When anaerobes encounter oxygen: mechanisms of oxygen toxicity, tolerance and defence

Zheng Lu[1] ✉ *and James A. Imlay*[2] ✉

Abstract | The defining trait of obligate anaerobes is that oxygen blocks their growth, yet the underlying mechanisms are unclear. A popular hypothesis was that these microorganisms failed to evolve defences to protect themselves from reactive oxygen species (ROS) such as superoxide and hydrogen peroxide, and that this failure is what prevents their expansion to oxic habitats. However, studies reveal that anaerobes actually wield most of the same defences that aerobes possess, and many of them have the capacity to tolerate substantial levels of oxygen. Therefore, to understand the structures and real-world dynamics of microbial communities, investigators have examined how anaerobes such as *Bacteroides*, *Desulfovibrio*, *Pyrococcus* and *Clostridium* spp. struggle and cope with oxygen. The hypoxic environments in which these organisms dwell — including the mammalian gut, sulfur vents and deep sediments — experience episodic oxygenation. In this Review, we explore the molecular mechanisms by which oxygen impairs anaerobes and the degree to which bacteria protect their metabolic pathways from it. The emergent view of anaerobiosis is that optimal strategies of anaerobic metabolism depend upon radical chemistry and low-potential metal centres. Such catalytic sites are intrinsically vulnerable to direct poisoning by molecular oxygen and ROS. Observations suggest that anaerobes have evolved tactics that either minimize the extent to which oxygen disrupts their metabolism or restore function shortly after the stress has dissipated.

Reducing equivalents
Chemical species that are capable of transferring the equivalent of one electron in redox reactions.

[1]*Department of Biology, Guangdong Provincial Key Laboratory of Marine Biotechnology, Institute of Marine Sciences, Shantou University, Shantou, China.*

[2]*Department of Microbiology, University of Illinois, Urbana, IL, USA.*

✉*e-mail: lzheng09@stu.edu.cn; jimlay@illinois.edu*

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The primordial atmosphere of Earth was virtually anoxic, containing less than 10^{-5} of the present level of molecular oxygen (O_2)[1,2]. It was in this circumstance that life appeared 3.8 billion years ago (Ga) and in which the basic biochemical mechanisms and metabolic pathways of organisms were established[3,4]. Photosystem II appeared approximately 3 Ga; it enabled ancient bacteria to acquire reducing equivalents from water, and thereby liberated photosynthetic microorganisms from a dependence upon electron sources such as hydrogen and hydrogen sulfide[5–7]. It also produced molecular O_2 as a by-product. Because O_2 crosses cell membranes at high rates, the early users of this photosystem did not suffer from elevated levels of intracellular O_2. Furthermore, the O_2 that diffused out of these cells was chemically scavenged by abiotic reactions with environmental reductants, notably dissolved ferrous iron and sulfur species, and for the next one thousand million years the seas and atmosphere remained hypoxic[6,7]. However, by ~2.5 Ga these reductants had largely been titrated, and molecular O_2 levels gradually rose. Atmospheric O_2 stabilized at ~10% of contemporary levels, before it finally rose again starting 0.8 Ga[8].

In response to this opportunity, anaerobic respiratory chains of some bacteria evolved such that they could exploit O_2 as a novel electron acceptor. Another population of microorganisms maintained their anaerobic styles of metabolism, and their contemporary descendants dwell in sediment and gut habitats that are hypoxic and anoxic zones. Such environments are effectively shielded from O_2 by the vigorous respiratory actions of adjacent layers of aerobic and facultative anaerobic bacteria[9–11]. These anaerobes are notable for their inability to sustain their core metabolism when they are exposed to O_2. It was tempting for microbiologists to conclude that these anaerobes failed to evolve in response to oxygenation of the planet, and that this failure is what constrains them to anoxic microenvironments. One of the aims of this Review is to correct that view: anaerobic microorganisms actually share most of the oxidative defences of their aerobic brethren. Instead, their persistent O_2 sensitivity is a by-product of the types of chemistry that optimize anaerobic energy production.

In this Review, we present evidence that anaerobes have evolved to tolerate — or even exploit — the

occasional incursion of low levels of O_2. However, excess O_2 arrests their growth, and we review in vitro and in vivo studies showing that O_2 does so by disabling a small group of specialized enzymes that have key roles in anaerobic metabolism. Both molecular O_2 itself and superoxide (O_2^-) that is derived from it are implicated. Oxic–anoxic interfaces are also likely sites of environmental hydrogen peroxide (H_2O_2) generation, and studies reveal that anaerobes respond robustly to protect themselves from it. Finally, we propose that anaerobes are likely to have well-developed strategies to resume growth after a period of O_2 exposure, suggesting that this is a prime topic for further exploration.

Anaerobes experience variable O_2

Dissolved O_2 levels plummet in habitats that contain substantial nutritional opportunities but lack convective systems to replenish the O_2 that is consumed by local microorganisms (FIG. 1). O_2 penetrates poorly into mud and sludge and sub-seafloor layers. Zones near hydrothermal vents are hypoxic both due to the insolubility of O_2 in the super-heated waters that feed them as well as chemical reactions between O_2 and the sulfur species that the vents discharge[12,13]. O_2 gradients have been especially well characterized in the mammalian gut. Although the diverse anatomical geography of the gut creates a range of O_2 microenvironments[10,14], in general O_2 levels rapidly decline between the gut epithelium, which receives an O_2 supply from the vasculature, and the lumen, where O_2 is completely consumed by facultative anaerobic bacteria[15]. Whereas the solubility of O_2 at 37 °C in laboratory culture media is ~210 μM, the vascularized submucosa and colonic muscle wall contain 60–120 μM O_2, the apical mucosal surface adjacent to it is limited to 1–10 μM and the bulk of the lumen is essentially anoxic[16,17] (FIG. 1). Accordingly, ~90% of enteric bacteria are categorized as obligate anaerobes. This label is functionally defined as denoting organisms that do not require O_2 to grow well, and whose growth is blocked by the levels of O_2 to which they were exposed in laboratory tests. However, this black-and-white definition turns out to obscure a substantial range of O_2 tolerance that is pertinent to the structures of microbial communities. For example, in the healthy gut *Bacteroides fragilis* is found in high numbers at the mucosal surface, where the O_2 concentration is elevated[18,19]. Although this bacterium cannot grow in fully air-saturated laboratory environments, it possesses both a classic anaerobic-type NrdD ribonucleotide reductase, which is poisoned by O_2, and an aerobic-type NrdAB enzyme that actually requires molecular O_2 for its activation[20]. Thus, the presence of NrdAB suggests some capacity to tolerate aeration. Indeed, after traumatic injury *B. fragilis* is frequently isolated from abscesses in the peritoneum, which is

