Enzymatic Antioxidant Systems in Early Anaerobes: Theoretical Considerations

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Abstract

It is widely accepted that cyanobacteria-dependent oxygen that was released into Earth's atmosphere *ca.* 2.5 billion years ago sparked the evolution of the aerobic metabolism and the antioxidant system. In modern aerobes, enzymes such as superoxide dismutases (SODs), peroxiredoxins (PXs), and catalases (CATs) constitute the core of the enzymatic antioxidant system (EAS) directed against reactive oxygen species (ROS). In many anaerobic prokaryotes, the superoxide reductases (SORs) have been identified as the main force in counteracting ROS toxicity. We found that 93% of the analyzed strict anaerobes possess at least one antioxidant enzyme, and 50% have a functional EAS, that is, consisting of at least two antioxidant enzymes: one for superoxide anion radical detoxification and another for hydrogen peroxide decomposition. The results presented here suggest that the last universal common ancestor (LUCA) was not a strict anaerobe. O₂ could have been available for the first microorganisms before oxygenic photosynthesis evolved, however, from the intrinsic activity of EAS, not solely from abiotic sources. **Key Words:** Archaea—Atmospheric gases—Evolution—H₂O₂ resistance—Oxygenic photosynthesis. *Astrobiology* 16, 348–358.

1. Introduction

FOR ITS DUAL ROLE in biological systems, oxygen has rightly been called a double-edged molecular sword. It is necessary for aerobic metabolism, and its deficiency hinders normal growth and development. But at the same time the reduction of molecular oxygen in biological systems results in the formation of reactive oxygen species (ROS). The most common ROS include singlet oxygen (¹O₂), the superoxide anion radical (O₂^{•−}), hydrogen peroxide (H₂O₂), and the hydroxyl radical (HO[•]). Uncontrolled ROS production can result in oxidation of various vulnerable molecules, disturbance in cellular homeostasis, and ultimately cell death (Benzie, 2000; Ślesak *et al.*, 2007). However, at early stages of Earth evolution, before the appearance of life *ca.* 3.8–3.5 billion years ago (Holland, 2006; Miller and Cleaves, 2007), the atmosphere was anoxic; thus it is widely accepted that the first living cells were strict anaerobes (Halliwell and Gutteridge, 1999). With this in mind, a number of paradigms concerning antioxidants and antioxidant metabolism have been accepted, as follows: (1) aerobic metabolism may not have occurred until after oxygen release into Earth's atmosphere *ca.* 2.7–2.2 billion years ago by cyanobacterial oxygenic photosynthesis; (2) the antioxidant cellular machinery evolved at the same time as aerobic metabolism and oxygenic

photosynthesis (Halliwell and Gutteridge, 1999; Alberts *et al.*, 2002; De Las Rivas *et al.*, 2004; Shaw, 2008). However, recently several lines of evidence suggest that the appearance of oxygenic photosynthesis preceded the Great Oxidation Event of Earth's atmosphere, and as a consequence, oxygen oases, possibly even with micromolar O₂ concentrations, were present in the Archean ocean (Olson *et al.*, 2013; Kaufman, 2014).

Aerobes are equipped with a sophisticated antioxidant defense system that involves ROS detoxification. It constitutes low molecular antioxidants (*i.e.*, glutathione, ascorbate, tocopherols, etc.) and antioxidant enzymes that belong to the enzymatic antioxidant system (EAS), which regulates the cellular ROS homeostasis (Benzie, 2000; Ślesak *et al.*, 2007; Foyer and Noctor, 2009; Schippers *et al.*, 2012; Karpiński *et al.*, 2013). The primary antioxidant enzyme that converts O₂^{•−} to H₂O₂ and O₂ is the superoxide dismutase (SOD; EC 1.15.1.1):

Four main classes of SOD, as indicated by metal ions present at the active site, have been identified: copper/zinc (Cu/ZnSOD), iron (FeSOD), manganese (MnSOD), and

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nickel (NiSOD) (Alscher *et al.*, 2002; Miller, 2012). The MnSOD and FeSOD are very similar in their primary and tertiary structures, whereas Cu/ZnSOD features structural differences (Fink and Scandalios, 2002; Wolfe-Simon *et al.*, 2005).

