Minireview

Responses of Clostridia to oxygen: from detoxification to adaptive strategies

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Summary

Clostridia comprise bacteria of environmental, biotechnological and medical interest and many commensals of the gut microbiota. Because of their strictly anaerobic lifestyle, oxygen is a major stress for Clostridia. However, recent data showed that these bacteria can cope with O$_2$ better than expected for obligate anaerobes through their ability to scavenge, detoxify and consume O$_2$. Upon O$_2$ exposure, Clostridia redirect their central metabolism onto pathways less O$_2$-sensitive and induce the expression of genes encoding enzymes involved in O$_2$-reduction and in the repair of oxidized damaged molecules. While *Faecalibacterium prausnitzii* efficiently consumes O$_2$ through a specific extracellular electron shuttling system requiring riboflavin, enzymes such as rubrerythrins and flavodiiron proteins with NAD(P) H-dependent O$_2$- and/or H$_2$O$_2$-reductase activities are usually encoded in other Clostridia. These two classes of enzymes play indeed a pivotal role in O$_2$ tolerance in *Clostridioides difficile* and *Clostridium acetobutylicum*. Two main signalling pathways triggering O$_2$-induced responses have been described so far in Clostridia. PerR acts as a key regulator of the O$_2$- and/or reactive oxygen species–defence

machinery while in *C. difficile*, σ^B, the sigma factor of the general stress response also plays a crucial role in O$_2$ tolerance by controlling the expression of genes involved in O$_2$ scavenging and repair systems.

Introduction

In the phylum of Firmicutes corresponding to low G + C Gram-positive bacteria, the class of Clostridia contains a large number of orders, families and genera. The class Clostridia includes ubiquitous bacteria of environmental, medical and biotechnological interest as well as many commensals of the gut microbiota and pathogens. This class likely appeared before the Great oxidation event (Paredes *et al*., 2005) and is characterized by one common feature, a strictly anaerobic lifestyle. Clostridia include many spore formers. Spores being the form of clostridial persistence resist to air in the environment (water, soils, hospital and industrial surfaces) and to oxidizing disinfectants such as hydrogen peroxide (H$_2$O$_2$) and hypochlorite used to eradicate pathogens on medical devices or contaminants in industrial processes. Clostridial vegetative cells are much more sensitive to oxygen (O$_2$) and are killed by prolonged air exposure (Fig. 1). However, vegetative cells may face transient exposure to air or to low O$_2$ tensions in their habitats, in a variety of situations. Indeed, O$_2$ is not completely absent from infected wounds where *Clostridium perfringens*, *Clostridium tetani* or *Clostridium botulinum* can develop, nor from food, which can be contaminated by anaerobes. In addition, anaerobic microorganisms can reach the microoxic edge of their niche in the environment. Water containing dissolved O$_2$ at various concentrations depending on temperature or salinity can also penetrate anoxic areas of the soils (Morris and Schmidt, 2013). Strict anaerobic conditions are also difficult to maintain during industrial processes that utilize solventogenic species (*Clostridium acetobutylicum*, *Clostridium beijerinckii*, *Clostridium pasteurianum*) or acetogenic species (*Clostridium ljungdahlii* and *Moorella thermoacetica*) (Tracy *et al*., 2012). Clostridia are also the most representative group of

Firmicutes in gut commensals. The gastrointestinal tract harbours a longitudinal decreasing gradient of O_2 tension from about 4% O_2 in the small intestine lumen to 0.1%–0.4% in the large intestine lumen (Keeley and Mann, 2019). A second increasing O_2 gradient is observed from the colon lumen towards the intestinal epithelium (Marteyn *et al*., 2011; Albenberg *et al*., 2014). These two intersecting O_2 gradients can be further altered by local and temporal fluctuations and notably by antibiotic treatments that disrupt the host intestinal flora resulting in an increased O_2 tension within the gut (Kelly *et al*., 2015; Rivera-Chavez *et al*., 2016). Unexpectedly, dysbiosis can instead favour the colonization of anaerobic clostridial enteropathogens in human or animals as demonstrated for *Clostridioides difficile*, which is the first cause of antibiotic-associated diarrhoea in adults. Dysbiosis of the intestinal microbiota leads to changes in the metabolite pool, particularly in bile acids that enable spore germination in the small intestine and thereafter colonization of the colon by vegetative *C. difficile* cells (Britton and Young, 2014; Abt *et al*., 2016). Additionally, dysbiosis of the gut microbiota is also associated with Inflammatory Bowel Diseases (IBD) that include Crohn's disease and ulcerative colitis even if it is not known whether dysbiosis is a cause or a consequence of inflammation (Fritsch and Abreu, 2019). It is worth noting that patients suffering from IBD are more at risk of developing infection by *C. difficile* with an increased morbidity and mortality (Fu and Wong, 2016). In patients with IBD, a reduction of obligate anaerobes from the phylum Firmicutes such as *Faecalibacterium prausnitzii* and an increase of facultative anaerobes are observed (Rigottier-Gois, 2013). It has been proposed that O_2 could play a role in the microbiota imbalance because not all commensals have the same adaptive capacity to resist O_2 (Rigottier-Gois, 2013; Henson and Phalak, 2017). In IBD, the possible entry of blood into the gastrointestinal tract observed during chronic inflammation could promote the release of O_2 from haemoglobin while the inflammation itself is associated with an oxidative burst, both impacting the O_2 level in the gut. Because of their anaerobic lifestyle, O_2 is a major stress for the vegetative cells of the different species among the class Clostridia (Fig. 1). However, data now available demonstrate that these obligate anaerobes can cope with O_2 better than expected thanks to their ability to scavenge and detoxify O_2 and also to repair damages of macromolecules following O_2 exposure.