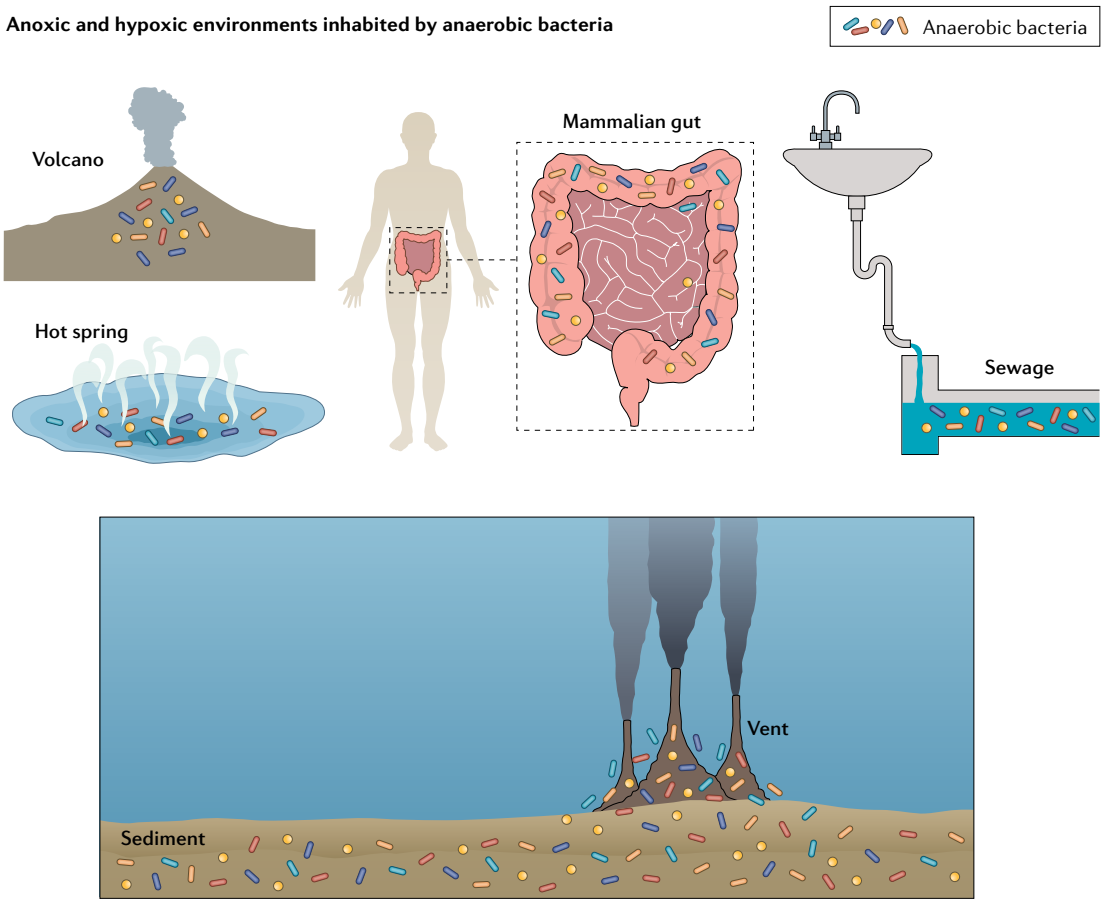

Fig. 1 | **Lifestyles of anaerobes.** Obligate anaerobes are principal occupants of diverse environments that have limited rates of convective oxygenation. Natural systems such as the intestinal tract, rumen, buried sediments and hot springs have slow oxygen (O_2) entry and rapid consumption by peripheral microbes, thereby establishing hypoxic or anoxic conditions.

Table 1 | **O_2 tolerance of obligate anaerobes**

Genus	Species	O_2 level (v/v)	Growth	Refs
Desulfovibrio	*D. vulgaris*	0.04%	Normal	29
		0.08%	Arrested	
Pyrococcus	*P. furiosus*	8%	Grew well	31
Geobacter	*G. sulfurreducens*	10% or less in the headspace	Grew with O_2 as a terminal electron acceptor	128
		Air	Tolerated at least 24 h of exposure	
Bacteroides	*B. caccae*, *B. distasonis*, *B. ovatus*, *B. thetaiotaomicron*, *B. uniformis*, *B. vulgatus*	0.03% (0.3 μM dissolved O_2)[a]	This level of O_2 had no inhibiting effect on growth	56
	B. fragilis	0.1% (1 μM dissolved O_2)[a]	Slow growth in the first 24 h; after that, growth at the anaerobic rate	56
		0.2% (2 μM dissolved O_2)[a]	Slow decrease in culture viability	
	B. oralis	0.4% or less	Grew	129
		Air	Tolerated 24 h	
	B. melaninogenicus	2.5% or less	Grew	129
		Air	Tolerated exposure (48–72 h)	
Faecalibacterium	*F. prausnitzii*	Sterile air	Formed a growth rim on agar media	130
Clostridium	*C. sordellii*, *C. putrificum*, *C. perfringens*	10% or less	Grew	129,131
		Air	Tolerated up to 72 h	
Peptostreptococcus, *Fusobacteria*	*P. elsdenii*, *F. nucleatum*	Air	Tolerated (48–72 h)	129

v/v, volume/volume. [a]Calculations were based on the concentration of saturated dissolved oxygen (O_2) in water at 37 °C (210 μM).

substantially oxygenated (6% O_2)[18]. By contrast, its relative *Bacteroides thetaiotaomicron*, another abundant gut resident, resides deeper in the lumen, lacks the NrdAB isozyme and is less likely to opportunistically infect aerated tissues. Other *Bacteroides* spp. — *Bacteroides ovatus*, *Bacteroides uniformis*, *Bacteroides vulgatus*, and so on — exhibit a range of O_2 sensitivities (TABLE 1).

Similarly, many *Clostridium* spp. can thrive in liquid media under conditions that are more hypoxic than anoxic. Members of this genus reside in low-O_2 soil, marine sediments and the human gut, where they ferment a broad array of organic compounds, including simple and complex carbohydrates and amino acids. However, although they were once regarded as stringent anaerobes, *Clostridium butylicum*, *Clostridium acetobutylicum* and *Clostridium aminocalericum* have been found to grow under continuous microoxic conditions; the acetogenic bacteria *Clostridium magnum* and *Clostridium glycolicum* can grow with 1–6% headspace O_2; and *Clostridium sordellii* and *Clostridium putrificum* tolerate up to 10% O_2 (REFS[21–25]). *Desulfovibrio* spp. thrive in a similarly wide range of habitats, primarily through the dissimilatory reduction of sulfur and nitrogen species; yet sulfate-reducing *Desulfovibrio vulgaris* Hildenborough appears to prefer 0.02–0.04% O_2 for growth[26–29]. *Thermotoga* spp. are bacterial thermophiles that grow well in anoxic cultures but can tolerate up to 0.5% O_2 in the gas phase[30] — which still pales relative to the anaerobic hyperthermophilic archaeon *Pyrococcus furiosus*, which grows well in the presence of 8% O_2 (REFS[31,32]). Implicitly, anaerobes experience — and even welcome — some degree of oxygenation.

Anaerobes have evolved to confront O_2

Recent studies confirm that anaerobes are highly adapted to O_2 exposure. Early surveys reported that superoxide dismutase activity is minimal or absent from many anaerobes[33,34]. However, subsequent work revealed that these microorganisms instead use superoxide reductases to degrade this O_2-derived species[31,35,36]. Intracellular O_2^- is generated as a by-product of metabolic activity in oxic environments, so the implication is that in many anaerobes some level of metabolism continues even when O_2 levels rise.

Even more strikingly, the advent of genomic sequencing revealed that a substantial number of supposedly anaerobic bacteria possess O_2-directed respiratory systems[37–39]. Two general types of respiratory systems have been found: soluble systems that use a cytoplasmic rubredoxin:oxygen oxidoreductase, and membrane-bound terminal oxidases that receive electrons from a classic quinone-based respiratory chain[40–43] (FIG. 2). Many anaerobes — including *Bacteroides* spp., *Desulfovibrio* spp. and the acetogen *Moorella thermoacetica* (originally *Clostridium thermoaceticum*) — have both[42–46].