Another key antioxidant enzyme involved in H_2O_2 scavenging is catalase (CAT; EC 1.11.1.6), which decomposes H_2O_2 to water and oxygen:

There are three classes of CATs: the first contains heme (typical monofunctional CATs, KatEs) in its active site; the second, heme-containing CATs with catalase-peroxidase activity (KatGs); and the non-heme CAT with Mn at the active site (MnCAT) (Zámocký *et al.*, 2012). Under certain conditions (low pH, low H_2O_2 concentration), KatGs may decompose H_2O_2 via peroxidation (Singh *et al.*, 2008; Ndontsa *et al.*, 2012; Eq. 3). Recently, peroxiredoxins (PXs) were recognized for their antioxidant properties. PXs are peroxidases with low substrate specificity, which are capable of reducing diverse peroxides, such as H_2O_2 , alkyl hydrogen peroxides, and peroxynitrite, to water and the corresponding alcohol, water, and nitrite, respectively. The general scheme of reactions catalyzed by PX with H_2O_2 as a substrate is

where AH_2 and A denote the reduced and oxidized acceptor, respectively. PXs are present in organisms from all three domains of life: Archaea, Bacteria, and Eukarya. They contain a conserved cysteine residue in the N-terminal region that is the primary site of oxidation by H_2O_2 (Horling *et al.*, 2002; Rhee *et al.*, 2005; Dietz, 2011). It was found that detoxification of $\text{O}_2^{\cdot -}$, unlike in the case of SOD, occurs via its reduction, a reaction catalyzed by a small non-heme Fe-containing enzyme superoxide reductase (SOR), where H_2O_2 is the only product (Nivière and Fontecave, 2004):

The presence of SOR has been mainly limited to strict anaerobes and microaerophiles that belong to Bacteria and Archaea (Imlay, 2002; Lucchetti-Miganeh *et al.*, 2011).

The growing number of gene and protein sequences from organisms that belong to the three domains of life (Woese *et al.*, 1990) facilitate the reconstruction of many cellular processes of the hypothetical last universal common ancestor (LUCA) of all modern organisms. The presence of SODs, the Fe and Mn forms in particular, CATs, and PXs in organisms from all three domains of life (Kornáš *et al.*, 2010) and ROS-removing reactions in strict anaerobes have been reported previously (Ślesak *et al.*, 2012). Thus, we made the simplified assumption that every enzyme that has archaeal, bacterial, and eukaryotic homologues was most likely present in LUCA. This postulation is allowed in the context of a general methodology used for the reconstruction of the LUCA genome (Ouzounis and Kyrpides, 1996; Mushegian, 2008). If present-day (modern) anaerobes have evolved as descendants of ancient forms, the following

questions need to be raised: (1) Could LUCA be an O_2 /ROS-tolerant organism possessing any primordial EAS? (2) What were the potential abiotic sources of ROS and oxygen on young Earth? Our results constitute additional biological support for the unorthodox hypothesis that EAS or a rudimentary equivalent may have been present in primordial organisms on early Earth, even before the appearance of oxygenic photosynthesis. Moreover, the theoretical considerations presented here paradoxically indicate that ROS-scavenging reactions could themselves be an intracellular net source of O_2 /ROS inside hypothetical LUCA protocells. Additionally, we hypothesize that the occurrence of iron ion binding sites at the active site of most analyzed antioxidant enzymes is an additional piece of evidence that suggests the incorporation of iron from the ancient oceans into key antioxidant enzymes, before oxygen accumulation in the atmosphere, presumably due to oxygenic photosynthesis.

2. Materials and Methods

2.1. The selection of obligate anaerobes

To verify oxygen tolerance of various microorganisms, a query was performed of the literature (*e.g.*, Vieira-Silva and Rocha, 2008) and available online resources, for example, the Genome List (www.ncbi.nlm.nih.gov/genomes/lproks.cgi), the European Bioinformatics Institute (www.ebi.ac.uk), and the DOE Joint Genome Institute (www.jgi.doe.gov).

2.2. Searching for components of EAS

Data indicating the presence of antioxidant enzymes, such as SOD, SOR, PX, and CAT in obligate anaerobic bacteria and archaea, were acquired from publicly available databases: STITCH 1.0 and later versions (Search Tool for Interactions of Chemicals; available at <http://stitch.embl.de>) and BioCyc (www.biocyc.org) (Caspi *et al.*, 2008; Kuhn *et al.*, 2008). Only complete genome representations were included in the present study. The completeness of each genome was verified by information from the following Web sites: www.ncbi.nlm.nih.gov/genome/browse/# and <http://www.ebi.ac.uk>, where full genome representation was shown at the complete genome (chromosomes and plasmids) assembly level. For *Pyrococcus abyssi*, full genome representation was presented at the chromosome level.