Impact of oxygen exposure on the physiology of Clostridia

Various levels of O_2 tolerance and air survival among Clostridia

The level of O_2 tolerance varies among clostridial vegetative cells and this tolerance is both strain- and medium-dependent within a species (Kawasaki *et al*., 1998; Hillmann *et al*., 2008; Edwards *et al*., 2016). Tolerance to O_2 with a maintained growth in liquid culture for several hours in the presence of low O_2 tension (0.2%–3%) has been described for *C. acetobutylicum* (O'Brien and Morris, 1971; Hillmann *et al*., 2008), *Clostridium butyricum* (Camerini *et al*., 2019) and *C. difficile* (Neumann-Schaal *et al*., 2018; Giordano *et al*., 2018b; Kint *et al*., 2020) even if a phase of latence is observed in *C. butyricum* (Kawasaki *et al*., 1998). Wound-colonizing clostridial species such as *C. perfringens* and *C. tetani* and *Clostridium tertium* isolated from gas gangrene wounds are considered to be aerotolerant (Trinh *et al*., 2000; Bruggemann *et al*., 2004; Fujitani *et al*., 2007). *Clostridium ljungdahlii*, an important gas fermenting acetogenic bacterium used in the biofuel industry is able to grow in mixotrophic conditions in the presence of 8% of O_2 (Whitham *et al*., 2015), while other acetogenic Clostridia are also described as aerotolerant (Kusel *et al*., 2001; Karnholz *et al*., 2002). Higher O_2 concentrations usually lead to growth inhibition and drastic changes in metabolism, even if both can resume when cells return to anaerobiosis as shown for *C. acetobutylicum* and *C. butyricum* (O'Brien and Morris, 1971; Kawasaki *et al*., 1998). *Clostridium aminovalericum* is unable to grow in the presence of 1% O_2 (Kawasaki *et al*., 2004). Air exposure leads to a drastic decrease in survival of several Clostridia (Hillmann
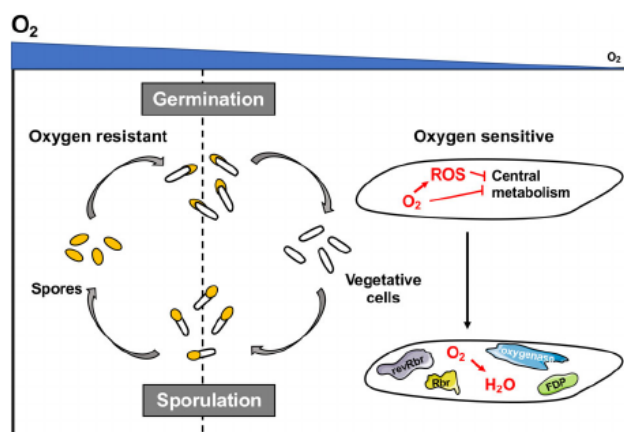

Fig 1. Oxygen tolerance in Clostridia. Scheme representing the different O_2 tolerance of the two cellular forms of Clostridia. Spores are fully oxygen resistant while after the germination process, the vegetative cells show various O_2 tolerance within clostridial species. The deleterious effect of O_2 or derived ROS on central metabolism is mitigated by the presence of various O_2 detoxification enzymes such as oxygenase, flavodiiron proteins (FDP), rubrerythrin (Rbr) and reverse rubrerythrin (revRbr) that reduce O_2 and/or H_2O_2 to water.