This discovery prompted two explanations as to why putative anaerobes would contain such enzymes. One idea is that these systems are defensive, scavenging O_2 in order to protect sensitive features of their metabolism from it. The other is that these bacteria consume O_2 for exactly the same reason as do their aerobic peers: to enhance energy conservation.

The O_2-depletion hypothesis seems obvious but, in fact, is plausible only in limited circumstances. Because

Proton motive force
The transmembrane electrochemical gradient, established by metabolic proton translocation, that powers the membrane proteins that synthesize ATP and import substrates.

Antibonding orbitals
High-energy molecular orbitals; they are typically incompletely filled and are the orbitals involved in electron-transfer reactions.

O_2 equilibrates almost instantly across membranes, respiratory chains cannot lower the O_2 concentration inside a bacterium below that of its immediate extracellular environment[47]. Therefore, the only way that respiration would shield cells from O_2 is if a community collectively cleared O_2 from its habitat. This idea has been proposed to explain the ability of *Bacteroides* spp. to create co-infections in erstwhile oxic tissues alongside more fastidiously anaerobic partners[48]. Similarly, because the viscosity of biofilms limits mixing with the macroenvironment, respiration in biofilm-forming organisms can establish local anoxia and support anaerobic processes such as nitrogen fixation[49,50]. However, when a bacterium is a minor member of a diverse consortium, as in the gut, one would not expect it to contribute resources for O_2 clearance.

The other possibility is that the benefit is energetic. The soluble rubredoxin system enhances ATP production by allowing more substrate to flow to ATP synthesis rather than into redox-balancing pathways[51–53]. This strategy resembles that of lactic acid bacteria that use O_2 as a direct acceptor for soluble lactate and pyruvate oxidases[54,55]. By contrast, the membrane-bound chain generates proton motive force by conventional proton translocation. In an important demonstration of this idea, a study showed that the cytochrome *bd* oxidase of *B. fragilis* can enhance its growth when at low but finite O_2 levels[56]. Accordingly, the bacterium was classified as a nanoaerophile. The presence of O_2-directed respiratory chains confirms that many 'anaerobes' can remain metabolically active in O_2-containing habitats — and it muddles the definition of anaerobe.

In short, both laboratory-culture experiments and genomic surveys show that O_2 sensitivity is a matter of degree. Microorganisms that cannot tolerate full aeration nevertheless have evolved strategies that protect themselves from a lower level of O_2, and many have enzymatic machinery that even allow them to exploit it. The evolution of these systems is an appropriate response to the complexity of natural environments.

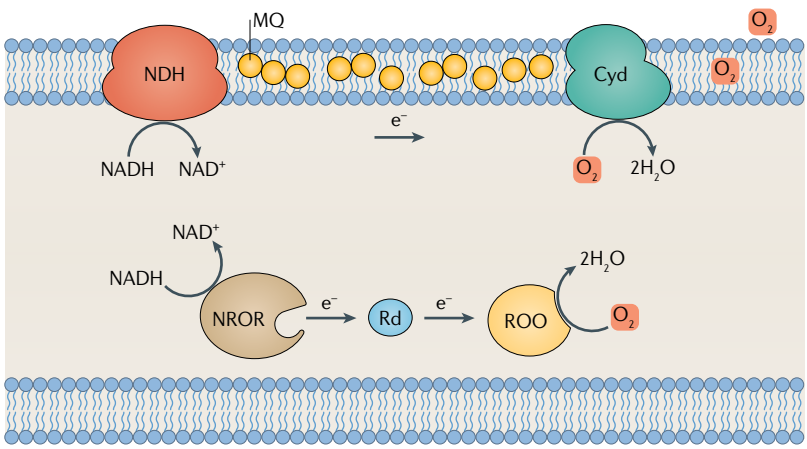

Fig. 2 | **O_2-dependent respiration in anaerobes.** Two types of oxygen (O_2)-directed respiration are found in anaerobes. The membrane-bound cytochrome *bd* oxidase (Cyd) receives electrons from menaquinone (MQ) and upstream dehydrogenases; and the soluble rubredoxin:oxygen oxidoreductase (ROO) obtains electrons from reduced rubredoxin. NDH, NADH dehydrogenase; NROR, NADH:rubredoxin oxidoreductase; Rd, rubredoxin.

O_2 toxicity and growth arrest

The question as to why air-level O_2 arrests the growth of many bacteria and archaea is long-standing. Microorganisms occupy their particular habitats because their central metabolism is well-suited to degrading nutrients that are found there; a reciprocal hypothesis is that anaerobes cannot occupy fully aerobic habitats because O_2 is somehow incompatible with their core metabolic strategies.

Those strategies, of course, are diverse. Anaerobic respiration is a catch-all phrase that denotes electron transfer from a range of reductants (for example, molecular hydrogen, lactic acid and hydrogen sulfide) to a similarly broad range of acceptors (for example, carbon dioxide, sulfate and ferric iron). The underlying molecular mechanisms are equally dissimilar. Fermenting organisms may specialize in catabolizing substrates such as short-chain fatty acids, or in degrading carbohydrates or peptides acquired from deceased primary producers. Yet despite the diversity of their metabolic machineries, biochemical studies support the view that the metabolic efficiency of these organisms hinges upon a few key enzymes that are O_2-sensitive. The energy-producing pathways of methanogens and Clostridia, for example, rely upon enzymes that are quickly poisoned by O_2 in aerobic buffers[57,58]. The study of anaerobic enzymology has thrived in recent years, and it has become clear that a few functional moieties are particular targets of O_2.

O_2 poisons some radical-dependent enzymes whereas others are protected. Anaerobic microorganisms derive most of their energy by splitting their growth substrates into smaller products. Carbohydrates and some amino acids are metabolized through glycolysis to pyruvate — which is subsequently decarboxylated to yield acetyl-CoA, an intermediate in the short pathway that produces acetate and ATP. In non-respiring organisms, the challenge is to split pyruvate without producing NADH: further NADH formation would require reduction of some acetyl-CoA in order to achieve redox balance, thereby cutting into the ATP yield (Supplementary Figure 1). In this regard, the anaerobic enzyme pyruvate formate-lyase (PFL) outperforms better-known pyruvate dehydrogenase, by converting pyruvate to acetyl-CoA and formic acid (FIG. 3). Splitting pyruvate so that the bonding electrons remain with the C1 carboxylate is contrary to the natural tendency of divalent chemistry, and so PFL circumvents this problem by engaging a univalent mechanism[59,60]. PFL is primed for this function by an activase that abstracts a single electron from a glycine residue, creating a resting-state glycyl radical species that initiates a radical-based cleavage[59,61]. This special enzyme improves ATP production during carbohydrate fermentations by 50% or more and enables growth on some substrates that would otherwise be unable to support growth.

