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MICROREVIEW

The selective advantage of facultative anaerobes relies on their unique ability to cope with changing oxygen levels during infection

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Abstract

Bacteria, including those that are pathogenic, have been generally classified according to their ability to survive and grow in the presence or absence of oxygen: aerobic and anaerobic bacteria, respectively. Strict aerobes require oxygen to grow (e.g., *Neisseria*), and strict anaerobes grow exclusively without, and do not survive oxygen exposure (e.g., *Clostridia*); aerotolerant bacteria (e.g., *Lactobacilli*) are insensitive to oxygen exposure. Facultative anaerobes (e.g., *E. coli*) have the unique ability to grow in the presence or in the absence of oxygen and are thus well-adapted to these changing conditions, which may constitute an underestimated selective advantage for infection. In the WHO antibiotic-resistant 'priority pathogens' list, facultative anaerobes are overrepresented (8 among 12 listed pathogens), consistent with clinical studies performed in populations particularly susceptible to infectious diseases. Bacteria aerobic respiratory chain plays a central role in oxygen consumption, leading to the formation of hypoxic infectious sites (infectious hypoxia). Facultative anaerobes have developed a wide diversity of aerotolerance and anaerotolerance strategies in vivo. However, at a single cell level, the modulation of the intracellular oxygen level in host infected cells remains elusive and will be discussed in this review. In conclusion, the ability of facultative bacteria to evolve in the presence or the absence of oxygen is essential for their virulence strategy and constitute a selective advantage.

Take Away

- Most life-threatening pathogenic bacteria are facultative anaerobes.
- Only facultative anaerobes are aerotolerant, anaerotolerant and capable of consuming O₂.
- Facultative anaerobes induce and are well adapted to cellular hypoxia.

KEYWORDS

facultative anaerobes, oxygen, hypoxia

1 | INTRODUCTION

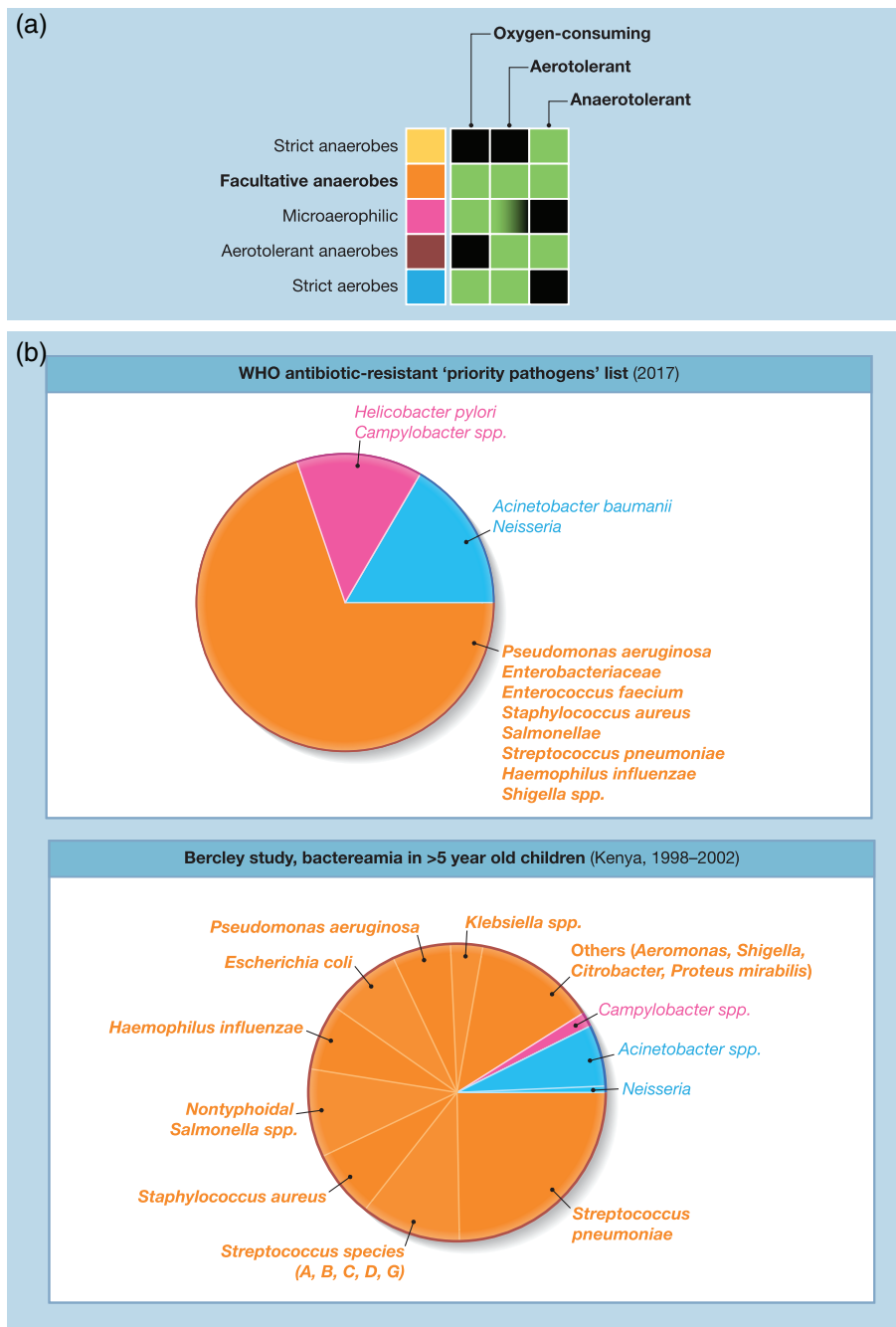
Bacteria, including those that are pathogenic, are generally classified according to their ability to survive and grow in the presence or absence of oxygen: aerobic and anaerobic bacteria, respectively. Strict aerobes require oxygen to grow (e.g., *Neisseria*), strict anaerobes grow

