Oxygen and Hydrogen Peroxide in the Early Evolution of Life on Earth: *In silico* Comparative Analysis of Biochemical Pathways

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Abstract

In the Universe, oxygen is the third most widespread element, while on Earth it is the most abundant one. Moreover, oxygen is a major constituent of all biopolymers fundamental to living organisms. Besides O₂, reactive oxygen species (ROS), among them hydrogen peroxide (H₂O₂), are also important reactants in the present aerobic metabolism. According to a widely accepted hypothesis, aerobic metabolism and many other reactions/pathways involving O₂ appeared after the evolution of oxygenic photosynthesis. In this study, the hypothesis was formulated that the Last Universal Common Ancestor (LUCA) was at least able to tolerate O₂ and detoxify ROS in a primordial environment. A comparative analysis was carried out of a number of the O₂- and H₂O₂-involving metabolic reactions that occur in strict anaerobes, facultative anaerobes, and aerobes. The results indicate that the most likely LUCA possessed O₂- and H₂O₂-involving pathways, mainly reactions to remove ROS, and had, at least in part, the components of aerobic respiration. Based on this, the presence of a low, but significant, quantity of H₂O₂ and O₂ should be taken into account in theoretical models of the early Archean atmosphere and oceans and the evolution of life. It is suggested that the early metabolism involving O₂/H₂O₂ was a key adaptation of LUCA to already existing weakly oxic zones in Earth's primordial environment. **Key Words:** Hydrogen peroxide—Oxygen—Origin of life—Photosynthesis—Superoxide dismutase—Superoxide reductase. *Astrobiology* 12, 775–784.

1. Introduction

OXYGEN is the third most abundant element in the Universe by mass (Trimble, 1997), and on Earth it is the most widespread element. It is a major constituent of all the compounds that are essential for living beings, such as water, carbohydrates, lipids, proteins, and nucleic acids. However, molecular oxygen is toxic to organisms called obligate anaerobes, and it is indispensable to the life of aerobes. It is widely accepted that Earth's primordial atmosphere was anoxic with *ca.* 10⁻⁵ of the present atmospheric level of molecular oxygen (Holland, 2006). The ancestral atmosphere without oxygen showed only poorly reducing character, and it probably contained predominantly carbon dioxide (CO₂) (Kasting, 1993; Maurel and Décout, 1999; Shaw, 2008). The time when the oxygenation of Earth's atmosphere took place

is still under debate, but the most widely accepted view locates the oxygenation event at *ca.* 2.4 Ga (Olson and Blankenship, 2004; Sessions *et al.*, 2009).

Nevertheless, there are some geochemical and geophysical data, theoretical model considerations, molecular phylogenetic and biochemical comparative analyses that suggest a higher oxygenation of both the young Earth's surface and atmosphere than is commonly accepted (McKay and Hartman, 1991; Castresana *et al.*, 1994; Towe, 1996; Draganić, 2005; Hoashi *et al.*, 2009; Kornaś *et al.*, 2010; Haqq-Misra *et al.*, 2011). The modeling of the photochemistry of some realistic atmospheres (including present and past Earth and Mars) has revealed O₂-rich atmospheres (up to 5%) as a result of abiotic H₂O and CO₂ photolysis (Selsis *et al.*, 2002). It has also been proposed that abiotically produced reactive oxygen species (ROS), such as H₂O₂, could be the primordial source

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of molecular oxygen (O_2). This assumption has been supported by experiments that have shown that pyrite/aqueous suspensions generate H_2O_2 in the absence of O_2 (Borda *et al.*, 2001). Additional arguments that suggest a more oxidized state of the young Earth may be adduced from astrophysical studies concerning the surface of Mars. The Viking mission in the 1970s showed that the surface of Mars is highly oxidized (Klein, 1978; Hartman and McKay, 1995), in contrast to its atmosphere, which contains only traces of molecular oxygen (*ca.* 0.13%) and a vast amount of CO_2 (*ca.* 95%) (Moroz, 1998; Selsis *et al.*, 2002). Recently, a geochemical model for the pyrite-dependent aqueous formation of ROS, such as $OH\cdot$ and H_2O_2 in the subsurface of Mars, has been presented (Davila *et al.*, 2008). Houtkooper and Schulze-Makuch (2007) suggested that hypothetical martian organisms might use a H_2O_2 - H_2O mixture as an intracellular fluid to be optimally adapted to the highly oxidized martian environment. Moreover, it has been reported that the surface of Europa, the icy satellite of Jupiter, contains H_2O_2 , and both radiolysis and photolysis of its icy surface leads to the formation of oxygen and ROS (Carlson *et al.*, 1999; Johnson *et al.*, 2003). Recently, the presence of abiotically formed O_2 has also been found in the atmosphere of Saturn's icy moon Rhea (Teolis *et al.*, 2010). These data are important in the context of hypothetical life-forms present on Europa (Chyba and Phillips, 2001), which could be equipped with some components of enzymatic antioxidant systems. All these very speculative models and some experimental data may suggest that the early stages of biological evolution on Earth occurred in a more oxic environment.