The drawback of radical-based chemistry is that radicals are incompatible with O_2. Molecular O_2 has a singular electronic structure: it possesses an even number of electrons, but its two outer-most electrons are spin-paired in separate antibonding orbitals[62]. That is, the molecule is a diradical (Supplementary Figure 2). Radicals react rapidly with other radicals, and so

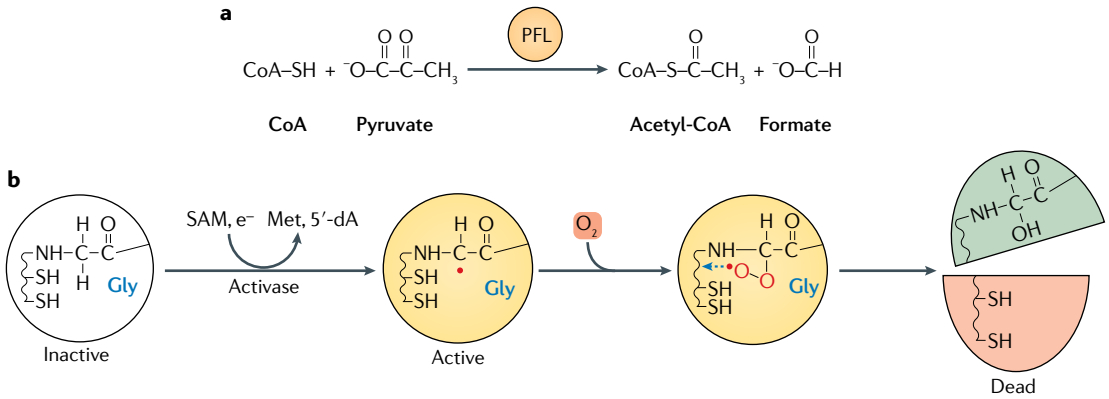

Fig. 3 | **O_2 inactivates pyruvate formate-lyase. a** | Pyruvate formate-lyase (PFL) catalyses pyruvate breakdown without producing NADH; this strategy is energetically economical because it avoids consuming acetyl-CoA to reoxidize NADH. Acetyl-CoA is then available for ATP synthesis (not shown). **b** | PFL is a prototype of the family of glycyl-radical enzymes. The glycyl radical is produced as a post-translational modification by PFL activating enzyme (activase), which activates PFL through a S-denosylmethionine (SAM)-dependent reaction, in which the monoelectronic reduction of SAM generates a 5-deoxyadenosyl radical (5′-dA) and methionine as by-products. Oxygen (O_2) inactivates PFL by adducting the glycyl radical, forming a hydroperoxyl radical species that finally cleaves the polypeptide.

O_2 reacts directly with PFL (FIG. 3). A peroxyl radical species is formed on the erstwhile glycyl radical, and then attacks the polypeptide chain and cleaves the protein backbone[63]. Not only is the catalytic radical lost, but the protein domain that carries the radical dissociates from the protein. Radical–radical reactions can occur at diffusion-limited rates, so even low levels of O_2 inactivate PFL in seconds[64], both in vitro and in vivo. In short, the fermentation strategy that optimizes ATP yield requires an enzyme mechanism that is intrinsically sensitive to molecular O_2.

The anaerobic NrdD ribonucleotide reductase is another glycyl-radical enzyme that conducts a difficult reaction, and it is similarly inactivated by O_2. However, unlike PFL activity, ribonucleotide reduction is also an essential function in aerated cells; therefore, alternative forms of the ribonucleotide reductase have evolved. The di-iron enzyme operative in mammals has an active site similar to that of NrdD — but instead of an exposed glycyl radical, it carries a resting-state tyrosyl radical that is buried deep within the polypeptide to ensure that it is inaccessible to O_2. Other aerobes rely upon a cobalamin cofactor whose initiating adenosyl radical appears and disappears only within the course of the reaction. The evolution of the latter isozymes represents evolutionary adjustments to the aeration of Earth.

O_2 disrupts low-potential metal centres. That O_2 has the capacity to oxidize biomolecules seems self-evident — but its diradical electronic structure prevents its reaction with most biomolecules. O_2 cannot receive a spin-paired electron pair, and most organic molecules are incapable of transferring a single electron. For this reason, O_2 cannot directly oxidize amino acids, carbohydrates, lipids or nucleic acids — the structural molecules from which cells are built. This oxidation barrier is why molecular O_2 was able to accumulate in a world full of reduced carbon.

However, the half-occupied orbitals of O_2 are capable of accepting single electrons. Its moderate univalent reduction potential (−160 mV) allows O_2 to oxidize a subset of biomolecules that are competent at low-potential univalent electron transfers: flavins, quinones and metal centres. These adventitious reactions[65] are not inherently destructive, as the oxidized forms of these moieties are part of their natural catalytic cycles. However, molecular O_2 can also oxidize the metal centres of certain enzymes to redox states that lie outside their normal catalytic valences, and such events can destroy the enzyme activity.

This effect was first documented for simple dehydratases of the aconitase family, which employ a $[4Fe–4S]^{2+}$ cluster to bind and withdraw a hydroxide anion from the substrate. Although the cluster has a non-redox role in this reaction, it can nevertheless be chemically oxidized by molecular O_2, converting the cluster to a $[4Fe–4S]^{3+}$ state that spontaneously degrades to an inactive $[3Fe–4S]^{+}$ form[66–68]. This reaction occurs quickly enough (the half-time is ~30 min) to frustrate study of these enzymes in oxic buffers, but is slow enough that the enzymes are functional in vivo — with further assistance from the cluster-rebuilding activities inside the cell.

Many anaerobes, however, use low-potential metals in enzymes that are crucial to their core metabolism — and over-oxidation of those metal centres can be catastrophic. Most anaerobes transfer electrons to low-potential acceptors: methanogens reduce CO_2 to methane, *Desulfovibrio* spp. reduce elemental sulfur to hydrogen sulfide and *Bacteroides* spp. reduce protons to H_2. To ensure that the terminal electron-transfer reaction is not energetically uphill, the electron movement from substrate to oxidant has to occur through metal centres with potentials that are similarly low — with the unfortunate consequence that their accidental electron leakage to O_2 will be strongly favoured and rapid. Pyruvate:ferredoxin oxidoreductase (PFOR) is an example that has been closely examined. PFOR is an alternative enzyme to PFL, dissimilating pyruvate to generate acetyl-CoA without NADH formation (Supplementary Figure 1); however, instead of generating formate, PFOR