exclusively without, and do not survive oxygen exposure (e.g., *Clostridia*) while aerotolerant anaerobes (e.g., *Lactobacilli*) are insensitive to oxygen exposure. Microaerophilic bacteria are adapted to sub-atmospheric levels of oxygen, but do not survive its absence (e.g., *Helicobacter pylori*, *Campylobacter jejuni*) (Bury-Moné et al., 2006; Krieg & Hoffman, 1986).

The objective of this review is to decipher how pathogenic bacteria cope with oxygen exposure when infecting human cells and tissues; we focus here on their survival capacity and potential strategies to manipulate oxygen levels to optimise their fitness within infectious sites, facing changing oxygen levels. For this purpose, we propose a new classification of pathogenic bacteria, in regards to their oxygen- or non-oxygen-consumption capacity, their aero- or anaero-tolerance and their aero- or anaero-sensitivity.

Aerotolerant bacteria are strict aerobes, facultative anaerobes, aerotolerant anaerobes and, to some extent, microaerophilic bacteria. Aerosensitive bacteria are only strict anaerobes. Anaerotolerant bacteria are strict anaerobes, facultative anaerobes and aerotolerant anaerobes. Oxygen-consuming bacteria are strict aerobes, facultative

anaerobes and microaerophilic bacteria. Non-oxygen-consuming bacteria are strict anaerobes and aerotolerant anaerobes (Figure 1a). This classification highlights the selective advantage of facultative anaerobes in regards to their survival capacity in the presence or absence of oxygen and their ability to consume it; facultative anaerobes are the lonely oxygen-consuming, aerotolerant and anaerotolerant bacteria (Figure 1a). As an illustration, among the 12 antibiotic-resistant 'priority pathogens' listed by the World Health Organisation (WHO) in 2017, 8 pathogens are facultative anaerobes (*Pseudomonas aeruginosa*, *Enterobacteriaceae*, *Enterococcus faecium*, *Staphylococcus aureus*, *Salmonellae*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Shigella* spp.) (Tacconelli et al., 2017) (Figure 1b). This proportion has been reported in various epidemiological studies, including the



Berkley clinical study performed in Kenya between 1998 and 2002, showing that 85% identified pathogens responsible of bacteraemia in children under the age of five were facultative anaerobes (including *Escherichia coli*, non-typhoid salmonella species, *Streptococcus pneumoniae*, *Haemophilus influenzae*) (Berkley et al., 2005) (Figure 2b).