CAT denotes sequences identified as monofunctional (KatE), bifunctional (KatG), and Mn-containing catalases (MnCAT). The enzymes' amino acid sequences and their accession numbers were obtained from GenBank (www.ncbi.nlm.nih.gov; Supplementary Table S2; Supplementary Data are available online at www.liebertonline.com/ast). We also made an arbitrary typology of enzymatic antioxidant systems (EASs). Typical "minimal" functional EASs should consist of at least SOD and/or SOR, converting $\text{O}_2^{\cdot -}$ to H_2O_2 , and enzymes, such as PX and/or CAT that decompose H_2O_2 to water and other compounds. For more clarity, we assumed that the majority of CATs decompose H_2O_2 to water and O_2 (Eq. 2), and the peroxidase-type reaction (Eq. 3) is negligible. In fact, H_2O_2 degradation, according to Eq. 2, is dominant in biological systems, but as was mentioned above, it depends *inter alia* on pH and H_2O_2 concentration. The functionality of selected combinations of EASs is based on the fact that reactive $\text{O}_2^{\cdot -}$ is reduced to the

less reactive H_2O_2 . Subsequently, H_2O_2 must be decomposed to neutral or less harmful compounds, that is, H_2O and O_2 , because it may react with $\text{O}_2^{\cdot-}$ and produce extremely reactive and dangerous ROS, that is, hydroxyl radical ($\text{HO}^\bullet$) (Ślesak *et al.*, 2007). Moreover, the deleterious effects of H_2O_2 ($\text{HO}^\bullet$ generation) depend on the presence of transition metals such as iron and copper in the environment (Halliwell and Gutteridge, 1999; Imlay, 2008a). In general, the functional EAS proposed above is typical for all aerobes (Benzie, 2000; Ślesak *et al.*, 2007; Schippers *et al.*, 2012). The lack of enzymes that remove $\text{O}_2^{\cdot-}$ (SOD, SOR) or H_2O_2 (PX, CAT) in an anaerobe was defined as “nonfunctional” EAS.

We selected eight functional EAS configurations: (1) SOD+SOR+PX+CAT, (2) SOD+PX+CAT, (3) SOD+PX, (4) SOD+CAT, (5) SOD+SOR+PX, (6) SOR+PX+CAT, (7) SOR+PX, (8) SOR+CAT. The nonfunctional EAS consists of (1) SOD, (2) SOR, (3) SOD+SOR, (4) PX, (5) PX+CAT, (6) CAT. Previous reports indicate that all antioxidant genes/enzymes in anaerobes are transcribed/active at about the same time, and the lack of compartmentalization of bacterial cells ensures that generation/detoxification of ROS occurs in the same intracellular area (*e.g.*, Brioukhanov *et al.*, 2006; Dolla *et al.*, 2006; Imlay, 2008b). Moreover, the analyzed antioxidant enzymes more or less differ in kinetic parameters, for example, the turnover number (k_{cat} [s^{-1}]), but it does not affect the stoichiometry and type of the net products (www.brenda-enzymes.org; Pratt and Cornely, 2014).

This allowed us to determine the number of obligate anaerobes that possess at least one of the above-mentioned enzymes and which of them have a functional antioxidant system.

2.3. Prediction of an ion metal at the active site of antioxidant enzymes

The SODa Web tool (<http://babylone.ulb.ac.be/SODa>) (Kwasigroch *et al.*, 2008) was used for prediction of a specific metal cofactor in the active site of the analyzed SODs. The prediction was shown as a score expressed in percent (Supplementary Table S3). The predictions for the occurrence of a metal in the active site of CATs were based on literature and GenBank (www.ncbi.nlm.nih.gov).

2.4. Determination of protein homology

Homology was inferred using standard protein BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) analysis. All sequences (of the same type of enzymes) were aligned with each other. The homology was estimated based on several parameters, describing the similarity of analyzed sequences: sequence amino acid identity (%) and expectation value (*E*-value) (Table 1). If pairwise sequence identity, for sequences longer than 100 amino acid residues, were higher than 20% ($>20\%$), and *E*-values were $\leq 10^{-5}$ ($E \leq 1\text{e-}05$), the sequences could be interpreted as homologues (Xiong, 2006; Pearson, 2013).