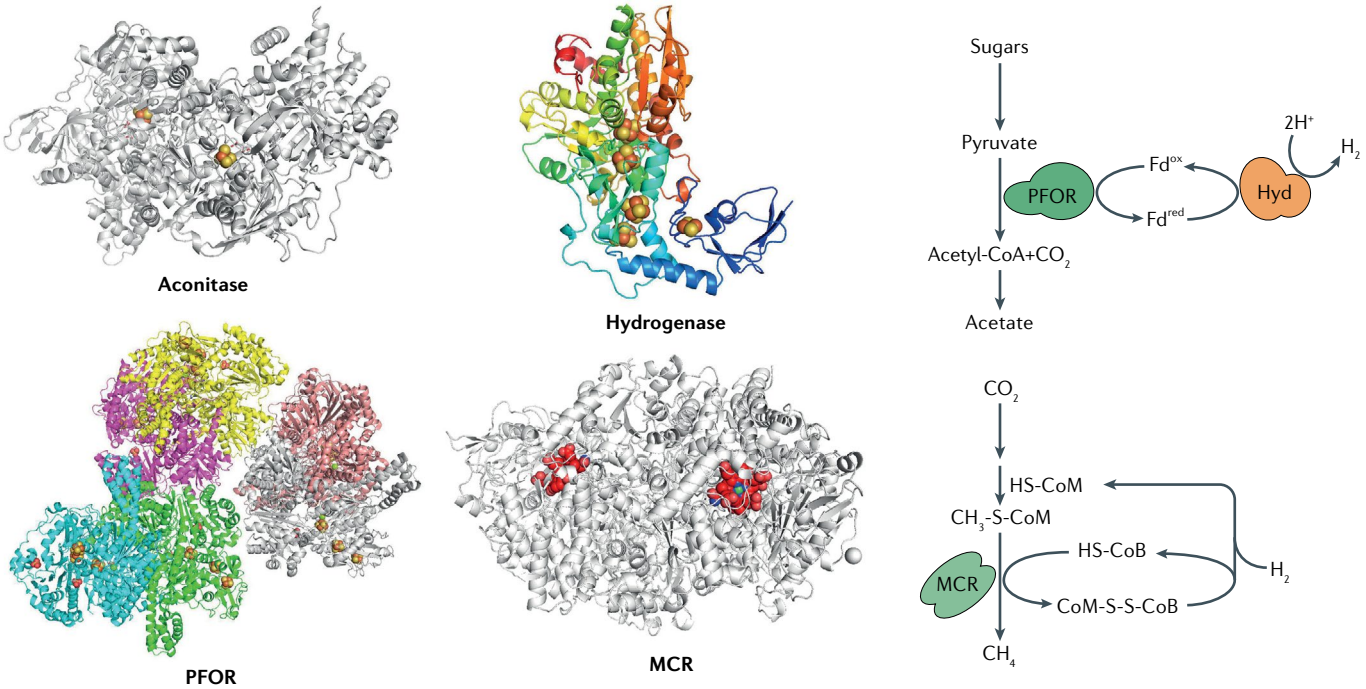

Fig. 4 | **Structures of O_2-sensitive metalloenzymes.** Molecular oxygen (O_2) directly inactivates diverse metalloenzymes featuring low-potential metal centres at or near the protein surface. The structures of representative O_2-sensitive metalloenzymes are shown (left), alongside their pivotal positions in anaerobic metabolism (right): aconitase B from *Escherichia coli* (Protein Data Bank (PDB) ID 1L5J); pyruvate:ferredoxin oxidoreductase (PFOR) from *Moorella thermoacetica* (PDB ID 6CIN); iron-only hydrogenase (Hyd) from *Clostridium pasteurianum* (PDB ID 1FEH); and methyl-coenzyme M reductase (MCR) from *Methanothermobacter marburgensis* Marburg (PDB ID 1MRO). Iron–sulfur clusters are represented by yellow and red balls, nickel-containing F430 of MCR is shown in red with nickel in green. CoM-SH, coenzyme M; CoB-SH, coenzyme B; Fd^{ox}, oxidized ferredoxin; Fd^{red}, reduced ferredoxin.

transfers the C1 bonding electrons to ferredoxin, a small iron–sulfur protein that is a general univalent electron carrier. Ferredoxin subsequently delivers the electrons to a hydrogenase. PFOR, like pyruvate dehydrogenase, uses thiamine to decarboxylate pyruvate, but the electrons then move through a series of iron–sulfur clusters to the enzyme surface, where ferredoxin binds[69,70]. When PFOR is mixed with oxic buffer, its activity quickly disappears. Its clusters are simultaneously bleached, suggesting that cluster over-oxidation by O_2 is responsible for the activity loss[70].

Similar events occur in vitro at the low-potential iron centres of hydrogenases, at both the molybdenum cofactor and iron enzyme of nitrogenase and at the Ni(I) centre of methyl-CoM reductase (FIG. 4): molecular O_2 inactivates them all, and spectroscopic signatures imply that metal oxidations are responsible[71]. The full details of the oxidation processes, and the disposition of the final enzymes, have not yet been demonstrated[72–74]. However, it seems a safe assumption that similar events transpire in vivo and are responsible for the cessation of hydrogen evolution, nitrogen fixation and methanogenesis, respectively, when these microorganisms are aerated.

Those enzymes all catalyse low-potential redox reactions. However, metal-centre oxidation is apparently also responsible for the O_2 sensitivity of a family of non-redox enzymes that are exclusively found in anaerobes. Clostridial consumption of amino acids occurs by Stickland fermentations[75], in which the exergonic oxidation of one amino acid must be redox-balanced by reduction of another to an aliphatic product, such as propionate. The latter process requires the dehydration of non-activated substrates. Dehydrations in aerobes invariably rely upon a carbonyl group adjacent to the proton that is to be removed; resonance provides enough anion stabilization that deprotonation is possible. However, anaerobes must operate upon more-reduced substrates, and some lack a stabilizing carbonyl or carboxylate moiety. To solve this problem, *Bacteroides* spp. employ a long carbon-shuffling pathway to activate the substrate for deprotonation[48]; the pathway is expensive, requiring not only ample synthesis of protein but also of biotin and cobalamin to activate key enzymes (Supplementary Figure 3). By contrast, Clostridia spp. employ a single-enzyme radical mechanism in which an iron–sulfur cluster injects a low-potential electron into the substrate and then withdraws it at the conclusion of the catalytic cycle[76]. The enzyme is initially activated by a separate [4Fe–4S]-containing activase, which receives an electron from ferredoxin and then uses ATP hydrolysis to drive the electron into the dehydratase cluster. Both clusters must operate at low potentials in order to push the electron onto the hydroxyacyl-CoA substrate — and therefore both centres are vulnerable to destructive oxidation by molecular O_2. Thus, low-potential metal centres may be requisite even for non-redox processes that operate upon the aliphatic, non-activated substrates that anaerobes must process.

Anoxic glove boxes
Boxes (chambers) that provide a strict anaerobic atmosphere of 0–5 ppm molecular oxygen (O_2). A palladium catalyst eliminates O_2 by using hydrogen gas as a co-reactant; a vacuum airlock reduces O_2 levels prior to transfer of items into the glove box.

Most enzymes in anaerobes are O_2-tolerant, as are the equivalent enzymes in aerobes. However, in studying the remarkable enzymatic functions of anaerobes — for instance, cleaving pyruvate without disturbing the redox balance or transferring electrons to low-potential oxidants — researchers found that the key enzymes retained activity only when maintained in anoxic glove boxes. Those in vitro studies provide compelling explanations for the O_2 sensitivity of anaerobes — but in vivo studies are needed to confirm that these same enzyme problems recur in vivo.

Studies of O_2 poisoning in vivo: confirmation and expansion of in vitro results. Some enzymes, such as radical *S*-denosylmethionine (SAM) and aconitase class enzymes, lose activity in oxic buffers but work perfectly well inside oxic cells. This observation warns against a simple extrapolation of in vitro events into the living cell. If the rate of enzyme oxidation is moderate, countervailing repair processes[77] may sustain high steady-state activity even when cells are O_2-replete. Furthermore, substrates generally stabilize the metal centres to which they bind[77], thereby likely lowering the rate of enzyme damage in vivo. These quantitative considerations influence the amount of O_2 exposure that an enzyme can tolerate in vivo. To date, there have been only limited physiological studies of how O_2 impinges upon the ongoing metabolism of various anaerobes.

The linkage between O_2 and metabolic dysfunction has been explored in a top-down way in the case of strains of *Bacteroides* and *Desulfovibrio* spp. Under anoxic conditions, the gut bacterium *B. thetaiotaomicron* efficiently ferments glucose to a mixture of succinate, propionate and acetate, but when it is aerated, glucose consumption stops[78]. Growth resumes after anoxia is restored. Its normal fermentation pathways are depicted in FIG. 5. Carbohydrate catabolism initially proceeds through glycolysis, with concomitant reduction of NAD^+. The NADH thus formed is reoxidized by reduction of phosphoenolpyruvate (PEP) to succinate. This process entails electron flow through an energy-conserving anaerobic respiratory chain. Residual PEP is converted to pyruvate and then decarboxylated, either by PFOR or by PFL[70,79] (FIG. 5).