As a conclusion, facultative anaerobes represent the most threatening pathogenic bacteria for humans.

2 | MOLECULAR MECHANISMS SUSTAINING FACULTATIVE ANAEROBES OXYGEN-CONSUMPTION

Facultative anaerobes constitute a unique bacteria group able to consume oxygen and to grow in the presence or absence of oxygen (Figure 1a), due to the expression of two respiratory chains using either oxygen or alternative

electron acceptors—referring to aerobic and anaerobic respiratory chains—as reviewed in Uden and Trageser (1991). On an evolutionary perspective, and in regards to oxygen exposure, facultative anaerobes may be considered as the most evolved bacteria species for their capacity to disseminate within any microenvironment encountered during infectious processes.

Bacterial infections start with the invasion of perfused organs, under normoxia, which may be associated with different oxygen levels, depending on the targeted organ. High oxygen levels are encountered in highly perfused organs (e.g., liver, kidneys) or relatively low levels in poorly perfused ones (e.g., colon).

Upon infection, the basal organ level of oxygen will be modulated due to recruited immune cells' need for oxygen and bacteria oxygen-consumption activity; their respective contribution to the induced infectious hypoxia remains currently debated.

The main cause of bacteria-mediated oxygen consumption is aerobic respiration, which is functional in facultative anaerobes, microaerophilic