2.5. Phylogenetic analysis

The molecular phylogenetic analysis was based on 16S rRNA sequences of anaerobes that contain at least SOD or SOR (Supplementary Table S2) and on 16 rRNA sequences

TABLE 1. PARAMETERS ESTIMATED BY PROTEIN BLAST SIMILARITY SEARCH (SEE ALSO MATERIALS AND METHODS)

Enzyme	Parameters		
	Range of amino acid sequence length	Range of amino acid identity (%)	Expectation value (<i>E</i> -value)
SOD	188–239	23–100	$E \leq 2\text{e-}08$
SOR	109–161	26–100	$E \leq 4\text{e-}06$
PX	158–235	22–100	$E \leq 4\text{e-}05^a$
KatE	478–801	38–100	$E \leq 1\text{e-}102$
KatG	712–741	52–87	$E = 0.000$
MnCAT ^b	190–228	38	$E = 1\text{e-}34$

Accession numbers below are listed according to www.ncbi.nlm.nih.gov.

^aFor aligned sequences: NP_349898, length = 164 amino acids (*C. acetobutylicum* ATCC 824) vs. WP_012882691, length = 213 (*D. mccartyi* VS): identity = 22%, $E = 0.003$ ($3\text{e-}03$).

^bTwo sequences were compared: AAM24993 (*T. tengcongensis* MB4) vs. YP_429873 (*M. thermoacetica* ATCC 39073).

of anaerobes that lack an EAS (Supplementary Table S1). All sequences were derived from SILVA (<http://www.arb-silva.de>), StrainInfo (<http://www.straininfo.net>), and GenBank (www.ncbi.nlm.nih.gov). The sequences were aligned with the default parameters of ClustalW implemented in MEGA6 (Tamura *et al.*, 2013). The evolutionary history was inferred by using the maximum likelihood method based on Kimura's 2-parameter model (Kimura, 1980). The bootstrap consensus tree was inferred from 100 replicates (Felsenstein, 1985). Initial tree(s) for the heuristic search were obtained by applying the neighbor-joining method to a matrix of pairwise distances estimated with the maximum composite likelihood approach. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.5461)). The rate variation model allowed for some sites to be evolutionary invariable ([+I], ~22% sites). The analysis involved 89 nucleotide sequences. All positions that contained gaps and were missing data were eliminated. There were a total of 479 positions in the final data set. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.*, 2013).

3. Results

3.1. EAS is quite common in strict anaerobes and determination of protein homology

We selected 100 obligate anaerobes, representative of the domains Archaea and Bacteria, and at least one antioxidant enzyme was found in 93% of species (Supplementary Table S2, Fig. 1a). According to the proposed typology (see Materials and Methods), both nonfunctional (43%) and functional (50%) EAS was present in species, with various configurations of enzymes detoxifying $\text{O}_2^{\cdot-}$ (SOD and/or SOR) and enzymes responsible for H_2O_2 elimination (PX and/or CAT) (Fig. 1b). In *Methanosarcina mazei* two sequences for heme CAT (KatG) were found, but in *Desulfotobacterium hafniense* two different types of CATs—KatE and KatG—were noted (Supplementary Table S2). In *Clostridium beijerinckii* and *Clostridium*

FIG. 1. Enzymatic antioxidant system (EAS) in analyzed obligate anaerobes. **(a)** The percent of organisms encoding an EAS. **(b)** The percent of organisms encoding functional and nonfunctional EAS.

novyi two SODs were identified (Supplementary Table S2). It is worth pointing out that a single antioxidant—SOD or SOR—was present in a large number of the anaerobes examined. In the case of Archaea, SORs were the predominant superoxide anion radical scavengers (Supplementary Table S2).

The existence of genes encoding related proteins does not provide final proof as to whether the EAS is really active. However, it gives strong support for *bona fide* EAS occurrence in an organism. In fact, the majority of available protein sequences have not been experimentally characterized; most sequences are annotated by computational analysis (Schnoes *et al.*, 2009). We hypothesized that every enzyme that has archaeal, bacterial, and eukaryotic homologues was likely present in LUCA. To test this hypothesis, it was necessary to estimate whether the analyzed sequences are homologues. Sequence homology allows one to conclude that there is a common ancestral relationship for all compared sequences drawn from comparisons when two sequences share a high-enough degree of similarity (Xiong, 2006). Therefore, annotations of all selected proteins were verified by the UniProt database (www.uniprot.org). The results indicate that the majority of the analyzed proteins: SODs, PXs, and various types of CATs were inferred from homology analysis. A small