Metabolic analysis of O_2-poisoned cells revealed two points of inhibition: at the decarboxylation of pyruvate by PFOR and PFL, and at the dehydration of malate to fumarate by fumarase[78,79]. Either block would be sufficient to prohibit glucose fermentation. A common model of obligate anaerobiosis posits that toxicity arises from the reactive oxygen species (ROS) O_2^- and/or H_2O_2 (REF.[33]); however, as predicted by in vitro studies, the inactivation of both PFOR and PFL was shown to be mediated directly by molecular O_2 itself[70,79,80]. In fact, PFOR cannot be oxidized by O_2^- or H_2O_2, because these species must bind directly to the metals that they oxidize — and those in PFOR are fully coordinated by polypeptide. By contrast, the electronic structure of O_2 allows electrons to hop to it by outer-sphere processes, and so can oxidize the metal centres of PFOR if it merely gets close to them.

PFL is hypersensitive to O_2, but even when cells were fully aerated it required tens of minutes for PFOR to lose activity[79]. The pace of inactivation depended upon O_2 concentration, with little activity loss at 5% O_2 and lower, indicating that PFOR may continue to support the metabolism of *B. thetaiotaomicron* in low-O_2 regions of the intestine.

Interestingly, the sediment-dwelling sulfate-reducing bacterium *Desulfovibrio africanus* also dissimilates pyruvate with PFOR — but its enzyme features a polypeptide extension that substantially augments its O_2 resistance even beyond that of the *B. thetaiotaomicron* PFOR. O_2 exposure triggers the formation of a disulfide bond that locks the extension over the PFOR clusters in a way that may shield them from the close approach of O_2 (REFS[81,82]). The enzyme remains active, albeit with a lower turnover number. Many *Desulfovibrio* spp. live in habitats with O_2 levels that are less controlled than those of the mammalian intestine, and so this structural alteration may enable the bacterium to tolerate oxidative conditions. No analogous adjustment is known that can improve the stability of PFL.

Endogenous ROS impose a second layer of oxidative stress. The other metabolic block in aerated *B. thetaiotaomicron* results from the deactivation of fumarase[68,78] (FIG. 5). However, in contrast to PFOR and PFL, the pathway block at fumarase cannot be ascribed to direct

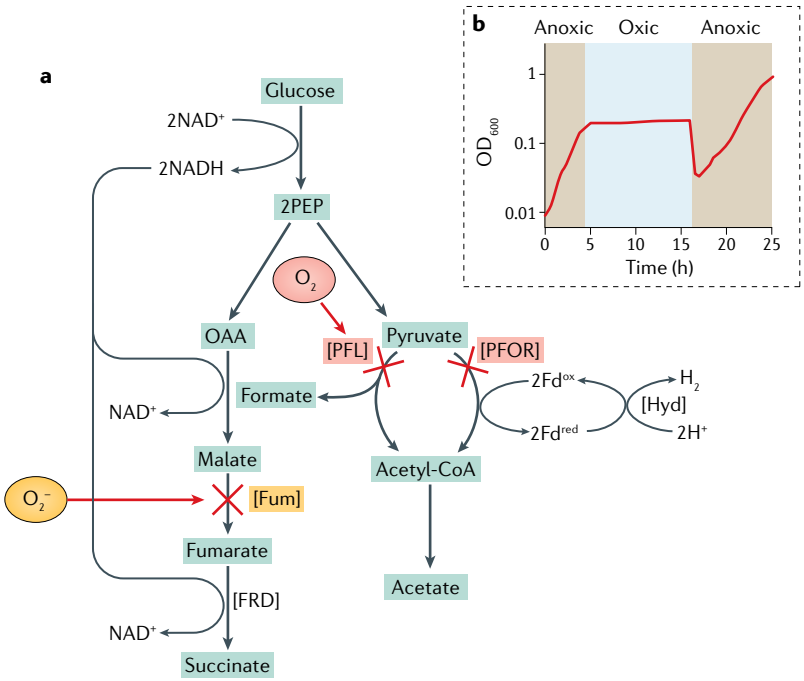

Fig. 5 | **Metabolism in *Bacteroides* spp. is blocked upon aeration and resumes in anoxia.** Oxygen (O_2) poisoning results from inactivation of several enzymes. **a** | In aerated cells, endogenous O_2^- inactivates fumarase in the redox-balancing succinate branch of central metabolism. At the same time, molecular O_2 directly inactivates both pyruvate formate-lyase (PFL) and pyruvate:ferredoxin oxidoreductase (PFOR), creating a bottleneck in the pathway leading to acetate. Either block is sufficient to prohibit growth. Enzymes indicated in square brackets. **b** | Cell viability is not lost during the period of O_2 exposure. When anoxia is restored, growth soon resumes (drop in OD_{600} reflects dilution into anoxic medium). Fd^{ox}, oxidized ferredoxin; Fd^{red}, reduced ferredoxin; FRD, fumarate reductase; Fum, fumarase; Hyd, hydrogenase; OAA, oxaloacetic acid; PEP, phosphoenolpyruvate. Part **b** reprinted from REF.[44], CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/).

damage by molecular O_2, and this observation suggests a second mechanism of oxidative poisoning. Although eukarotes and some bacteria employ metal-independent fumarases, most anaerobes — including *Bacteroides* spp. — have a class I fumarase of the aconitase class with a [4Fe–4S] cluster as its catalytic cofactor. The cluster of these enzymes protrudes into the active site so that an iron atom can directly bind the substrate, and this solvent exposure makes it vulnerable to the approach of oxidants[83–87] (Supplementary Figure 4a). Molecular O_2 can slowly oxidize the fumarase cluster, but it does so on a long timescale that has little biological impact[68]. However, studies with the enzymes of *Escherichia coli* established that O_2^- and H_2O_2 can avidly bind and oxidize the cluster. The oxidized cluster is unstable and loses its catalytic iron atom, and the enzyme is inactivated. Other hydratases and dehydratases of the same catalytic class are also vulnerable to these oxidants[83,88,89]. The failure of fumarase in aerated *B. thetaiotaomicron* suggested that O_2^- and H_2O_2 rise to toxic levels. Indeed, it was shown that endogenous O_2^- is the culprit, and that the [4Fe–4S] enzymes aconitase and isopropylmalate isomerase are similarly poisoned[68].

According to sequence analyses, O_2-sensitive [4Fe–4S] dehydratases are common to other anaerobes too, including the genera *Desulfovibrio*, *Clostridia* and *Thermoanaerobacter*[90]. These enzymes are indispensable for the biosyntheses of amino acids belonging to branched-chain (Leu, Ile and Val) and α-ketoglutarate (Glu, Gln, Pro and Arg) families, suggesting that the metabolic failures observed in *Bacteroides* spp. may occur generally whenever enough O_2 encroaches upon anaerobic communities.

It is notable that whereas some microorganisms — and higher organisms — have acquired oxidant-resistant versions of fumarase, others continue to rely upon more vulnerable isozymes. Why is this? The apparent answer is that the cluster-free fumarases are less catalytically efficient, analogous to the *D. africanus* PFOR[91,92]. On balance, high-turnover — albeit oxidant-sensitive — isozymes may be the better choice for microorganisms that dwell in niches that are rarely oxygenated.