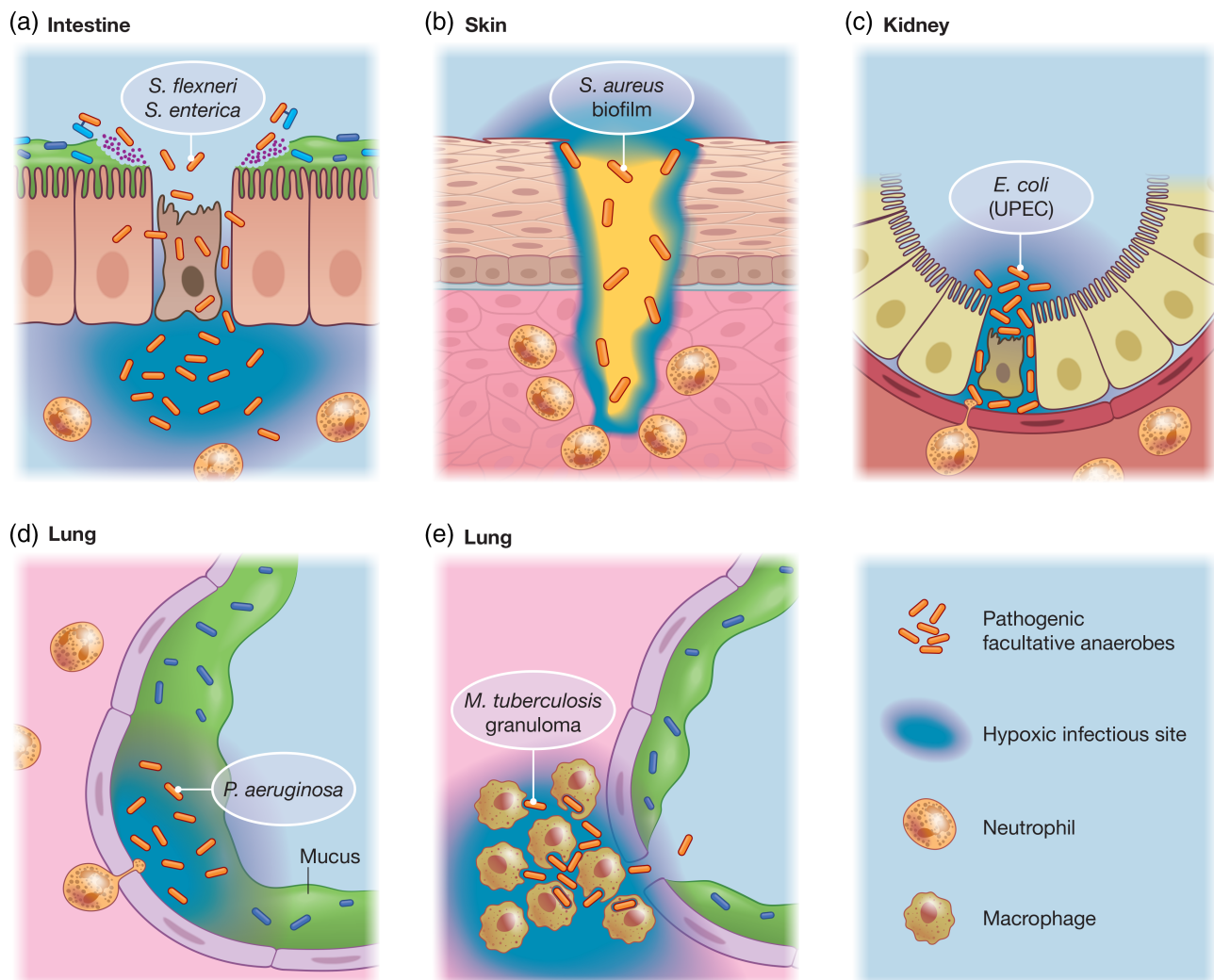


FIGURE 2 Hypoxic infectious foci induced during facultative anaerobes. Hypoxic foci of infection were reported upon (a) intestine infections by *Salmonella enterica* (Jennewein et al., 2015) and *Shigella flexneri* (Arena, Tinevez, Nigro, Sansonetti, & Marteyn, 2017; Tinevez et al., 2019), (b) dermal infections, within biofilms formed by *Staphylococcus aureus* (Lone et al., 2015a; Lone et al., 2015b), (c) kidney infections by *Escherichia coli* (Melican et al., 2008) and (d) pulmonary infections by *Pseudomonas aeruginosa* (Worlitzsch et al., 2002) and (e) *Mycobacterium tuberculosis* granuloma (Klinkenberg, Sutherland, Bishai, & Karakousis, 2008; Peterson et al., 2020)

bacteria and strict aerobes. Aerobic respiration relies on electron transfer from various substrates through protein complexes located in the bacteria inner membrane encompassing dehydrogenases, electron carriers such as cytochrome C and Quinol, leading to dioxygen reduction by the last complex of the respiratory chain belonging to the terminal oxygen reductase family. The biological function, composition and regulation of bacteria aerobic respiratory chains are complex and diverse. Here, the diversity of oxygen reductases, which are directly involved in oxygen consumption, will be described. For other aspects, we refer to recent and complete reviews on the bacteria aerobic respiratory chain (Cook, Greening, Hards, & Berney, 2014; Kracke, Vassilev, & Krömer, 2015; Melo & Teixeira, 2016). Terminal oxygen reductases are reduced by cytochrome C (one-electron donor) or quinol (QH₂, two-electron donor) to allow the four-electron reduction of dioxygen to water, defining three families of oxygen reductases: the heme-copper oxygen reductase family (HCO), the alternative oxidase family (AOX) and the cytochrome *bd* family (Borisov, Gennis, Hemp, & Verkhovskiy, 2011). HCO is composed of cytochrome *c* oxidases and quinol oxidases. AOX and cytochrome *bd* oxidases receive electrons from quinol, not cytochrome *c*. To our knowledge, no AOX has been identified in major human pathogenic bacteria.

HCO are related to the mitochondrial complex IV. HCOs contain a high-spin heme (*a*₃, *o*₃ or *b*₃) and a copper ion at the oxygen-reduction site. Three HCO groups have been characterized: cytochrome *aa*₃ oxidase (HCO Type A oxygen reductase), cytochrome *ba*₃ and *b(o)a*₃ oxidases (HCO Type B oxygen reductases), and cytochrome *cbb*₃ and *bcc*₃ oxidases (HCO Type C oxygen reductase) (Pereira, Santana, & Teixeira, 2001; Sousa et al., 2012). Cytochrome *bo*₃ oxidases use quinol as an electron donor, *cbb*₃, *bcc*₃ and *aa*₃ oxidases are reduced by cytochrome *c* (Cook, Greening, et al., 2014). All pathogenic bacteria express at least one HCO (see below).

