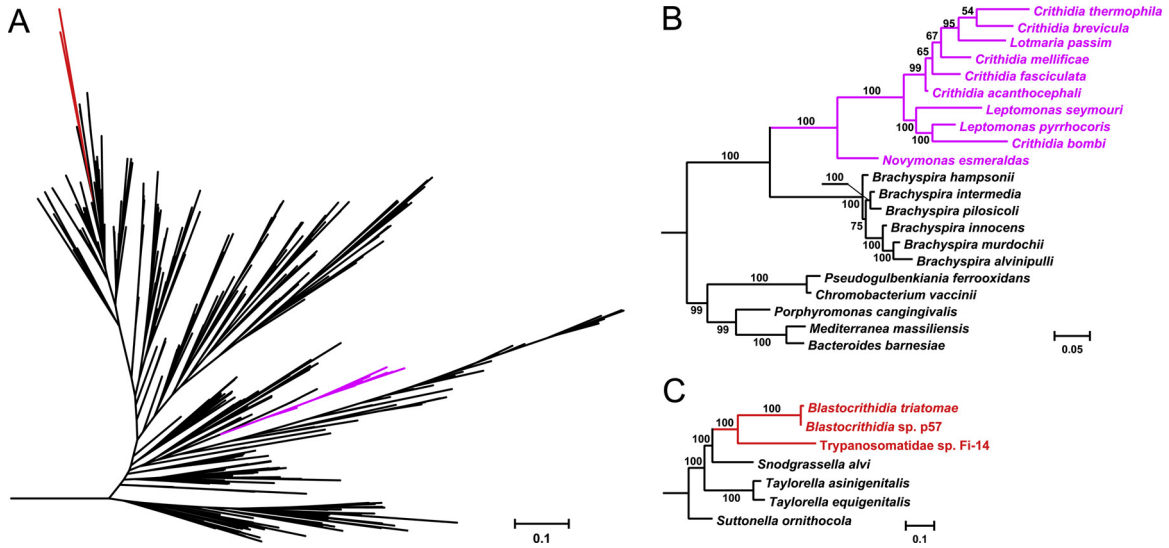

Fig. 2. Catalase phylogeny reconstructed in IQ-TREE based on amino acid alignment prepared in MAFFT and trimmed in trimAl. The dataset was obtained using a BlastP search with the catalase sequence of *Blastocrithidia* sp. p57 as a query against NCBI nr database. It was then purged from duplicates (multiple sequences for one species), closely related sequences for prokaryotic species of one genus, and sequences of unidentified organisms. Numbers at branches are ultrafast bootstrap supports; the scale bar corresponds to the number of the substitutions per site. A – a subtree containing catalases of trypanosomatids, demonstrating their evolutionary remoteness and independent origin of this enzyme in *Blastocrithidia* / "*jaculum*" and Leishmaniinae clades; B – a subtree with the catalases of Leishmaniinae; C – a subtree with the catalases of *Blastocrithidia* spp. and Trypanosomatidae sp. Fi-14.

two related evolutionary questions worth considering in this regard: i) why catalase has been acquired only in some trypanosomatid groups?, and ii) why has it been retained in *Blastocrithidia* / "*jaculum*" lineage, while it does not appear to be important for oxidative stress protection in these flagellates? The acquisition of such a robust biochemical system to combat harmful molecules [14] should be advantageous for the majority of these parasites. However, many trypanosomatid lineages do not have it, yet they survive in the same conditions using alternative detoxification pathways. Some of them can even loose catalase secondarily, as exemplified by *Leishmania* spp., which have disposed of this enzyme in evolution when it has become incompatible with their dixenous life cycle [4]. The key to answer this question may be the peculiarity of catalase as compared to other enzymes used for H_2O_2 decomposition: it is the only one producing O_2. And while Leishmaniinae are very diverse in terms of host adaptation, *Blastocrithidia* appear to be more uniform. We speculate that *Blastocrithidia* spp. have retained catalase to sense the gradient of hydrogen peroxide by the mean of the produced oxygen. H_2O_2 is produced by the cells of the insect intestinal wall to control microflora [15] and sensing it may be important for proper localization of trypanosomatids. In contrast to *L. pyrrhocoris*, which resides in the midgut lumen and never attaches to enterocytes [16], *Blastocrithidia* spp. tend to reach and anchor on the epithelium of the midgut wall and/or Malpighian tubules [17–19]. In these conditions, sensing peroxide molecules might help in orientation of the flagellates. Other potential explanations for the observed phenomenon can be proposed, and more work is needed to understand the biological role of catalase trypanosomatids.

A

	fmol O_2/cell/min	heme b (pmol/10^8 cells)	heme a (pmol/10^8 cells)
Leptomonas pyrrhocoris H10	8.97 ± 0.57	817.0 ± 61.9	94.6 ± 7.5
Blastocrithidia sp. p57	1.93 ± 0.55	164.8 ± 11.2	13.6 ± 1.4

B

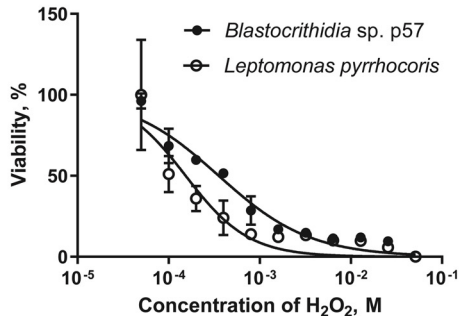

Fig. 3. Oxygen production upon addition of H_2O_2, levels of heme a and b (A) and cell survival after exposure to hydrogen peroxide (B). Numbers in A were measured as in [11]. For cell survival analysis, 10^6 parasites in 100 µl medium were exposed to a serial dilution of H_2O_2, starting from 51.6 mM. The plates were incubated for 1 h at room temperature, and then 10 µl of 5% resazurin solution was added to each well. After 47 h incubation at 23 °C, fluorescence (excitation wavelength 540 nm, emission wavelength 590 nm) was measured. Data from three independent biological replicates are presented in both panels.

Author contributions

VY conceived the study; VY and JL supervised the study; NK, EH and AYK designed experiments; CB, NK, KZ, EH, and RS performed experiments; CB, AYK, NK, EH, and RS analyzed data; CB and VY wrote the manuscript; JL, AYK, NK, and EH made manuscript revisions.

Acknowledgements

We thank members of our laboratories for stimulation discussions. This work was supported by the Grant Agency of Czech Republic (grant 17-10656S); Russian Science Foundation (grant 19-15-00054, bioinformatics analysis of *Blastocrithidia* catalase) to VY; European Research CouncilCZ LL1601 (JL); European Regional Funds (project "Centre for Research of Pathogenicity and Virulence of Parasites" CZ.02.1.01/0.0/0.0/16_019/0000759 to VY, JL, NK, AYK, and EH); and a grant from the University of Ostrava (SGS08/PrF/2019 to CB and VY). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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