ROS studies in *E. coli* have determined that excess O_2^- can also inactivate a family of enzymes that use a single Fe(II) cofactor to catalyse non-redox reactions[93,94] (Supplementary Figure 4b). In fact, two representatives of this family — ribulose-5-phosphate 3-epimerase and peptide deformylase — also lost activity when *B. thetaiotaomicron* was fully aerated[68].

Why is O_2^- toxic in anaerobes? So, although molecular O_2 directly mediates some cell injuries to anaerobes, its derivative O_2^- can also disrupt metabolism. Yet the enzymes that O_2^- inactivates in aerated *B. thetaiotaomicron* remain fully functional in aerated *E. coli*. Why the difference?

The superoxide-sensitive enzymes of *Bacteroides* spp. are no more intrinsically reactive with O_2^- than are their homologues from facultative and aerobic bacteria. Indeed, when *E. coli* and *B. thetaiotaomicron* enzymes were swapped between the two species, both enzymes remained active in aerated *E. coli*, but both quickly lost activity in *B. thetaiotaomicron*[68]. This result led to the recognition that ROS are simply generated in higher volume in the anaerobe.

The rate of ROS formation in a bacterium can be discerned by tracking the release of H_2O_2 from a mutant whose scavenging systems are knocked out[95]. Because O_2^- and H_2O_2 are made by similar processes[65], these measurements provide predictions about O_2^- production as well. In one study, a *B. fragilis* mutant was constructed that has little ability to scavenge H_2O_2, and the authors found that when the strain was aerated, H_2O_2 effluxed from these cells several-fold faster than it effluxes from non-scavenging *E. coli* mutants[43]. A similar experiment showed that aerated *B. thetaiotaomicron* produced H_2O_2 ten times more quickly than *E. coli* strains did[96]. Thus, it is not surprising that the aeration of *Bacteroides* spp. causes superoxide-sensitive pathways to fail and growth to stop.

In general, when cells grow in oxic environments, molecular O_2 adventitiously abstracts electrons from the reduced flavins or metal centres of some redox enzymes, thereby forming O_2^- and H_2O_2 (REFS[44,97,98]). Because these events occur in proportion to collision frequency, the rate of ROS production is proportionate to the O_2 concentration. Indeed, the rate of superoxide-mediated enzyme damage in *B. thetaiotaomicron* is modest at low O_2 concentrations but is crippling upon full aeration[68].

The source of all these ROS is not certain. *B. thetaiotaomicron* has an anaerobic respiratory chain that carries large fluxes of electrons through its flavins and metal centres, but follow-up work indicated that this system is unlikely to be the primary producer of ROS[34,98,99]. The deletion of the rubredoxin-dependent electron chain (FIG. 2) caused only a modest drop in H_2O_2 production[44]. One attractive candidate is the ferredoxin-mediated flow of electrons from pyruvate to hydrogenase (FIG. 5). The PFOR–ferredoxin–hydrogenase pathway involves a series of low-potential ferredoxin-type metal centres. The reduction potentials of ferredoxins typically range from –350 to –455 mV, which makes electron transfer to molecular O_2 (–160 mV) favourable[100,101]. Indeed, biochemical reports have shown that O_2 oxidizes the ferredoxins of *Clostridium pasteurianum* and spinach, generating O_2^- (REF.[102]). For now, this point remains unsettled. The primary ROS source remains of interest, as its identity might shed light on the possibility that endogenous ROS-mediated enzyme damage is a common phenomenon when anaerobes encounter O_2.

Defences against environmental H_2O_2

Damage to enzymes from molecular O_2 or endogenous O_2^- can block the growth of anaerobes, but this damage is not lethal: once anoxia is restored, enzymes can be repaired or replaced, and growth can resume. However, DNA damage is potentially deadly. DNA oxidation is precipitated by the Fenton reaction, in which cellular ferrous iron transfers an electron to H_2O_2. The resultant hydroxyl radical can oxidize both nucleotide bases and sugars, creating impassible lesions that block DNA replication.

Facultative anaerobic bacteria activate H_2O_2 stress responses only when H_2O_2 flows into the cell from the

Box 1 | **Oxidative stress in hypoxic environments**

Natural anoxic environments may be intermittently oxygenated, leading to the formation of reactive oxygen species (ROS). At the periphery of deep-sea vents, cool oxygenated seawater mixes with hydrothermal vent fluid; oxygen (O_2) oxidizes hydrogen sulfide (H_2S) and forms extracellular superoxide (O_2^-) and hydrogen peroxide (H_2O_2). This ROS-forming reaction also occurs at the interface of sediment and water in marine environments. In the mammalian gastrointestinal tract, oxygenation can be enhanced by disruptive stresses. For example, inflammatory bowel diseases, including Crohn's disease and ulcerative colitis, are associated with dysbiosis of the colon microbiota that may be mediated by enhanced oxygenation. Oxygenation can also increase upon changes in the host physiology that accompany antibiotic treatment[15,120,132,133] or by the presence of pathogens. This O_2 plausibly triggers several sources of colonic H_2O_2. Sulfide that is generated by luminal sulfur-reducing bacteria may react abiotically with O_2 that it encounters at the oxic–anoxic interface, producing H_2O_2 (REFS[134–136]). Gut inflammation releases H_2O_2 that is produced by host macrophages[121,122,137]. Some intestinal commensal bacteria even actively induce ROS production, mainly in the form of H_2O_2 within enterocytes. For example, members of the genus *Lactobacillus* produce and shed small formylated peptides, which are perceived via formyl peptide receptors localized on the apical surface of gut epithelia. Those receptors then activate NADPH oxidases (NOX) that generate ROS. To deal with perturbations such as these, anaerobes possess inducible defences against exogenous oxidants.

environment. By contrast, full aeration is sufficient to activate those stress responses in many anaerobes, presumably due to the high rate of internal H_2O_2 formation from the autoxidation of their low-potential electron carriers. One study showed that *B. fragilis* responds to aeration by inducing catalase, the alkyl hydroperoxidase (AhpC), thioredoxin peroxidase (Tpx) and rubrerythrin peroxidases[103]. A similar response was observed for *B. thetaiotaomicron*[96]. *C. acetobutylicum* represses H_2O_2-scavenging enzymes using PerR, a transcriptional repressor that is deactivated when H_2O_2 oxidizes its Fe(II) cofactor; this system, too, is activated by simple aeration[104]. If this response is not triggered quickly enough, internal Fenton chemistry is lethal.

Surprisingly, various evidence suggests that bacteria may need to deal with H_2O_2 even when bacteria are still in the anaerobic gut. For example, the enteric bacterium *E. coli* induces a cytochrome *c* peroxidase (Ccp) upon H_2O_2 exposure, but only when O_2 is absent[105]. This arrangement refutes the idea that H_2O_2 stress only occurs in highly oxygenated habitats. The apparent purpose of the membrane-bound enzyme is to enable the bacterium to use H_2O_2 as an alternative respiratory electron acceptor[106]. *Bacteroides* spp. are among the many other bacteria that carry Ccp homologues[103].