A view commonly presented in handbooks is that primordial organisms were strict anaerobes (Halliwell and Gutteridge, 1999; Alberts *et al.*, 2002; De Las Rivas *et al.*, 2004; Shaw, 2008). At present, there is a growing number of fully sequenced genomes of organisms that belong to three domains of life (Archaea, Bacteria, and Eukarya). This has allowed comparative analysis of the biochemical processes of the hypothetical Last Universal Common Ancestor (LUCA) and the metabolic background of presently living organisms (Peregrin-Alvarez *et al.*, 2003; Ouzounis *et al.*, 2006). Currently, we have access to primary databases of the small-molecule metabolism (*e.g.*, BioCyc, KEGG), containing many experimentally verified metabolic pathways, along with enzyme information derived from the scientific literature (Caspi *et al.*, 2008; Kanehisa *et al.*, 2008).

Anaerobes, mainly prokaryotic organisms, still exist today; but as a rule, their growth is inhibited, or they are killed by exposure to the present O_2 atmospheric level (Halliwell, 2006). Nevertheless, the available literature and data stored in public domains indicate that components of antioxidant response systems are present in strict and in facultative anaerobes (Brioukhanov and Netrusov, 2004; Imlay, 2008). With this in mind, we have formulated the following hypotheses: (1) LUCA was not an obligate anaerobe, but it had genes that encoded enzymes that utilized ROS, for example, H_2O_2 , and the earliest protocells were able to tolerate O_2 ; (2) on the ancient surface of the young Earth, *ca.* 3.5 Ga, micro-environments could have existed where minor, but sufficient, amounts of ROS and O_2 were generated in the generally anoxic environment of early Earth. *In silico* analysis of selected O_2 / H_2O_2 -dependent pathways deposited in the

primary BioCyc database (Caspi *et al.*, 2008) was used to test whether the above hypothesis could be a promising pointer for further studies.

2. Materials and Methods

The comparative analysis was performed according to BioCyc (BioCyc.org 13.0 and 15.0 Web site), the primary database collection of pathway/genomes. Of interest was the total number of reactions, where O_2 and H_2O_2 appear as a reactant in a diverse range of roles from biosynthesis to electron transfer, along with detoxification reactions in organisms with different oxygen requirements, that is, obligate anaerobes (called hence anaerobes), facultative anaerobes, and aerobes. The information concerning oxygen tolerance by various organisms was found and verified by consulting the literature (*e.g.*, Vieira-Silva and Rocha, 2008) and Web pages, for example, the GeneBank (www.ncbi.nlm.nih.gov/genomes/lproks.cgi), European Bioinformatics Institute (www.ebi.ac.uk), and Doe Joint Genome Institute (www.jgi.doe.gov). The search procedures of the primary BioCyc database and other secondary databases are shown in Fig. 1. The groups of obligate anaerobes, facultative anaerobes, and aerobes selected for analysis are presented in Table 1. The organisms were also classified according to their photosynthetic activity (anoxygenic photosynthesis, oxygenic photosynthesis, no photosynthesis) and as belonging to the three main domains of life (Bacteria, Archaea, Eukarya, Table 1). The data were collected and calculated for each organism as follows. The mean number and percentage of O_2 / H_2O_2 -involving reactions (reactions where O_2 / H_2O_2 are involved as substrates or products in obligate anaerobes, facultative anaerobes, and aerobes) are described by the following formulas:

The mean number of O_2 / H_2O_2 -involving reactions

$$= \frac{\sum_{i=1}^n x_i}{n} \quad (1)$$

Percentage [%] of O_2 / H_2O_2 -involving reactions

$$= \frac{\left(\sum_{i=1}^n \frac{x_i}{x_{t(i)}} \right)}{n} 100 \quad (2)$$

where x_i is the number of O_2 / H_2O_2 -involving reactions for the i^{th} organism derived from databases (Table 1), $x_{t(i)}$ is the total number of all enzyme-catalyzed reactions that utilize unique small molecules for the i^{th} organism, and n is the entire number of analyzed organisms of each group: anaerobes, facultative anaerobes, and aerobes (Table 1). Moreover, two crucial biochemical pathways where O_2 / H_2O_2 is involved were selected: (1) aerobic respiration, (2) removal of ROS. Simplified criteria for linking the above pathways to the organisms listed in Table 1 were used. In this study, the aerobic metabolism as a process of electron transport to molecular oxygen as a final acceptor was defined. Finally, all the anaerobes and facultative anaerobes that have the terminal electron acceptors known as dioxygen reductases were classified as organisms that show aerobic respiration. The reduction of O_2 to water is catalyzed by dioxygen reductases belonging to two superfamilies, cyt. *bd* oxidases and heme-copper oxidases, for example, cytochrome *c* oxidases (Pereira *et al.*, 2001; Brochier-Armanet *et al.*, 2009), according to the reaction:

FIG. 1. Two schemes (a, b) of searching algorithms in which BioCyc (<http://biocyc.org>) was used as the primary database.

^aSecondary databases: AraCyc (www.arabidopsis.org/biocyc/index.jsp), CalbiCyc (<http://pathway.candidagenome.org/>), DictyCyc (http://dictybase.org/Dicty_Info/dictycyc_info.html), FungiCyc (<http://fungicyc.broadinstitute.org:1555/>), MedicCyc (<http://mediccy.noble.org/>), MouseCyc (<http://mousecyc.jax.org/>), RiceCyc (<http://www.gramene.org/pathway/>), SolCyc (<http://solcyc.solgenomics.net/>), Yeast Biochemical Pathways (<http://pathway.yeastgenome.org/biocyc/>).

^bExcluding the following compounds, which are extremely common in all organisms: H₂O, H⁺, ATP, ADP, AMP, phosphate, diphosphate, NAD⁺, NADH, NADP⁺, NADPH, an oxidized electron acceptor, a reduced electron acceptor.

Among analyzed obligate and facultative anaerobes (Table 1), the percentage of organisms possessing at least one type of dioxygen reductase was derived not only from the BioCyc database but also from molecular phylogenies obtained previously by Blank (2009) and Brochier-Armanet *et al.* (2009). A ROS removal system was defined by the occurrence of at least one of the antioxidant enzymes, superoxide dismutase (SOD), superoxide reductase (SOR), or

catalase (CAT). These enzymes are responsible for the detoxification of ROS, such as superoxide anion radical and H₂O₂, in the following reactions:

In light of this classification, “complete” aerobes are organisms having both aerobic respiration and ROS removal pathways.

This estimation was used to clarify the data presentation and to discuss the obtained results. It was assumed that all the analyzed aerobes take advantage of aerobic respiration and a ROS removal system. In the case of anaerobes and facultative anaerobes, the percentage of all organisms having these pathways was determined by the equation:

$$\text{Organisms possessing a pathway } [\%] = (n_p/n) \cdot 100 \quad (7)$$

where n_p is the number of analyzed organisms possessing the components of a pathway, that is, aerobic respiration or/and a ROS removal pathway, and n is the total number of analyzed organisms, anaerobes, facultative anaerobes, and aerobes (listed in Table 1).

Statistical analysis was performed by using a nonparametric Kruskal-Wallis test. Dunn’s test was used as a *post test*.

3. Results

3.1. Mean number of reactions involving O₂ and H₂O₂

An analysis of 105 different organisms was performed, including 40 obligate anaerobes, 31 facultative anaerobes, and 34 aerobes (Table 1).

The global comparison of total amount of O₂- and H₂O₂-involving reactions showed that aerobes perform significantly more reactions where O₂ and H₂O₂ are engaged than obligate anaerobes (Fig. 2). On the other hand, no significant differences were found between facultative anaerobes and aerobes, as far as reactions involving O₂/H₂O₂ are concerned (Fig. 2a, 2c). If the comparative analysis was carried out with use of the percentage of O₂-involving processes, a significant increase in such reactions was observed between all the analyzed groups of organisms (Fig. 2b). A significantly higher number of H₂O₂-involving reactions identified in aerobes in comparison to obligate anaerobes was observed only when the data were expressed as a percentage of all reactions involving H₂O₂ in each group of analyzed organisms (Fig. 2d).