Cytochrome *bd* oxidases are quinol oxidases, which may use ubiquinol or menaquinol as substrates, and are phylogenetically distinct to mitochondrial enzymes. They do not contain copper or non-heme iron and are composed of two subunits (I and II); hemes *b*₅₉₅ and *d* are associated at the dioxygen reduction site (Borisov et al., 2011). Cytochrome *bd* oxidases are expressed by most pathogenic bacteria, some expressing several; their expression is induced under low-oxygen or stressful conditions, which may have a significant relevance during the infectious process.

Among oxygen-consuming bacteria listed as 'priority pathogens' by the WHO, *Pseudomonas aeruginosa* expresses 5 oxygen reductases (*aa*₃, *cbb*₃ [x2], *bo*₃, and *bd*) (Arai, 2011; Yamanaka, Fukumori, Yamazaki, Kato, & Nakayama, 1985) (Hirai, Osamura, Ishii, & Arai, 2016), Enterobacteriaceae express 3 complexes (*bo*₃ and *bd* [x2]), including *Salmonella* (Jo, Cortez, Cornell, Price-Whelan, & Dietrich, 2017) (Jünemann, 1997). *Campylobacter* spp. and *Staphylococcus aureus* express two complexes (*bd/bcc*₃ and *aa*₃/*bd*, respectively) (Jackson et al., 2007) (Smith, Finel, Korolik, & Mendz, 2000) (Hammer et al., 2013), similarly to *Shigella* spp. (*bdl* (CydABX) (VanOrsdel et al., 2013) and *bdII* (AppBC) (Tinevez et al., 2019). Other pathogenic bacteria express only one oxygen reductase, including *Neisseria* (*cbb*₃), *Helicobacter pylori* (*bcc*₃) and *Enterococcus faecium* or *Streptococcus pneumoniae* (*bd*) (Nagata, Tsukita, Tamura, & Sone, 1996) (Deeudom, Koomey, &

Moir, 2008) (Hopper, Tovell, & Cole, 2009) (Ekici, Pawlik, Lohmeyer, Koch, & Daldal, 2012) (Winstedt, Frankenberg, Hederstedt, & von Wachenfeldt, 2000). *Mycobacterium tuberculosis* expresses two oxygen reductases (*bd* and the super-complex *bcc-aa*₃) (Cook, Hards, Vilchère, Hartman, & Berney, 2014).

Listing oxygen reductases from the bacterial genome is probably insufficient to assess their function and role during infectious processes. It has been demonstrated that pathogenic *E. coli* aerobic respiration is required to colonise mice. ATP synthase defective mutants or cytochrome *bd* oxidase mutants fail to colonise; those with defective nitrate reductase or fumarate reductase have the same phenotype. These results highlight the importance of the combination of aerobic and anaerobic respiration—a 'respiratory flexibility'—for bacteria virulence (Jones et al., 2007).

Other bacteria-signalling pathways are associated with oxygen consumption, such as the Nitric Oxide (NO) synthesis, catalysed by the bacterial bNOS, as reviewed elsewhere (Hutfless, Chaudhari, & Thomas, 2018). However, the oxygen-consumption rate associated to this reaction seems to be negligible compared to the aerobic respiration. As an illustration, a mutation in *Shigella flexneri* cytochrome *bdl* oxidase (CydAB) totally abolishes its ability to consume oxygen, turning a facultative anaerobe into an aerotolerant strict anaerobe (Tinevez et al., 2019).

More recently, an extracellular electron transfer (EET) chain has been identified in several Gram-positive bacteria, including *Listeria monocytogenes* (Light et al., 2018), supporting the idea that alternative oxygen-consuming signalling pathways remain to be discovered. EET allows electron transfer from the cytosol to extracellular Iron (from NAD⁺ to DMK and PplA), supporting *L. monocytogenes* growth under anaerobic conditions. This alternative electron transfer pathway has been elegantly minded and described. However, it remains for now unclear whether oxygen may be a substrate for EET; its potential contribution to oxygen depletion during bacterial infection should be carefully investigated.