et al., 2008; Edwards *et al.*, 2016; Camerini *et al.*, 2019). *F. prausnitzii*, a gut commensal of the *Clostridium leptum* group is extremely O_2 sensitive and a brief air exposure prevents its subsequent anaerobic growth (Duncan *et al.*, 2002), although this bacterium can adapt to O_2 under particular growth conditions (see below). *Clostridium neonatale*, responsible for necrotizing enterocolitis of premature neonates, is more resistant to 24 h of air exposure than the closely related *C. butyricum* (Schonherr-Hellec *et al.*, 2017). It is worth noting that the addition of antioxidant compounds such as reducing agents (thioglycolate or cysteine), riboflavin, ascorbic or uric acid can drastically increase O_2 tolerance (Khan *et al.*, 2014; Kint *et al.*, 2017; Million *et al.*, 2020). A common feature of several Clostridia discussed below is their ability to reduce and efficiently consume O_2 (Hillmann *et al.*, 2008; Whitham *et al.*, 2015) (Table 1).

The global response to O_2 or air exposure in Clostridia

To study physiological adaptation to low O_2 tension or air exposure, transcriptomic or proteomic studies have been performed in *C. acetobutylicum* (1 h exposure to about 0.5% O_2), *C. butyricum* (growth in the presence of air) and *C. ljungdahlii* (growth at 8% O_2) (Hillmann *et al.*, 2008; Whitham *et al.*, 2015; Camerini *et al.*, 2019) (Table 1). In *C. difficile*, transcriptomic or multiomic studies were performed in 630 strains grown in complex media in the presence of 2% O_2 or after brief aeration as well as in the 630Δerm strain exposed to 5% O_2 in minimal medium (Emerson *et al.*, 2008; Neumann-Schaal *et al.*, 2018; Giordano *et al.*, 2018a). Adaptation to growth in the presence of O_2 or air leads to changes in the expression of genes encoding proteins involved in energy, carbon, amino-acid, sulfur, iron and/or cofactor metabolisms as well as an induction of systems implicated in O_2 and reactive oxygen species (ROS) scavenging/detoxification systems and the repair of molecules damaged by oxidation such as thiols and DNA.

Changes in metabolism upon O_2 exposure in Clostridia

The toxicity of O_2 may arise directly from itself, due to unspecific oxidation of low-reduction potential cofactors, or from its derived species as a result of its incomplete reduction, the so-called ROS: the superoxide anion radical ($O_2^{-\cdot}$), H_2O_2 or the hydroxyl radical ($HO\cdot$). O_2 or ROS reacts unspecifically with a plethora of redox enzymes/proteins leading to their inhibition as a result of the damaged catalytic centres or accessory metal or flavin cofactors. These damages, in many cases irreversible, ultimately impair multiple pathways. For this reason, ROS are used as chemical weapons by the human innate immune system to fight pathogens, namely, phagocytic

cells that produce ROS through NAD(P)H:O_2 oxidoreductases (Winterbourn *et al.*, 2016). Paradigmatic examples of enzymes poisoned by O_2 are those involved in key metabolic steps of energy conversion in anaerobes such as pyruvate ferredoxin oxidoreductase (PFOR), pyruvate formate lyase, CO-dehydrogenases or 4-hydroxy-butyryl-CoA dehydratase. Indeed, enzymes and proteins containing low potential iron–sulfur (Fe-S) clusters that play a key role in clostridial metabolism (Meyer, 2000) or glycyl radical-dependent enzymes are especially O_2 sensitive (Buckel and Golding, 2006; Imlay, 2006; Hong and Pachter, 2012; Imlay *et al.*, 2019; Khademian and Imlay, 2020a, 2020b). To escape this bottleneck, anaerobes redirect when possible their carbon and energy metabolisms onto pathways more resistant to O_2 that vary depending on the specificity of their metabolism. In addition, the enzymes involved in O_2 detoxification (see below) require NAD(P)H as electron source. Therefore, to maintain a sufficient pool of these reduced molecules, the cells must be able to produce NAD(P)H continuously or to limit their consumption in the presence of O_2 (Whitham *et al.*, 2015; Neumann-Schaal *et al.*, 2018). Several Clostridia likely involve the Rex regulator in these metabolic switches [see below, (Zhang *et al.*, 2014)]. Thus, Clostridia reorganize their metabolism to limit the use of O_2 sensitive enzymes and to supply enough NAD(P)H and the cofactors required for O_2 scavenging by different ways. A downregulated pathway in *C. acetobutylicum*, *C. butyricum* and *C. difficile* upon O_2 exposure is the one converting acetyl-CoA to butyrate, which is associated with a reduced amount of detected butyrate in *C. butyricum* and *C. difficile* in the presence of O_2 (Hillmann *et al.*, 2009a; Neumann-Schaal *et al.*, 2018; Camerini *et al.*, 2019) and an arrest of butyrate production in *C. acetobutylicum* (O'Brien and Morris, 1971). By contrast, acetate production, which does not require reducing equivalents, is maintained or even increased as observed in *C. butyricum* and *C. acetobutylicum* (O'Brien and Morris, 1971; Neumann-Schaal *et al.*, 2018; Camerini *et al.*, 2019). This rerouting of metabolism might be related to the poisoning by O_2 of the above-mentioned key enzymes, such as PFOR and 4-hydroxybutyryl-CoA dehydratase (Buckel and Golding, 2006). In *C. acetobutylicum*, an increased excretion of pyruvate is also detected under aerobic conditions (O'Brien and Morris, 1971). Under microaerophilic conditions, a higher ethanol and a lower acetate productions are observed in *C. ljungdahlii* that is likely associated with an increased level of synthesis and activity of an aldehyde oxidoreductase (Whitham *et al.*, 2015). In *C. difficile*, an exposure to 5% O_2 is followed by a decreased expression of genes encoding proteins involved in the proline and glycine reductive pathways of the Stickland reaction, resulting in an accumulation of