3.2. Reactions involving O₂ and H₂O₂ in Bacteria, Archaea, and Eukarya

Calculations analogical to those presented in Fig. 2, but independent for Bacteria, Archaea, and Eukarya, were carried out (Fig. 3). The results show that obligate anaerobic bacteria have a significantly lower number and percentage of O₂-involving reactions compared to those of aerobic bacteria and eukaryotes (Fig. 3a, 3b). Anaerobic archaea have less O₂-dependent reactions than facultative anaerobic bacteria, aerobic bacteria, and aerobic eukaryotes. The analysis

TABLE 1. THE LIST OF ORGANISMS ANALYZED

Bacteria	Archaea	Eukarya
Obligate anaerobes¹		
<i>Anaeromyxobacter dehalogenans</i> 2CP-C	<i>Archaeoglobus fulgidus</i> DSM 4304	
<i>Bacteroides fragilis</i> NCTC 9343	<i>Methanocaldococcus jannaschii</i> DSM 2661	
<i>Bacteroides fragilis</i> YHC46	<i>Methanosarcina acetivorans</i> C2A	
<i>Bacteroides thetaiotaomicron</i> VPI-5482	<i>Methanosarcina barkeri</i> Fusaro	
<i>Bifidobacterium longum</i> NCC2705	<i>Methanosarcina mazei</i> Go1	
<i>Moorella thermoacetica</i> ATCC 39073	<i>Methanospaera stadtmanae</i> DSM 3091	
<i>Mycoplasma mycoides mycoides</i> SC str. PG1	<i>Methanothermobacter thermautotrophicus</i> Delta H	
<i>Streptococcus thermophilus</i> LMG 18311	<i>Thermococcus kodakarensis</i> KOD1	
<i>Clostridium acetobutylicum</i> ATCC 824	<i>Pyrococcus abyssi</i> GE5	
<i>Clostridium perfringens</i> 13	<i>Pyrococcus furiosus</i> DSM 3638	
<i>Clostridium tetani</i> E88	<i>Methanococcoides burtonii</i> DSM 6242	
<i>Syntrophus aciditrophicus</i> SB	<i>Methanococcus maripaludis</i> S2	
<i>Thermoanaerobacter tengcongensis</i> MB4	<i>Methanopyrus kandleri</i> AV19	
<i>Pelobacter carbinolicus</i> DSM 2380		
<i>Desulfitobacterium hafniense</i> Y51		
<i>Desulfotalea psychrophila</i> LSv54		
<i>Desulfovibrio desulfuricans</i> G20		
<i>Desulfovibrio vulgaris vulgaris</i> Hildenborough		
<i>Fusobacterium nucleatum nucleatum</i> ATCC 25586		
<i>Geobacter metallireducens</i> GS-15		
<i>Geobacter sulfurreducens</i> PCA		
<i>Zymomonas mobilis</i> ZM4		
<i>Carboxydotherrmus hydrogenoformans</i> Z-2901		
<i>Chlorobium tepidum</i> TLS ^{ap}		
<i>Chlorobium chlorochromatii</i> CaD3 ^{ap}		
<i>Pelodictyon luteolum</i> DSM 273 ^{ap}		
<i>Porphyromonas gingivalis</i> W83		
Bacteria: 27	Archaea: 13	
	Total no. of organisms: 40	
	Organisms performing anoxygenic photosynthesis: 3	
Facultative anaerobes		
<i>Haemophilus influenzae</i> Rd KW20	<i>Thermoplasma volcanium</i> GSS1	<i>Saccharomyces cerevisiae</i> S288C
<i>Bacillus anthracis</i> Ames	<i>Pyrobaculum aerophilum</i> IM2	<i>Schizosaccharomyces pombe</i>
<i>Salmonella enterica enterica</i> serovar Paratyphi A str. ATCC 9150	<i>Halobacterium</i> sp. NRC-1	<i>Chlamydomonas reinhardtii</i>
<i>Bacillus subtilis subtilis</i> 168		<i>Fusarium oxysporum</i> sp. <i>lycopersici</i> 4286
<i>Bacillus thuringiensis</i> serovar konkukian str. 97-27		
<i>Lactococcus lactis lactis</i> II1403		
<i>Shewanella oneidensis</i> MR-1		
<i>Staphylococcus aureus aureus</i> COL		
<i>Brucella melitensis</i> biovar Abortus		
<i>Streptococcus pyogenes</i> M1 GAS		
<i>Mycoplasma genitalium</i> G37		
<i>Chromobacterium violaceum</i> ATCC 12472		
<i>Chromohalobacter salexigens</i> DSM 3043		
<i>Photobacterium profundum</i> SS9		
<i>Thiobacillus denitrificans</i> ATCC 25259		
<i>Vibrio cholerae</i> O1 biovar eltor str. N16961		
<i>Escherichia coli</i> W3110		
<i>Yersinia pestis</i> KIM		
<i>Ralstonia eutropha</i> JMP134		
<i>Rhodopseudomonas palustris</i> BisB5 ^{ap}		
<i>Rhodospirillum rubrum</i> ATCC 11170 ^{ap}		
<i>Rhodobacter sphaeroides</i> 2.4.1 ^{ap}		
<i>Rhodospirillum rubrum</i> T118		

(continued)

TABLE 1. (CONTINUED)