number was additionally verified experimentally (see www.uniprot.org). Moreover, BLAST analysis indicated that SOD, SOR, PX, KatE, KatG, and MnCAT sequences showed that similarity indicators—sequence identity level (22–100%) in relation to the protein sequence length and *E*-values ($E \leq 4e-05$) (Table 1)—were in the range that allows one to conclude that compared sequences are homologues (Xiong, 2006; Pearson, 2013). Two PXs, however, were exceptions to this: (1) from *C. acetobutylicum* (NP_349898), predicted as atypical 2-Cys peroxiredoxin, and (2) from *D. mccartyi* (WP_012882691), described as 1-Cys peroxiredoxin (www.ncbi.nlm.nih.gov). After BLAST alignment, a 22% value of identity between these sequences and $E = 0.003$ (Table 1) was near (% of identity) or below (*E*-value) the threshold values accepted as indicators of sequence homology (see Materials and Methods). Such results, mainly *E*-value in the range of 10^{-1} to 10^{-5} , may indicate that the compared sequences are distant homologues (Xiong, 2006). It has been shown, however, with active site structure and amino acid residue analysis, that these PXs belong to one family of peroxiredoxins (Nelson *et al.*, 2011). In other words, all the studied sequences share homology; thus it can be assumed that it is most likely the ancestral form of the analyzed antioxidant enzyme that was present in LUCA.

3.2. The functional and nonfunctional EASs are net producers of O₂/ROS

The following reactions describe the conversion of ROS in hypothetical EAS.

3.2.1. Functional EASs

(1) SOD + SOR + PX + CAT:

where AH₂ and A denote a reduced and an oxidized acceptor, respectively.

(2) SOD + PX + CAT:

(3) SOD + PX:

(4) SOD + CAT:

(5) SOD + SOR + PX:

(6) SOR + PX + CAT:

(7) SOR + PX:

(8) SOR + CAT:

3.2.2. The nonfunctional EASs

(1) SOD:

(2) SOR:

(3) SOD + SOR:

(4) PX:

(5) PX + CAT:

(6) CAT:

As a result, we were able to identify the net products of all the reactions. O₂/ROS are marked in bold. No net O₂/ROS products were found for the functional combination of two enzymes SOR and PX (Eq. 11) and the PX-containing variant for a nonfunctional system (Eq. 16). EAS: SOR + PX and the single PX were present in only 9% of the analyzed anaerobes (Fig. 1b). In fact, the results showed the net generation of O₂ (Eqs. 5, 6, 7, 8, 9, 10, 12, 17, 18), O₂ and H₂O₂ (Eqs. 13, 15), or H₂O₂ only (Eq. 14) in both functional and nonfunctional EAS combinations, which were identified in analyzed anaerobes. It should be emphasized that, in most EASs, O₂ was the only net product, and O₂^{·-} and H₂O₂, as unstable and highly reactive intermediates, were not the net

final products in most cases. These results suggest that the activity of antioxidant enzymes, annotated for each anaerobe (Supplementary Table S2), could be an intracellular source of molecular oxygen.

3.3. Molecular phylogenetic analysis

To simplify a test with regard to the hypothesis concerning the loss of EAS (functional or nonfunctional), we selected anaerobes that contained at least one of the most commonly annotated antioxidant enzymes, that is, SOD or SOR (Supplementary Table S2, Fig. 1b), and reconstructed a phylogenetic tree based on the 16S rRNA sequences from these anaerobes and those without EAS (Supplementary Table S1). According to the tree (Fig. 2), non-EAS anaerobes, which include *Methanobrevibacter smithii*, *Methanopyrus kandleri*, *Treponema* sp. ZAS-2 (alias *T. primitia* ZAS-2), *Bifidobacterium adolescentis*, *Bifidobacterium dentium*, *Carboxydotherrmus hydrogenoformans*, and *Caldicellulosiruptor saccharolyticus*, are, in general, scattered among various groups with EAS. It seems that most analyzed anaerobes, in relation to their ancestors, lost two antioxidant enzymes that constitute functional EAS. Moreover, the results might suggest that an occurrence of EAS was a primordial attribute of so-called obligate anaerobes, which was accidentally lost in progeny lineages (Fig. 2).