Table 1. Main studies on oxygen tolerance in Clostridia.

Clostridial species	Response to O_2 or air	Regulatory mechanisms	Targeted functions involved in O_2 tolerance	References
Clostridium acetobutylicum	Growth arrest in the presence of 3% O_2 Response to 1 h exposure to 0.5% O_2	Role of PerR Possible role of Rex	Function of FDP, NROR, Rd, revRbr and Rbr	O'Brien and Morris (1971), Hillmann *et al*. (2008, 2009a) and Hillmann *et al*. (2009b); Riebe *et al*. (2009); Kawasaki *et al*. (2007) and Kawasaki *et al*. (2009); May *et al*. (2004), Zhang *et al*. (2014)
Clostridium aminovalericum	Absence of growth in 1% O_2 Induction of an adaptive response after microoxic stress	Unknown	Function of a FDP	Kawasaki *et al*. (2004) and Kawasaki *et al*. (2005)
Clostridium butyricum	Growth in the presence of 3% O_2 Growth resumes after air exposure Transcriptional response after 5 h aerated cultures	Unknown	NADH oxidase activity in crude extracts	Kawasaki *et al*. (1998); Camerini *et al*. (2019)
Clostridium neonatale	High level of resistance to 24 h exposure to air	Unknown		Schonherr-Hellec *et al*. (2017)
Clostridium ljungdahlii	Growth at 8% O_2 and transcriptional response	Unknown		Whitham *et al*. (2015)
Clostridium perfringens	Survive in a growth-arrested stage in the presence of O_2 Induction of an adaptive response	Unknown		Trinh *et al*. (2000)
Clostridium tetani	Considered as aerotolerant	Unknown	Function of a Heme oxygenase	Bruggemann *et al*. (2004)
Clostridioides difficile	4.5 h exposure to 2% O_2, 15–60 min exposure to 5% O_2, 15 min exposure to air	Role of PerR Role of σ^B	Function of Fdp, revRbr Role of IscS and HemA	Giordano *et al*. (2018a) and Giordano *et al*. (2018b); Emerson *et al*. (2008); Neumann-Schaal *et al*. (2018); Kint *et al*. (2017) and Kint *et al*. (2020), Boekhoud *et al*. (2020); Troitzsch *et al*. (2021); Knippel *et al*. (2020)
Faecalibacterium prausnitzii	Loss of viability after 2 min air exposure Growth at the air interface in presence of riboflavin	Unknown	Extracellular electron shuttle to reduce O_2 in presence of riboflavin	Duncan *et al*. (2002) Khan *et al*. (2012a), Khan *et al*. (2012b) and Khan *et al*. (2014)

extracellular proline (Neumann-Schaal *et al*., 2018). In addition, the expression of genes encoding proteins involved in the leucine reductive pathway is also transiently decreased. It is worth noting that one enzyme of this pathway, the 2-hydroxycaproyl-CoA dehydratase (HadBC), a radical enzyme containing a Fe-S cluster (Knauer *et al*., 2011), is known to be especially O_2-sensitive (Kim *et al*., 2004; Buckel and Golding, 2006). The metabolism of cofactors also shifts to less O_2 labile reactions. In *C. difficile*, the synthesis of thiamine and cobalamin cofactors decreases. A temporary induction of the synthesis of riboflavin is also detected possibly due to an increased demand for flavin cofactors (FMN or FAD) in redox reactions that may substitute for the O_2-