3 | ANAEROTOLERANCE FAVOURS BACTERIA SURVIVAL TO INFECTIOUS HYPOXIA

Anaerotolerance may be defined by the ability of bacteria to survive to the absence of oxygen. *Stricto sensu*, anaerotolerance may be defined by the ability of bacteria to grow in the absence of oxygen or to survive oxygen exposure. Facultative anaerobe's anaerotolerance is crucial for pathogenic bacteria belonging to this family since infectious hypoxia induction has been reported to be induced during their colonisation of host organs (*Salmonella enterica*, *Shigella flexneri*, *Staphylococcus aureus*, UPEC, *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis*) (Jennewein et al., 2015; Lone et al., 2015a; Lone et al., 2015b; Peterson et al., 2020; Tinevez et al., 2019; Worlitzsch et al., 2002).

Bacteria growth under anoxic conditions essentially relies on the expression of an anaerobic respiratory chain composed of terminal electron acceptor complexes such as nitrate reductase, nitrite

reductase or fumarate reductase, which ultimately mediate electron transfer to nitrate, nitrite or fumarate (among others), respectively (reviewed in (Cook, Greening, et al., 2014; Cook, Hards, et al., 2014)). Energy production is therefore independent of oxygen availability. Facultative anaerobes, aerotolerant anaerobes and strict anaerobes express a functional anaerobic respiratory chain supporting their anaerotolerance and their growth in the absence of oxygen. Conversely, anaerobic microaerophilic bacteria or strict aerobes do not express a functional anaerobic respiratory chain.

Other anaerotolerance strategies have been developed among pathogenic bacteria, which have the ability to evolve and propagate under hypoxia. *L. monocytogenes*' extracellular electron transfer (EET) may be considered as an alternative anaerotolerance strategy (Light et al., 2018), together with alternate respiratory polyketide quinones in *Mycobacterium tuberculosis*, specifically expressed in biofilms and other oxygen-limited conditions (Anand et al., 2015). This pathogen is an obligate aerobe but colonises the host and persists under an oxygen-resistant latent state within hypoxic granulomas (Tsai et al., 2006) (Klinkenberg et al., 2008) (Figure 2). *Mycobacterium tuberculosis* do not grow in the absence of oxygen; this dormant state should rather be defined as an 'anaeroprotective' strategies to survive in the absence of oxygen (reviewed in Singh, Ghosh, Islam, Dasgupta, and Kirsebom (2010)) and Rustad, Sherrid, Minch, and Sherman (2009).

4 | BACTERIA AEROTOLERANCE STRATEGIES TO SURVIVE O₂ AND ASSOCIATED ROS EXPOSURE

Dioxygen is not toxic for bacteria per se. However, its presence is associated with the formation of highly toxic derivatives such as reactive oxygen species (ROS), including oxygen-superoxide radical (O₂^{•-}), hydrogen peroxide (H₂O₂) or hydroxyl radical (HO^{*}). These reactive molecules may damage bacteria components (DNA, peptides and lipids) if not detoxified, and may be converted within infectious sites into more stable and more potent microbicidal molecules, such as hypochlorous acid (HOCl) by host immune cells, including polymorphonuclear neutrophils (Klebanoff, Kettle, Rosen, Winterbourn, & Nauseef, 2013).

The dissemination of oxygen-consuming bacteria, such as facultative anaerobes, is anticipated to contribute to the limitation of oxygen levels at infectious sites, hence limiting ROS production. We recently demonstrated that *Shigella flexneri* aerobic respiration mediates oxygen depletion in vivo (Arena et al., 2017; Tinevez et al., 2019) (Figure 2).

In addition, bacteria have evolved to detoxify ROS produced by host immune cells during the inflammatory response (reviewed in (Khademian & Imlay, 2021)). Several ROS detoxification pathways have been identified in bacteria, such as hydrogen peroxide-inducible genes activator (OxyR) (Choi et al., 2001) in response to H₂O₂ in *Shigella flexneri*, *Salmonella* (Jennewein et al., 2015) or *Pseudomonas aeruginosa* (Wei et al., 2012). The combination of the superoxide dismutase (SOD) and catalase activities mediates the detoxification of

O₂^{•-} to produce H₂O and O₂. Strict anaerobes, such as *Clostridium difficile*, may resist ROS with a key role by the sigma-B factor (Kint et al., 1933) and further adapt to higher oxygen levels by forming spores (reviewed in (Dürre, 2014)), stating as an 'aeroprotection' strategy. It was further reported that *Salmonella typhimurium* may exploit ROS production for its own respiration. It has been shown that tetrathionate was formed in a ROS-dependent manner and that its respiration increases *Salmonella typhimurium* abundance in vivo (Winter et al., 2010).