3.4. Prediction of the specificity of the iron metal present in the active site of antioxidant enzymes

We predicted the presence of iron only in SOD and CAT active sites, because all SORs belong to non-heme proteins and PXs are not metalloenzymes (see Introduction). Among all SODs, 49 were predicted as Fe-SODs, 14 putatively contained Mn, one was probably Fe/Mn-SOD, and for one enzyme (accession number NP_349176, Supplementary Table S3) the SODa bioinformatic tool did not identify a metal cofactor (Fig. 3a), though GenBank classified it as a protein belonging to the Fe/Mn-SOD family (Supplementary Table S3). The detailed values concerning metal prediction are shown as score expressed in percent (Supplementary Table S3). Among 29 CATs (Supplementary Table S2), 16 were putatively identified as monofunctional (typical) heme-containing CATs (KatEs), 11 as catalase-peroxidases (KatGs), and 2 presumably are Mn-containing enzymes (MnCAT) (Fig. 3b).

4. Discussion

Most likely, life on Earth evolved under anoxic conditions, with the hypothetical LUCA being an anaerobe. In general, prebiotic chemistry for synthesis of many biomolecules is impossible in the presence of molecular oxygen (e.g., Lazcano and Miller, 1996; Miller and Cleaves, 2007). However, reanalysis of samples from Miller's spark discharge experiment has shown the occurrence of hydroxylated compounds in samples (Johnson *et al.*, 2008), suggesting the generation of ROS, such as hydroxyl radicals ($\text{HO}^\bullet$), which may have reacted with amino acid precursors or amino acids themselves (Ring and Miller, 1984; Johnson *et al.*, 2008). Nevertheless, the concentration of molecular oxygen in the atmosphere of early Earth was estimated to be very low, *ca.* 10^{-5} of the present atmospheric level, only with traces of O_2 in shallow oceans (Holland, 2006). However, the timing of the rise of O_2 in the ancient atmosphere is

still under vigorous debate (Yamaguchi, 2005; Kump, 2008; Shaw, 2008; Lyons *et al.*, 2014). Numerous reports suggest that local areas with increased O_2 concentration appeared several hundred million year before the Great Oxidation Event (Crowe *et al.*, 2013; Olson *et al.*, 2013; Lyons *et al.*, 2014; Planavsky *et al.*, 2014); therefore, many ancient anaerobes had already been adapted to O_2 /ROS.

4.1. Modern anaerobes

Anaerobes, mainly prokaryotes, are defined as organisms that do not require oxygen to obtain metabolic energy for growth. Among them, obligate or strict anaerobes never use O_2 in their metabolism (Sanz, 2011). It is most likely that such a broad criterion was used for classification of most modern obligate anaerobes described in databases and literature. Nevertheless, no habitat is free even from an occasional increase of O_2 , and so-called obligate anaerobes share similar microbiota as facultative anaerobes. Moreover, the adaptability of obligate anaerobes to O_2 might have been overlooked because laboratory conditions failed to mimic the dynamic of aeration in natural environments (Imlay, 2008b). In fact, in modern anaerobes, components of aerobic respiration, and EAS, such as various cytochrome oxidases (Baughn and Malamy, 2004; Das *et al.*, 2005; Dolla *et al.*, 2006), NADH oxidases (Yang and Ma, 2007), SODs (Gregory *et al.*, 1978; Dos Santos *et al.*, 2000; Brioukhanov and Netrusov, 2004; Dolla *et al.*, 2006; Horne and Lessner, 2013), SORs (Nivière and Fontecave, 2004; Dolla *et al.*, 2006), and CATs (Brioukhanov and Netrusov, 2004; Dolla *et al.*, 2006; Mishra and Imlay, 2013) have been identified previously. According to other reports, enzymes and metabolic pathways that are directly or indirectly oxygen-dependent are present in many anaerobes (Peregrin-Alvarez *et al.*, 2003; Freitas *et al.*, 2005; Raymond and Segrè, 2006; Passardi *et al.*, 2007; Ślesak *et al.*, 2012). Moreover, according to theoretical studies, Fe/MnSOD and CAT would have been required for basic metabolism of the hypothetical LUCA (Ouzounis *et al.*, 2006).