sensitive FeS proteins, such as the ferredoxins involved in multiple electron transfer pathways and in O_2 detoxification reactions (see below) (Neumann-Schaal *et al*., 2018). The synthesis of molybdopterin cofactor is repressed in *C. acetobutylicum* while genes likely involved in metal cofactor synthesis or repair are induced upon O_2 exposure in *C. ljungdahlii* (Whitham *et al*., 2015). Besides these genes, ferritin and ferric uptake regulation protein (Fur) are upregulated upon O_2 exposure in *C. ljungdahlii* (Whitham *et al*., 2015). This may indicate not only a protection mechanism focused on the control of iron availability inside the cell by Fur, repressing the genes involved in iron uptake, but also an alternative and complementary route to remove O_2 while

scavenging iron via ferritin ferroxidase activity (Bradley et al., 2020), thus contributing to prevent ROS formation through Fenton chemistry. Fe-S clusters, especially of the $[4Fe4S]^{2+/1+}$ type, are highly sensitive to O_2 and/or ROS. The expression of the *suf* operon is induced upon O_2 exposure in *C. acetobutylicum* (Hillmann et al., 2009a; Camerini et al., 2019). It is worth noting that the Suf system is favoured over Isc under stressful conditions especially oxidative stress in *Escherichia coli* (Mettert and Kiley, 2015). An induction of genes encoding proteins involved in cysteine synthesis and/or thiol homeostasis is also observed in most studies on clostridial O_2 responses. It is associated with an important increase of cysteine oxidation in proteins in micro-aerophilic growth conditions in *C. difficile* (Neumann-Schaal et al., 2018). In addition, the introduction of a glutathione pathway in *C. acetobutylicum* increases its aerotolerance showing the crucial importance of thiol protection when cells are exposed to O_2 (Zhu et al., 2011). An induction is also observed for key steps of nucleotide synthesis (purine metabolism, ribonucleotide diphosphate or triphosphate reductase) and for DNA repair (UvrAB, RecA, RecO, Nth) (Emerson et al., 2008; Hillmann et al., 2009a; Neumann-Schaal et al., 2018).

O_2 and ROS responses are also related to nitric oxide (NO) responses. NO, a radical molecule, affects many of the O_2/ROS targets, namely, metalloproteins, and is also used by the innate immune system to fight pathogens. Particularly important for the immune response is the production of peroxynitrite, a potent oxidant and bactericidal compound, resulting from the reaction of the superoxide anion produced by the phagocytic NADPH:O_2 oxidoreductases with the NO produced by the respective NO synthases. Therefore, some bacterial responses to NO involve the same regulators as those for oxidative stress response (Kint et al., 2017) and include also some of the same responsive enzymes, such as the flavodiiron proteins (FDP) (see below) (Martins et al., 2019).

Enzymes involved in oxygen scavenging or protection

All organisms have enzymes to deal with the toxicity of O_2 and ROS, such as O_2 reducing enzymes, superoxide dismutases (SOD) or reductases (SOR), peroxidases and catalases. In Clostridia, among the most induced genes in the presence of low O_2 tensions or after air exposure, are always the genes encoding rubrerythrins (Rbr), reverse rubrerythrins (revRbr) and/or FDP (Kawasaki et al., 2005; Kawasaki et al., 2007; Emerson et al., 2008; Hillmann et al., 2009b; Whitham et al., 2015; Camerini et al., 2019; Kint et al., 2020). We will focus particularly on these recently identified enzymes employed by anaerobes among others to deal with O_2 exposure

situations. Of note, some Clostridia may also contain Fe- and Mn-SODs heme- and Mn-catalases as well as 1Fe or 2Fe-SORs that reduce the superoxide anion to H_2O_2 (Sheng et al., 2014).

The most direct way to counteract O_2 is to eliminate it, thereby avoiding its direct toxicity and ROS formation. This can be accomplished by the membrane-bound O_2 reductases or the FDPs. Membrane-bound O_2 reductases are rarely found in the genomes of Clostridia, and, among those, only enzymes of the cytochrome *bd* type are present in few species, i.e. heme-copper O_2 reductases are apparently absent in these bacteria. The cytochrome *bd* O_2 reductases have a high-affinity for O_2 and, when present in anaerobes, are generally believed to confer protection to O_2 sensitive enzymes, such as nitrogenases. Since these enzymes are electrogenic, it remains to be clarified whether they also contribute to energy conservation concomitantly to their protective function in anaerobes [for an update discussion see Borisov et al., 2020]. A few other enzymes contributing to O_2 scavenging have been described such as a heme oxygenase that consumes O_2 to oxidize heme in *C. tetani* (Bruggemann et al., 2004). A heme oxygenase is also present in the *C. perfringens* genome. A recent work indicates that the membrane-associated HsmA protein sequesters heme leading to a decrease of ROS production upon O_2 exposure in *C. difficile* (Knippel et al., 2020).