Another aeroprotection strategy consists in bacteria extracellular colonisation on host tissues or within biofilms, thus avoiding oxygen diffusing within host organs. This strategy is used by various pathogenic enterobacteria such as *E. coli* (EPEC), Shiga toxin-producing *E. coli* (STEC), enterotoxigenic *E. coli* (ETEC), diffusely adherent *E. coli* (DAEC) or Enteroaggregative *E. coli* (EAEC). Their virulence requires their adherence to the host intestine epithelium, where microcolonies (e.g., EPEC) or biofilms (e.g., EAEC) are formed within the anaerobic luminal compartment, avoiding further oxygen exposure (reviewed in (Croxen et al., 2013)). A similar strategy is exploited by *Helicobacter pylori*, which remains mainly extracellular in a microaerophilic microenvironment, potentially within protective biofilms (Hathroubi, Servetas, Windham, Merrell, & Ottemann, 2018). It has been suggested that *Helicobacter pylori* may have an intracellular life (Dubois & Borén, 2007), although this point remains to be discussed.

Biofilm formation is another aeroprotection strategy used by pathogens. Oxygen diffusion within biofilms is limited, shaping an oxygen-deprived niche in the heart of the structure, while oxygen may diffuse in its periphery (Stewart et al., 2016). Biofilms are formed by several life-threatening pathogens, such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, pathogenic *E. coli*, *Klebsiella pneumoniae* or *Haemophilus influenzae*, among others (reviewed in (Jamal et al., 2018)) (Figure 2). Most of biofilm-forming bacteria are facultative anaerobes, which may evolve in this heterogeneous and complex microenvironment.

It is now well accepted that hypoxia is induced within host tissues during bacterial infection (infectious hypoxia). The invasion of host epithelial cells by bacteria stands as crucial early step of the infection process. The impact of bacteria invasion on host cell oxygenation status has been poorly investigated so far.

5 | INTRACELLULAR OXYGEN LEVEL DECREASES UPON BACTERIAL INFECTION

Oxygen availability plays a central role in host cell invasion by bacteria (reviewed in (Marteyn, Scorza, Sansonetti, & Tang, 2011)). Under anaerobic conditions, it has been shown that bacteria invasion efficiency was increased using *Shigella* (Marteyn et al., 2010) or *Salmonella* (Ernst et al., 1990) as facultative anaerobe models.

To better appreciate the importance of bacteria oxygen consumption, aerotolerance and anaerotolerance on their ability to survive within invaded host cells, the modulation of the intracellular oxygenation status remains to be minded. How the partial pressure of oxygen within infected cells evolves during infection remains elusive. The

development of innovative technologies, such as the FLIM combined with O₂ probes based on Pt-porphyrin (Sud, Zhong, Beer, & Mycek, 2005) (Papkovsky & Dmitriev, 2018) (Penjweini et al., 2020), is anticipated to provide these quantitative data. It has to be noticed that such experiments should be done under controlled low pO₂ to avoid experimental and non-physiological diffusion of atmospheric oxygen.

Nevertheless, there are indirect evidences that oxygen abundance varies in infected cells, which may have an important impact on the host cell function, metabolism or survival. Bacteria virulence mechanisms may be regulated by oxygen availability. Jones & Falkow have shown in *S. typhimurium* that *orgA* (encoding for the oxygen-regulated invasion protein OrgA) was over-expressed under low-oxygen conditions (Jones & Falkow, 1994). It has been demonstrated that *S. typhimurium* Δ *orgA* mutant is unable to invade and destroy M cells in Peyer's patches in vivo, indicating that OrgA is essential for host cells entry, although its precise function was not elucidated. *Shigella* Type III secretion system is not functional under anaerobic conditions (Marteyn et al., 2010) and has been reported to be inactive within infected cells (Nigro et al., 2019), strongly suggested that intracellular oxygen level is reduced upon *Shigella* infection and replication. In addition, it has been reported that the expression of several genes encoding for proteins involved in the adaptation of *Shigella* to changes in oxygen level such as *hemB* (porphobilinogen synthase) and *ppC* (phosphoenolpyruvate carboxylase) is overexpressed intracellularly (Bartoleschi et al., 2002).