The minority of the analyzed anaerobes have a double set of antioxidant enzymes that perform the same functions: SOD + SOR and CAT + PX (Supplementary Table S2, Fig. 2). It cannot be excluded that such redundancy is, in part, a result of horizontal gene transfer. The presence of EAS in contemporary obligate anaerobes from the domains Archaea and Bacteria raises questions concerning the evolution of antioxidant cellular pathways. Only 7% of the analyzed anaerobes do not have antioxidant enzymes (Fig. 1a). The simplest explanation for the variable absence of SOD and SOR genes on the 16S rRNA tree is that these genes were present in their common ancestor but were later lost in some lineages (Fig. 2). Based on this result, the reductive genome evolution could be considered to be a possible explanation of the selective loss of SOD/SOR genes in some analyzed anaerobes. Reductive evolution is related to genome reduction, which occurs via two modes: (i) the neutral gene loss ratchet and (ii) adaptive genome streamlining, which dominates in parasitic and symbiotic organisms. Reductive evolution has also been observed in several organisms that evolved a commensal lifestyle (Wolf and Koonin, 2013). The examples in our study are bifidobacteria (*B. dentium*, *B. adolescentis*) and *M. smithii*, which inhabit human gut and intestines (Samuel *et al.*, 2002; Klijn *et al.*, 2005). It is

FIG. 2. Unrooted 16S rRNA tree (cladogram) for anaerobes possessing at least SOD or SOR. Species labeled with black circles have no antioxidant response system EAS (Supplementary Table S1). Red squares indicate lineages that possibly lost at least SOD and/or SOR genes. All antioxidant enzymes encoded in genome of each organism are presented in parentheses. Numbers at nodes represent bootstrap values (based on 100 replications).

FIG. 3. The number of various classes of SODs (a) and CATs (b) predicted by SODa Web tool (<http://babylone.ulb.ac.be/SODa>) in analyzed anaerobes. For more details see Results. Fe-SOD, iron-containing SOD; Mn-SOD, manganese-containing SOD; Fe/Mn-SOD, manganese/iron-containing SOD; KatE (Fe), iron-containing monofunctional CATs; KatG (Fe), iron-containing catalase-peroxidases; MnCAT, manganese CAT.

interesting to note that no genes for SOD have been identified in bifidobacteria, but divalent metal ions (Fe^{2+} and Mn^{2+}) may play the role of SOD (Chang and So, 1998). Moreover, a loss of various genes was previously suggested for the catalase (*katG*) gene even in oxygen-producing cyanobacteria (Morris *et al.*, 2012). If the modern anaerobic metabolism of obligate anaerobes is representative for their ancestors, our analyses strongly suggest that EAS appeared at the very early period of Earth evolution, yet before the evolution of oxygen-producing cyanobacteria. Subsequently, components of EAS were lost in selected microbial lines. According to our typology (based on aerobes' antioxidant machinery), 43% of EAS found were nonfunctional (Fig. 1b); however, we have scarce data confirming the nonfunctionality of such enzyme combinations in specific anaerobic species.

It should be emphasized that, in the case of the evolution of oxygenic photosynthesis, we have the “chicken-and-the-egg problem”. Photosystem II (PSII)-mediated O_2 evolution requires antioxidant enzymes that limit O_2 derivatives, that is, ROS toxicity, while antioxidant enzymes cannot evolve without O_2 /ROS (McKay and Hartman, 1991; Kirschvink and Kopp, 2008). Therefore, if the unorthodox

respiration-early hypothesis (Castresana and Saraste, 1995) is true, SOD, SOR, PX, and CAT were key enzymes of EAS, which evolved before the oxygenic photosynthesis-dependent rise of atmospheric O_2 . Nevertheless, the verification of such a hypothesis requires the identification of early nonphotosynthetic (abiotic) O_2 sources.