Bacteria	Archaea	Eukarya
<i>Lactobacillus acidophilus</i> NCFM		
Bacteria: 24	Archaea: 3	Eukarya: 4
Total no. of organisms: 31		
Organisms performing anoxygenic photosynthesis: 3		
Aerobes		
<i>Bacillus cereus</i> W	<i>Sulfolobus acidocaldarius</i> DSM 639	Plants ^{OP} :
<i>Burkholderia thailandensis</i> E264	<i>Sulfolobus solfataricus</i> P2	<i>Arabidopsis thaliana</i> col
<i>Candidatus Pelagibacter ubique</i>	<i>Sulfolobus tokadaii</i> 7	<i>Solanum lycopersicum</i>
HTCC1062		<i>Capsicum annum</i>
<i>Nitrosomonas europaea</i> ATCC 19718		<i>Medicago truncatula</i>
<i>Oceanobacillus iheyensis</i> HTE831		<i>Solanum tuberosum</i>
<i>Cytophaga hutchinsonii</i> ATCC 33406		<i>Coffea canephora</i>
<i>Thermus thermophilus</i> HB27		<i>Oryza sativa japonica</i> Nipponbare
<i>Deinococcus radiodurans</i> R1		<i>Sorghum bicolor</i> BTx623
<i>Geobacillus kaustophilus</i> HTA426		<i>Nicotiana tabacum</i>
Cyanobacteria ^{OP} :		Animals/Fungi:
<i>Anabaena variabilis</i> ATCC 29413		<i>Homo sapiens</i>
<i>Nostoc</i> sp. PCC 7120		<i>Mus musculus</i>
<i>Gloeobacter violaceus</i> PCC 7421		<i>Bos taurus</i>
<i>Prochlorococcus marinus</i> MIT 9312		<i>Drosophila melanogaster</i>
<i>Synechococcus elongatus</i> PCC 6301		<i>Neurospora crassa</i>
<i>Thermosynechococcus elongatus</i> BP-1		<i>Dictyostelium discoideum</i> AX4
		<i>Candida albicans</i> SC5314
Bacteria: 15	Archaea: 3	Eukarya: 16
Total no. of organisms: 34		
Organisms performing oxygenic photosynthesis: 15		

¹The classification of organisms by oxygen tolerance was derived from database NCBI Microbial Genome Project—<http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi>—and from the literature (Vieira-Silva and Rocha, 2008; Mentel and Martin, 2008).

^{OP}Organisms performing anoxygenic photosynthesis.

^{OP}Organisms performing oxygenic photosynthesis.

showed no significant differences in the mean number and percentage of O₂-involving reactions between all groups of Archaea (Fig. 3a, 3b). For H₂O₂, the common tendency toward an increase of reactions involving H₂O₂ was similar to that for O₂ (Fig. 3c). Moreover, the mean number of H₂O₂-involving reactions was significantly higher only between obligate anaerobic Bacteria, Archaea, and eukaryotic aerobes (Fig. 3c). If the results are presented as a percentage of reactions involving H₂O₂, significant differences were found between anaerobic Archaea and both facultatively anaerobic and aerobic Bacteria (Fig. 3d). It should be noted that the lowest mean number of O₂/H₂O₂-involving reactions was very similar for strictly anaerobic, facultatively anaerobic, and aerobic archaea (Fig. 3d).

3.3. The relationship between the mean number of reactions involving O₂ and H₂O₂ and oxygenic photosynthesis

The data show a significant increase in the number of O₂-involving reactions only between strict anaerobic bacteria and plants (Fig. 4a, 4b). Unexpectedly, oxygen-producing cyanobacteria showed a significantly lower percentage of reactions involving O₂ than plants (Fig. 4b). Moreover, no significant distinctions in the quantity of O₂-involving reactions between photosynthesizing obligate anaerobes, facultative anaerobes, and cyanobacteria were noted (Fig. 4a, 4b). For reactions involving H₂O₂, no significant differences between organisms were observed (Fig. 4c, 4d).

3.4. Aerobic respiration and ROS removal

The percentage of ROS removal systems and aerobic respiration pathways found for aerobes was set to 100% (Fig. 5). Among obligate anaerobes and facultative anaerobes, 50% and 90%, respectively, possess both oxic pathways: aerobic respiration and ROS-removing reactions (Fig. 5c). If the pathways are analyzed separately, aerobic respiration and ROS removal pathways are found in *ca.* 55%/85% and 90%/93% of obligate anaerobes and facultative anaerobes, respectively (Fig. 5a, 5b).