How does H_2O_2 arise in hypoxic habitats (BOX 1)? Reduced sulfur species are released by sulfate-reducing bacteria in the intestinal lumen, and they may converge with O_2 that seeps in from the epithelium, triggering abiotic ROS formation at the oxic–anoxic interface. Furthermore, some lactic acid bacteria that thrive at such zones generate H_2O_2 as a stoichiometric product of their central metabolism[55,107]. Similar events are likely at analogous interfaces in soil and elsewhere. The importance of all this H_2O_2 is underscored by the ubiquity and high titres of bacterial defences against it. Experimental studies and genomic sequence data have revealed that anaerobes protect themselves from environmental H_2O_2 by deploying most of the same defensive tactics that were originally identified in aerobes. *Bacteroides*, *Desulfovibrio*, *Pyrococcus* and *Clostridium* spp. synthesize both NADH peroxidase (Ahp) and rubrerythrins to scavenge H_2O_2; *Bacteroides and Desulfovibrio* spp. have catalase in addition[21,25,26,31,32,96,103,108,109].

Anaerobes sense H_2O_2 and launch these responses using the same ROS-sensing transcription factors that were originally discovered in aerobes. OxyR is the paradigmatic H_2O_2-sensing transcription factor[110]. It was originally identified and characterized in *E. coli*, but is broadly distributed among anaerobic bacteria[103,111,112]. The greatest threat of H_2O_2 is that it will react with intracellular Fe(II) to generate lethal DNA damage; therefore, although the full regulon can vary from one bacterium to another, it generally includes enzymes that scavenge H_2O_2 (catalase and peroxidases) and that sequester intracellular Fe(II) (the mini-ferritin Dps)[113,114].

Other bacteria employ the iron-based sensing system of PerR[115,116] to regulate their H_2O_2 stress responses. This regulon is distributed among some anaerobes and has been characterized in *C. acetobutylicum* and *D. vulgaris* Hildenborough[104,117,118]. It includes many of the same genes that OxyR controls in other bacteria, including Ahp, catalase and Dps. Therefore, our view today is almost fully reversed from that of earlier days: anaerobes are obviously subjected to H_2O_2 stress in their lifestyle, and they have evolved a full suite of strategies to defend themselves against it.

It is worth noting that early work may have led researchers to overestimate the lethal effects of aeration upon anaerobes. Laboratory experiments that tested this phenomenon often abruptly introduced O_2 directly into erstwhile anoxic media; such media often contained high levels of reduced sulfur species, including cysteine, sulfide or even dithionite. Consequent redox reactions would have generated unnaturally high concentrations of H_2O_2, which could diffuse into cells and produce lethal DNA damage[119].

Reduction potential and growth

It is sometimes asserted that even in the absence of O_2 species some anaerobes cannot grow in an environment with a high redox potential. This formulation obscures more than illuminates: no single reduction potential accurately describes an environment. The redox partners that comprise growth substrates — for example, H_2 and CO_2 for methanogens, or lactate and nitrate for denitrifiers — are not in thermodynamic equilibrium with one another, and in fact the organism derives energy from catalysing their approach towards equilibrium. Instead, the statement that a high redox potential interferes with the growth of an anaerobe typically means that a key reduced growth substrate is insufficiently available. In natural mixed populations, the addition of sulfate, for example, can suppress the growth of methanogens because it favours sulfate-reducing bacteria in their mutual competition for H_2 (REF.[110]). In pure cultures, some microorganisms require hydrogen sulfide, rather than sulfate, as their source of essential sulfur atoms; others may be able to import only ferrous iron rather than its ferric form. It is preferable to identify the specific problem rather than to ascribe it to a putative overarching reduction potential.

How do anaerobes rebound from aeration?

Recovery from oxygenation is likely to be a key event in the lifestyle of anaerobes — whether for *Desulfovibrio* spp. that dwell near oxic interfaces or for the methanogens that cope with cycles of desiccation and rainfall that bring oxygenated water into soil crevasses. The issue is highly pertinent to the establishment and stability of the gut microbiome: not only do these microorganisms encounter stress when transiting through the aerobic world from one host to another, but disruptive antibiotic treatments and inflammation events increase the penetration of O_2 into the intestinal lumen[120–122]. Opportunistic pathogens such as *B. fragilis* and *Clostridium perfringens* must survive aeration when they disseminate into oxic tissues. However, our understanding of how cells recover from oxidative stress is more advanced in aerobes and facultative anaerobic bacteria. A take-home lesson from that work is that those bacteria employ various strategies to repair and reactivate enzymes that were damaged by O_2^- or H_2O_2. Iron–sulfur clusters are repaired with the help of inducible cluster-assembly systems[78,87,123,124], and mononuclear Fe(II) enzymes are re-metallated, either with iron itself or with oxidant-resistant manganese[93,125]. The same reactivations occur when stressed anaerobes return to anoxic habitats.

So, it seems logical that anaerobes must also have evolved systems that allow them to cope with O_2-mediated injuries to glycyl-radical enzymes and metalloproteins. The speed with which growth resumes after periods of aeration (FIG. 5) tends to support this conjecture. Ironically, one elegant tactic to restore the activity of O_2-cleaved PFL was actually discovered in *E. coli*, a facultative bacterium that uses PFL in anoxic environments. After a period of O_2 exposure, the resumption of anoxia stimulates the synthesis of GrcA, a small protein that physically resembles the glycyl-radical domain of PFL that is lost upon aeration[126]. GrcA binds to the remaining PFL fragment and, in collaboration with the PFL-activating enzyme, regenerates an active radical-containing complex. However, GrcA seems to be restricted to facultative bacteria — organisms whose movement between anoxic and oxic habitats is likely to be frequent. GrcA is absent from anaerobes that do not tolerate full aeration. So, do they, too, have mechanisms that allow them to recover after O_2 exposure?

It has been pointed out that an individual bacterium that colonizes a new habitat likely spends as much time in transit as it does in its preferred niche[127]. Therefore, it is almost certain that evolution has equipped anaerobes not only to survive O_2 stress but also to recover swiftly from it. The tactics by which they do so are largely unstudied, and we regard this as the next challenge in this field.

Conclusions

As obligate anaerobes have become genetically accessible, and as researchers have become much better at investigating their physiologies and the biochemical mechanisms of O_2-sensitive enzymes, the field has finally acquired enough knowledge to put together the puzzle of why O_2 is so disruptive to them. The goal is both to illuminate the molecular bases of their incapacity to grow in oxic environments and to reveal the adaptations that provide them with some level of O_2 tolerance.

The notion that anaerobes simply neglected to evolve antioxidant defences has been refuted: these bacteria possess the majority of the same antioxidant defences and regulatory systems that are found in facultative anaerobic bacteria and aerobes. Instead, their intractable problem with O_2 derives from the chemical mechanisms by which they optimize their anaerobic success. Low-potential electron flow is necessary to achieve redox-balanced fermentations and to effect anaerobic respiration, but it relies upon univalent carriers that are naturally vulnerable to adventitious oxidation by molecular O_2. The over-oxidation of metal centres to unstable valences is destructive to the key enzymes that underlie the redox processes that are fundamental to much of anaerobic physiology. Those oxidation reactions are also likely responsible for the excessive ROS that are formed when anaerobes are aerated; such ROS cause another layer of damage. Meanwhile, many anaerobes also employ free-radical mechanisms to enable difficult chemistry — and the free-radical nature of O_2 causes it to disrupt those catalysts by reacting directly with them. Thus, it seems unavoidable that the most effective anaerobes — those that predominate in anoxic habitats — are incapable of thriving when exposed to O_2.

We have moved a long way from the early hypothesis that anaerobes shelter in environments from which O_2 and ROS are completely excluded. What remains to be determined are details of the injuries, measurements of damage rates and elucidation of as yet unknown defensive tactics. Ultimately, we wish to identify the circumstances and dynamics by which O_2 disrupts and reshapes important microbial communities.

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