4.2. Formation of oxygen and related species on early Earth

Here, we have identified potential intracellular sources of O_2 /ROS as by-products of net EAS activity in a hypothetical ancestor of anaerobes. Nevertheless, we are unable to expand upon our current understanding of the mechanisms of abiotic O_2 /ROS production in early Earth's atmosphere; a reminder of the possible nonbiological sources of O_2 /ROS is necessary for the clarity of our argumentation. In ancient Earth's atmosphere, *ca.* 3.85–2.45 O_2 /ROS could have been produced abiotically by water photolysis (UV radiation), radiolysis, and CO_2 photolysis (Halliwell and Gutteridge, 1999; Baumstark-Khan and Facius, 2002; Azrague *et al.*, 2005; Draganić, 2005; Haqq-Misra *et al.*, 2011). All these processes were present before oxygenic photosynthesis. Moreover, some of the O_2 / H_2O_2 molecules would have been produced in cosmic space and then imported to Earth's atmosphere by comets and other icy interstellar bodies (Zheng *et al.*, 2006; Cooper *et al.*, 2008). Recently, ROS generation at the oxide/water interface has also been reported (Xu *et al.*, 2013). All these hypothetical scenarios strongly indicate that prebiotic ROS could have been, at least to some extent, a primordial source of O_2 . This assumption has been supported by experimental evidence, which has shown that pyrite/aqueous suspensions generate H_2O_2 , via hydroxyl radicals ($\text{HO}^\bullet$), in the absence of O_2 (Borda *et al.*, 2001; Cohn *et al.*, 2006). Moreover, this fact corresponds to the theory that the early events in the evolution of life took place in iron-rich environments containing iron-sulfide minerals (Martin and Russell, 2003; Wächtershäuser, 2007) and that Fe was abundant mainly in the reduced soluble form Fe^{2+} (Saito *et al.*, 2003). According to this, it would seem that Fe^{2+} was initially the most probable transition metal in the active site of the first SOD (Bannister *et al.*, 1991; Asada, 2000). In a similar way, the evolution of catalases, from free Fe^{2+} through iron-heme complexes to the formation of the active site of the CAT protein, was proposed by Calvin (1969). Iron is still present in many enzymes of contemporary organisms (Dupont *et al.*, 2006; Nitschke *et al.*, 2013) and can be treated as a vestige of ancient Fe-containing proteins. The increase of oxygen in the atmosphere resulted in a number of immediate geochemical effects. O_2 reacted with soluble iron (Fe^{2+}) in the ancient water reservoirs to form layers of insoluble iron oxides (Fe^{3+}), now known as banded iron formations (Bekker *et al.*, 2010); therefore, the incorporation of Fe^{2+} into the active sites' enzymes was hindered after the release of major amounts of O_2 into the atmosphere. The presence of iron in active sites of analyzed antioxidant enzymes may suggest that they appeared at very early stages of life evolution yet before the release of O_2 into the atmosphere. The results obtained from the ion metal prediction in SOD and CAT active sites clearly indicate that putative iron-metalloenzymes dominate among analyzed CATs and SODs (Fig. 2). Only a minority of them have been predicted to be manganese-containing enzymes,

which suggests that Mn ions were less available in ancient oceans, as has been suggested in previous geochemical studies (Saito *et al.*, 2003).

According to available reports and our *in silico* analyses presented here, a common strategy for dealing with oxidative stress exists not only in aerobes but also in anaerobes. Obligate anaerobes also neutralize harmful ROS by a series of reactions where the product is always less toxic than the substrate, but inevitably the net by-product of such a series of events is molecular oxygen or rarely H₂O₂. Molecular oxygen is less toxic than its derivatives (ROS), but O₂ itself is an intracellular source of ROS. Recently, metagenomic studies revealed another intracellular O₂-generating pathway in the anaerobic denitrifying bacterium *Candidatus Methylopirabilis oxyfera* (Ettwig *et al.*, 2010). The results presented here clearly indicate that abiotically derived O₂ was not the only source of O₂ available for the first microorganisms before oxygenic photosynthesis evolved. These organisms probably account for the availability of endogenous, metabolically derived O₂.

4.3. Conclusions

Given the outcome of this study, we draw the following conclusions. The presence of iron-containing antioxidant enzymes in many modern strict anaerobes may be an evolutionary vestige inherited from early ancestors of anaerobic prokaryotes, which had to have had the ability to cope with ROS. Nevertheless, our data strongly suggest that the occurrence of EAS in obligate anaerobes could have appeared at an early stage of life evolution. Besides environmental abiogenic sources of O₂/ROS, rudimentary EASs could also have been endogenous net producers of O₂/ROS in LUCA. This hypothesis may explain why harmful effects of cyanobacterial-produced O₂ were restricted for their producers. Ancient non-photosynthesizing cyanobacterial ancestors may have already been equipped with some key antioxidant protoenzymes inherited from O₂/ROS-tolerant ancestors.

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Abbreviations Used

CAT = catalase
EAS = enzymatic antioxidant system
LUCA = last universal common ancestor
PX = peroxiredoxin
ROS = reactive oxygen species
SOD = superoxide dismutase
SOR = superoxide reductase