4. Discussion

4.1. Reactions involving O₂

In general, as expected, the mean number of O₂-involving reactions and the percentage of reactions where O₂ is engaged increased from strict anaerobes to obligate aerobes (Fig. 2a, 2b). Moreover, the results concerning O₂/H₂O₂-involving reactions in Bacteria, Archaea, and Eukarya obtained during the analysis might indicate that obligate anaerobes belonging to Archaea are “more obligate” anaerobes than anaerobic bacteria. In this case, the mean number of O₂/H₂O₂-involving reactions of anaerobic archaea is *ca.* 3- and 2-fold lower for reactions involving O₂ and H₂O₂, respectively, compared to anaerobic bacteria (Fig. 3a, 3c). These results are surprising, because all the analyzed obligate anaerobes actually have metabolic pathways/reactions involving O₂. If contemporary anaerobic pathways of

FIG. 2. Reactions involving O₂/H₂O₂. (a) the mean number of O₂-involving reactions, (b) the percent of O₂-involving reactions, (c) total mean number of H₂O₂-involving reactions, (d) the percent of H₂O₂-involving reactions. The values were calculated according to Eqs. 1 and 2 (Materials and Methods). Values represent means $\pm$ SD; n = number of species, obligate anaerobes: n = 40, facultative anaerobes: n = 31, aerobes: n = 34; see also Table 1. The same letters above bars indicate no significant differences between means, at least at the level of P < 0.05 (Kruskal-Wallis test and Dunn's test as a post test).

obligate anaerobes are represented in their anaerobic ancestors, the occurrence of various O₂-involving reactions/pathways in present obligate anaerobes might suggest that O₂, as a metabolic reactant, appeared very early in the evolution of life. The results support the previously unorthodox hypothesis that photochemically produced molecular oxygen and aerobic metabolism were already present on early Earth and in primordial organisms, respectively, yet before the evolution of oxygen-producing cyanobacteria (Towe 1978, 1981, 1990; Castresana *et al.*, 1994; Pereira *et al.*, 2001). Recent studies have shown that dioxygen reductases, such as cytochrome oxidases type A (*e.g.*, the contemporary mitochondrial form) preceded other cytochrome oxidases. In this context, the respiration-early hypothesis (Castresana and Saraste, 1995) that early microbiota contained low concentrations of O₂ could not be ruled out (Brochier-Armanet *et al.*, 2009). However, many reactions involving O₂ may be re-

FIG. 3. Reactions involving O₂/H₂O₂ in organisms belonging to the three domains of life, Bacteria, Archaea, and Eukarya. (a), (b), (c), (d) description is the same as in Fig. 2. The values were calculated according to Eqs. 1 and 2 (Materials and Methods). Values represent means $\pm$ SD; n = number of species, obligate anaerobes: n = 27 (Bacteria), n = 13 (Archaea), facultative anaerobes: n = 24 (Bacteria), n = 3 (Archaea), n = 4 (Eukarya), aerobes: n = 15 (Bacteria), n = 3 (Archaea), n = 16 (Eukarya). The same letters above bars indicate no significant differences between means, at least at the level of P < 0.05 (Kruskal-Wallis test and Dunn's test as a post test).

placed by analogous O₂-independent (anaerobic) reactions, as it has been previously described (Raymond and Blankenship, 2004). Nevertheless, it is most likely that the majority of O₂-dependent reactions, including O₂ reduction by dioxygen reductases and ROS-detoxifying pathways (see below), cannot be simply substituted by anaerobic equivalents. The analysis of obligate and facultative anaerobes revealed the occurrence of aerobic respiration components in many obligate anaerobes (Fig. 5). It might be suggested that O₂ reduction (Eq. 3) is not exclusively limited to strict aerobes only. Moreover, dioxygen may not be the main oxic compound that leads to the evolution of O₂-dependent reactions. It cannot be excluded that one class of dioxygen reductases originally reduced other compounds, for example, nitric oxide (for a review see Ducluzeau *et al.*, 2009). Recently, it has been found that facultative anaerobe *E. coli* is

FIG. 4. Reactions involving O₂/H₂O₂ in organisms performing anoxygenic photosynthesis (Bacteria), oxygenic photosynthesis (cyanobacteria and plants), and in non-photosynthesizing organisms (animals and fungi). (a), (b), (c), (d) description is the same as in Fig. 2; o. an—obligate anaerobes, f. an—facultative anaerobes. The values were calculated according to Eqs. 1 and 2 (Materials and Methods). Values represent means $\pm$ SD; n =number of species, obligate anaerobes: $n=3$ (Bacteria), facultative anaerobes: $n=3$ (Bacteria), aerobes: $n=6$ (cyanobacteria), $n=9$ (plants), $n=7$ (animals and fungi). The same letters above bars indicate no significant differences between means, at least at the level of $P < 0.05$ (Kruskal-Wallis test and Dunn's test as a post test).