Host cells' metabolic shifts occurring during bacterial invasion seem to indicate that a decrease of the intracellular oxygen level is induced during bacterial infection. Hypoxia-inducible factor (HIF-1 α) is a well-characterized transcriptional regulator, which is stabilised in the absence of oxygen. It has been shown that upon *Clostridium difficile* infection of Caco-2 cells, a significant increase of HIF-1 α transcription and protein levels is observed (Hirota et al., 2010). Similar results were observed in Hep-2 cells infected by *Chlamydia pneumoniae*, at least during the early phase of infection (Rupp et al., 2007). However, it has to be noticed that HIF stabilisation does not necessarily reflect hypoxia induction during bacterial infection since it has been demonstrated that *E. coli* lipopolysaccharide (LPS) alone induces HIF-1 α mRNA expression in monocytes (Frede, Stockmann, Freitag, & Fandrey, 2006) and in many other cell types. HIF degradation process is initiated by its hydroxylation catalysed by prolyl-hydroxylases (PHDs) in the presence of oxygen, ascorbate and 2-oxoglutarate. It has been demonstrated that various metabolic compounds such as succinate, fumarate and other molecules such as iron (FeIII), ROS or NO inhibit PHDs activity, leading to HIF stabilisation, even in the presence of oxygen (Selak et al., 2005) (Metzen, Zhou, Jelkmann, Fandrey, & Brüne, 2003) (Siegert et al., 2015).

Several studies report that host cell metabolism is regulated during bacterial infection, mainly through oxygen-independent glycolysis (reviewed in (Eisenreich, Rudel, Heesemann, & Goebel, 2019)). *Mycobacterium tuberculosis* induces such a metabolic shift in macrophages, repressing the oxidative phosphorylation and activating glycolysis (Gleeson et al., 2016) (Cumming, Addicott, Adamson, & Steyn, 2018). *Pseudomonas aeruginosa* quorum-sensing molecule 3-oxo-C12-HSL induces mitochondria dysfunction in bronchial epithelial cells (Maurice et al., 2019),

while *Chlamydia pneumoniae* infection increases monocyte glucose uptake (Rupp et al., 2007) and mitochondrial functions, favouring bacteria intracellular growth (Käding et al., 2017). It has been shown that under hypoxia, macrophage HIF-1 α was stabilised, and the TCA metabolites availability was reduced. In this context, it was reported that the intracellular replication of *Coxiella burnetii*, an aerobic obligate intracellular pathogen causing Q fever, was drastically repressed, although its viability was not impeded (Hayek et al., 2019). Hayek and colleagues demonstrated that *Coxiella burnetii* requires citrate under hypoxia, while its abundance is decreased in a HIF-1 α -dependent manner. Limiting citrate availability in macrophage infected by *Coxiella burnetii* stands as a novel defence mechanism to decrease its intracellular replication, although persistence is observed. Further investigations are required to define whether this metabolic adaptation occurs also under anoxia and to decipher how *Coxiella burnetii* use citrate as a carbon source and why it is essential for its proliferation under hypoxia.

6 | CONCLUDING REMARKS

In this review, we propose to better take into account bacteria oxygen consumption, aerotolerance and anaerotolerance to understand and characterize their ability to invade, survive and disseminate within host cells and tissues. Concomitantly, quantifying oxygen level within infected cells or infectious sites (in situ, with non-disruptive methods) will be the next challenge to confirm the importance of this question in vivo, requiring important technological developments to allow in situ pO₂ quantification using non-disruptive methods.

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DATA AVAILABILITY STATEMENT

No new data is generated

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