Flavodiiron proteins involved in O_2 reduction

While present in the three domains of life, including multicellular eukaryotes, the first FDP was discovered in the anaerobic sulfate-reducing bacterium, *Desulfovibrio gigas*, where it was proposed to confer resistance to O_2, yet without allowing its growth in the presence of O_2 (Chen et al., 1993; Fareleira et al., 2003). Since then, other FDPs have been isolated from a variety of prokaryotic and eukaryotic anaerobes, including several Clostridia. FDPs are soluble metalloenzymes generally considered to be cytoplasmic, endowed with *bona fide* O_2- and/or NO-reductase activities, converting them into H_2O or the innocuous nitrous oxide, N_2O respectively [for recent reviews see Romao et al., 2016a and Martins et al., 2019]. FDPs either display a selective preference for one of the two main substrates, NO or O_2, or have a dual behaviour. Although the three-dimensional crystallographic structures for each type of FDPs are already known, the determinants for substrate selectivity are still elusive, as the amino acid ligands of the catalytic site are conserved. The prototype of a NO selective FDP is the flavorubredoxin (also called NorV, from the name of its encoding gene) from *E. coli*, while O_2 selective FDPs are, for example, those from *C. acetobutylicum*, *C.*

aminovalericum, C. difficile (see below) and the protozoan *Entamoeba histolytica* (Kawasaki *et al*., 2009; Hillmann *et al*., 2009b; Vicente *et al*., 2012; Folgosa *et al*., 2018b; Kint *et al*., 2020). FDPs have as minimal functional unit a head-to-tail homodimer, in which each monomer (400 amino acids) is built by two consecutive domains: (i) a metallo-β-lactamase like domain, which harbours the catalytic site, a diiron centre of the histidine-carboxylate type, having one- or two μ-hydroxo species bridging the iron ions (Fig. 2), and (ii) a small-flavodoxin-like domain, harbouring one flavin mononucleotide, FMN. The head-to-tail quaternary structure leads to a close proximity of the diiron site of one monomer to the FMN of the other, thus allowing fast intramolecular electron transfer between the electron-accepting site, the FMN and the catalytic centre. The most abundant FDPs, class A FDPs, have only the two core domains (Fig. 2). To function, these FDPs need to receive electrons from redox partners such as rubredoxins (Rds), which in turn are reduced by NAD(P)H:Rd oxidoreductases (NROR). In *C. acetobutylicum*, a Rd and a NROR are present and form, *in vitro*, an electron transfer chain together with its class A FDP, linking NAD(P)H oxidation to O_2 reduction (Kawasaki *et al*., 2009). However, in many genomes encoding FDPs, genes coding for Rds are missing, i.e. alternative electron shuttles remain to be identified. An example is *C. difficile* class A FDP (FdpA), an O_2 selective enzyme, for which the physiological electron donor remains unknown. Its activity was determined using the *E. coli* FDP-Rd domain and the associated NADH oxidoreductase as electron partners (Kint *et al*., 2020). There are also many examples of much more complex FDPs with extra modules following the flavodoxin-like domain, and up to three additional extra domains putatively containing multiple redox cofactors have thus far been identified (Romao *et al*., 2016a; Martins *et al*., 2019). Interestingly, the most complex FDPs are mainly found in Clostridiales. Those extra domains appear to act as electron shuttles through redox centres such as the Rd-type (FeCys$_4$), FMN, FAD and Fe-S clusters (Folgosa *et al*., 2018a; Martins *et al*., 2019), but other functional roles cannot be excluded at present. The well-studied examples of complex FDPs are the *E. coli* FDP NO-reductase (flavorubredoxin), which contains an extra Rd domain (Romao *et al*., 2016b), and the Class F FDP from *C. difficile* (FdpF) that has an extra short-chain Rd-domain, followed by an FAD containing NROR-like domain (Fig. 2) (Folgosa *et al*., 2018b). Bioinformatic analysis shows that Class F FDPs are also found in the genomes of other Clostridia, such as *C. perfringens*, *C. beijerinckii*, *C. pasteurianum*, *C. butyricum*, *C. tertium*, *Paeniclostridium sordellii* and *Paraclostridium bifermentans* (unpublished analysis). FdpF, the most complex FDP thus far studied, is a standalone FDP,