able to perform aerobic respiration at extremely low (≤ 3 nM) concentration of O₂. If the primordial Earth atmosphere contained $ca. 10^{-5}$ of the present atmospheric level of O₂ (see Introduction), such a concentration of oxygen could have supported aerobic respiration, even before photosynthetically induced vast amounts of O₂ arose in the atmosphere $ca. 2.4$ Ga (Stolper *et al.*, 2010). During recently performed metagenomic studies, a new intracellular aerobic pathway in apparently anaerobic denitrifying bacterium *Candidatus Methyloirabialis oxyfera* was identified (Ettwig *et al.*, 2010). This result reveals that O₂ could have been available to the first microorganisms not only from abiotic sources but also from internal biochemical pathways even before oxygenic photosynthesis evolved (Ettwig *et al.*, 2010).

FIG. 5. The presence of reactions/pathways representative of aerobes in obligate anaerobes and facultative anaerobes. (a) the percentage of organisms performing aerobic respiration, (b) the percentage of organisms performing ROS removal reactions, (c) the percentage of organisms where both aerobic respiration and ROS removal reactions are present. The values were calculated according to Eq. 7 (Materials and Methods). The dashed lines represent a reference value for aerobes=100%; n =number of species, $n=40$ (obligate anaerobes), $n=31$ (facultative anaerobes).

The number of reactions involving O₂ in organisms that perform anoxygenic photosynthesis (strict and facultative anaerobic bacteria) and oxygenic photosynthesis (cyanobacteria, plants), and in eukaryotic organisms that use O₂ for respiration (animals/fungi), could indicate that evolution of cyanobacteria, as compared to strict and facultative anaerobes, was very slow and that the core of O₂-involving reactions/pathways remained similar in photosynthesizing anaerobes and facultative anaerobes. Even heterotrophic aerobes, as “consumers” of O₂, showed an insignificant quantity of reactions involving O₂ in comparison to other photosynthetic organisms (Fig. 4). In contrast, plants that perform the same oxygenic photosynthesis are considerably more “overloaded” by O₂-involving reactions/pathways than cyanobacteria (Fig. 4a, 4b). The differences between cyanobacteria and plants, mentioned above, require further study (see discussion below).

4.2. Reactions involving H₂O₂

Although it is not possible to separate O₂-utilizing and ROS-removing reactions completely, in that some of these reactions overlap with each other (*e.g.*, see Eqs. 4 and 6), we observed differences between the amount of O₂-and

H₂O₂-involving reactions for various groups of organisms. In general, the data concerning H₂O₂-utilizing reactions are fairly uniform (Fig. 2c, 2d; Fig. 3c, 3d) when compared to that of O₂-utilizing reactions (Fig. 2a, 2b; Fig. 3a, 3b). This phenomenon was most pronounced when anaerobes that perform anoxygenic photosynthesis were compared to oxygen-producing cyanobacteria and plants (Fig. 4c, 4d; see also discussion below). There were no differences in the amount of H₂O₂-utilizing reactions between cyanobacteria/plants and the other organisms analyzed (Fig. 4c, 4d), which strongly indicates the early evolution of reactions utilizing H₂O₂, as has been suggested previously (McKay and Hartman, 1991). These results might also suggest that cyanobacteria evolved at the same time and under the same conditions as obligate anaerobes. In light of recent geochemical studies that focused on hematite minerals, cyanobacteria might have even appeared earlier than anaerobes, at least in some isolated areas (Hoashi *et al.*, 2009). Recently, the complete genome of the anaerobic and thermophilic bacterium *Candidatus Desulfurudis audaxviator* was sequenced. Although this organism lives in anoxic and hot conditions (48–62°C) at a depth of *ca.* 2.8 km below Earth's surface, it shows the presence of a sequence for Mn/FeSOD (Chivian *et al.*, 2008). In line with the presented model, the generation of a superoxide anion radical, a substrate for SOD, might be induced by the radioactive decay of uranium present in the natural environment of this organism (Chivian *et al.*, 2008). The production of H₂O₂ at the rock-water interface has also been reported, which suggests that ROS must have been generated long before the rise of free O₂ in the atmosphere (Balk *et al.*, 2009). The occurrence of ROS removal systems in so many strict anaerobes (Fig. 5) suggests that the production of ROS was unavoidable even under the globally anoxic environment of early Earth.

It cannot be excluded from these results that ROS, such as H₂O₂, a hydroxyl radical, and a superoxide anion radical, could also have affected the evolution of oxic pathways. The suggestion of ROS control before the development of oxygenic photosynthesis and before the photosynthetic rise of atmospheric O₂ is strictly in line with theoretical data showing that photochemistry could produce ROS, mainly H₂O₂ on primordial Earth (Towe, 1978; McKay and Hartman, 1991; Liang *et al.*, 2006; Haqq-Misra *et al.*, 2011). Moreover, the analyses performed here strongly suggest that, even if the primordial organism (LUCA) was not able to use O₂ as the final electron acceptor (aerobic respiration) or if O₂ utilization was limited, it tolerated a local increase in O₂ concentration as a result of ROS detoxification, for example, due to CAT activity (Eq. 6).

4.3. The alternative hypothesis and horizontal gene transfer (HGT)

The commonly accepted explanation of processes involving oxygen and ROS removal in strict anaerobes is that they have acquired O₂/ROS-utilizing enzymes as a specific adaptation to an environment where oxygen sporadically occurred. It cannot be excluded that genes for O₂/ROS metabolism and antioxidant enzymes were acquired by obligate anaerobes from aerobes later during the evolution of life as a kind of preadaptation to possible future transient exposure to oxygen or ROS, or both (Imlay, 2002; Briou-

khanov and Netrusov, 2004). In this context, our conclusion concerning the presence of antioxidant enzymes in LUCA might be of less importance if horizontal gene transfer (HGT) had occurred many times during evolution between three domains of life as has been suggested for many other genes (Gribaldo and Brochier, 2009). Interdomain HGT events have been proposed recently for some classes of cytochrome oxidases (Brochier-Armanet *et al.*, 2009). On the other hand, HGT strongly depends on the environmental conditions, such as temperature, pH, salinity, and so on, where organisms live. For example, prokaryotes preferentially acquire genes from other prokaryotes when both share the same aerobic/anaerobic environments (Jain *et al.*, 2003). As evidence for this hypothesis, Passardi *et al.* (2007) indeed found that HGT for "antioxidant genes" encoding catalase-peroxidases occurred between prokaryotes showing similar O₂ tolerance. It should be noted that even if HGT is an important evolutionary phenomenon, it does not affect all genes to the same extent (Gribaldo and Brochier, 2009). In our opinion, such a collective and synchronized transfer of the genes of enzymes of aerobic respiration and antioxidant enzymes (*e.g.*, SOD, CAT) between aerobes and strict anaerobes was considerably less probable than, for example, between aerobes only. Among antioxidant enzymes, superoxide reductase (SOR, Eq. 5) has been identified predominantly in those strict anaerobes and microaerophiles that belong to Bacteria or Archaea (Imlay, 2002; Lucchetti-Miganeh *et al.*, 2011). The very rare occurrence of SORs in aerobic organisms indicates generally that anaerobes could not have acquired these enzymes by recent HGT from aerobes, because they occupied different habitats. Moreover, it suggests that these enzymes could appear long before the rise in O₂ in Earth's atmosphere. Based on this, we surmise that the HGT of many "oxic genes" between aerobic and anaerobic Archaea and Bacteria was not essentially responsible for the relatively wide spread of different O₂-utilizing reactions and ROS removal pathways in contemporary anaerobes.

One possible explanation of such surprising results is the fact that the analysis did not take into consideration all the strict anaerobes, facultative anaerobes, and aerobes whose genomes have been sequenced and annotated to date. Moreover, not all metabolic pathways are empirically verified, nor are all databases currently updated. Nevertheless, we suggest that an extrapolation made on the basis of ~100 genomes reveals real differences between these groups of organisms, and it offers a new path for studies concerning the earliest events during the evolution of living beings and for speculation on the attributes of LUCA.

4.4. Conclusions and implications for further research

The presented data suggest that the hypothetical LUCA was "equipped" with reactions involving O₂/H₂O₂. Based on this, the presence of low concentrations of ROS, such as H₂O₂, and O₂ should be taken into account in theoretical models of the early evolution of Archean oceans/atmosphere and life. However, no possible sources of ROS were identified. Nevertheless, the obtained results might indicate that the ability of ROS detoxification did not emerge as a response to an increase in O₂ level produced by cyanobacteria but that it was a crucial adaptation to weakly oxic (ROS)

primordial microenvironments as a result of abiotic photochemical processes on early Earth. This adaptation could have allowed for the very early appearance of oxygenic photosynthesis. Thus, the hypothesis concerning the occurrence of ROS-utilizing genes/enzymes and at least O₂ tolerance by the first living organisms cannot be excluded and requires further study.

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Abbreviations

CAT, catalase; HGT, horizontal gene transfer; LUCA, Last Universal Common Ancestor; ROS, reactive oxygen species; SOD, superoxide dismutase (Fe/MnSOD, iron/manganese form of SOD); SOR, superoxide reductase.

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