since it has, in a single polypeptide chain, all the necessary cofactors and domains to receive electrons from NADH (its preferred electron donor) and transfer them to the catalytic centre (Folgosa *et al*., 2018b). *In vitro* studies demonstrated that O_2 is the preferential substrate for this enzyme, with a considerably high turnover rate for its complete reduction to H_2O in comparison to other FDPs with preferential O_2 reductase activity (Vicente *et al*., 2012). The turnover rate observed was negligible when NO was used as substrate. These results pointed towards the participation of this protein in the O_2 scavenging system of *C. difficile* (Folgosa *et al*., 2018b; Kint *et al*., 2020). It was also found out that FdpA and FdpF from *C. difficile* exhibit an H_2O_2-reductase activity *in vitro*. While the turnover rate observed with FdpA is relatively low, 0.27 s^{-1} (this value may be considerably underestimated as the natural electron donor is unknown), the one observed with FdpF that accepts electrons directly from NADH, 2.4 s^{-1}, is comparable to those of rubrerythrins. Many organisms contain multiple genes (up to seven, in *Anabaena*) encoding FDPs, either of the same type (e.g. two class A FDPs in *C. acetobutylicum*) or of different classes (e.g. *C. difficile*, with a Class A, FdpA and a Class F, FdpF) (Fig. 2).

Rubrerythrins involved in H_2O_2 and O_2 reduction

Rubrerythrins are another type of enzyme involved in response to oxidative stress conditions, widespread in anaerobes and in particular in Clostridiales but, as the FDPs, not restricted to them (Fig. 2). Rubrerythrins were also first discovered in the anaerobic sulfate-reducing bacteria of the *Desulfovibrio* genus and were so named because they contained a Rd-like centre plus a diiron site reminiscent of those present in hemerythrins, which are O_2 binding proteins from worms (LeGall *et al*., 1998). The minimal functional unit of these proteins ($\sim$200 amino acids), now called erythrin, is a four-helix bundle harbouring the diiron catalytic centre, also of the histidine-carboxylate type (Fig. 2) but distinct from that of the FDPs. Erythrins and derived proteins are part of a large superfamily of proteins, which include, among others, the iron storage proteins ferritins, heme(bacterio) ferritins and DNA binding proteins upon starvation, and were proposed to be the evolutionary ancestors of the ferritin family (Andrews, 2010). Erythrins appear in general associated with other structural modules and also with extra redox cofactors (Andrews, 2010; Pinto *et al*., 2011; Cardenas *et al*., 2016), the most frequent being either short-chain or canonical Rd sites. Therefore, like the FDPs, rubrerythrins are another example of modular oxidoreductases. The Rd domains may be present in the C-terminal part of the protein in the 'canonical' rubrerythrins (Rbr) or at the N-terminal region, the so-called
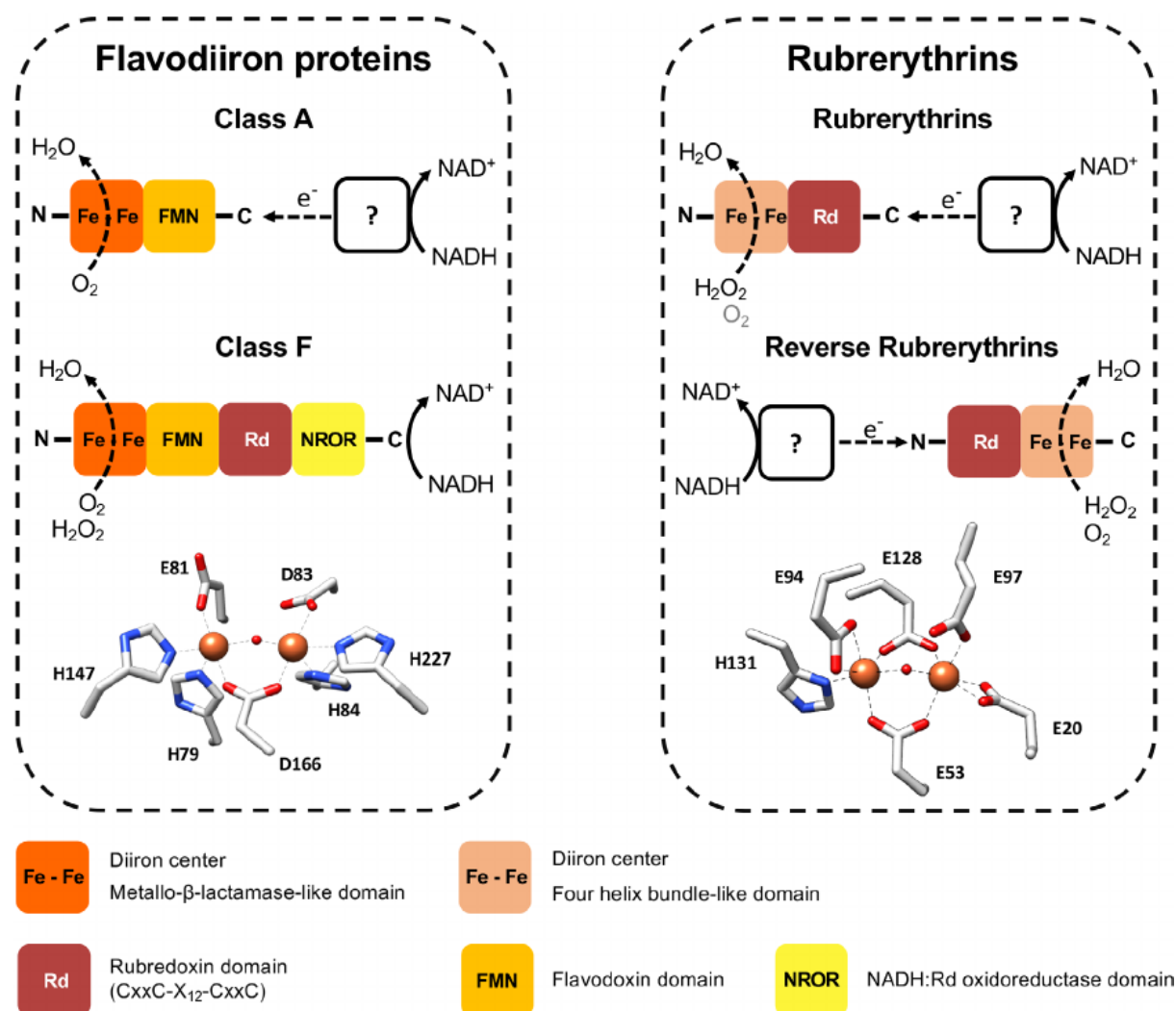
Fig 2. Rbr and FDP proteins. Representation of the flavodiiron proteins and rubrerythrins found in several clostridial genomes. The substrates and products displayed are represented for the respective proteins and are based on those determined by *in vitro* experiments with the enzymes of *C. difficile* and *C. acetobutylicum* (Kawasaki *et al.*, 2009; Riebe *et al.*, 2009; Folgosa *et al.*, 2018a; Folgosa *et al.*, 2018b; Kint *et al.*, 2020) except for those shown for canonical rubrerythrins that are determined only for *C. acetobutylicum* (Riebe *et al.*, 2009). The diiron catalytic centres of both canonical FDPs and Rbrs are shown in the correspondent panel. Iron atoms are represented as orange spheres and O_2 as red smaller spheres. The numbers shown on the amino acid ligands refer to the numbering of the structures of *E. coli* FDP (PDB 4D02) and *D. vulgaris* Rbr (PDB 1LKM) that were used as examples for each protein. Both structures are for the oxidized forms. The description of each domain is presented below the panels.

reverse rubrerythrins (revRbr) (Fig. 2). Rbrs and revRbrs are widely distributed in Clostridiales, and in many of them, the two types of rubrerythrins coexist.

Rbrs and revRbrs are considered to be cytoplasmic, and the most consensual enzymatic activity is that of H_2O_2 reductases (Coulter *et al.*, 1999; Kurtz Jr., 2006; Kawasaki *et al.*, 2009; Riebe *et al.*, 2009). In this case, the turnover rates are in the range of 2–5 s^{-1} as described for the *Pyrococcus furiosus*, *Desulfovibrio vulgaris* and *C. perfringens* Rbrs (Coulter *et al.*, 1999; Weinberg *et al.*, 2004). In line with these results, the H_2O_2-reductase activity of the two revRbr from *C. difficile*, revRbr1 and revRbr2 was determined as 2.1 and

7.7 s^{-1} respectively (Kint *et al.*, 2020); bearing in mind that the physiological electron donors in *C. difficile* are still unknown, and these activities may be underestimated. The revRbr of *C. acetobutylicum* also functions as an efficient NAD(P)H-dependent H_2O_2 reductase (Kawasaki *et al.*, 2009; Riebe *et al.*, 2009). However, the revRbrs of *C. difficile* and *C. acetobutylicum* have also O_2-reductase activity even if H_2O_2 seems to be the preferred substrate, especially in *C. acetobutylicum* (Kawasaki *et al.*, 2009; Riebe *et al.*, 2009; Kint *et al.*, 2020). Interestingly, the catalytic site of rubrerythrins is very similar to that of the so-called alternative oxidases, also imbedded in a four-helix bundle structure,

and indeed rubrerythrins were hypothesized to have been 'primitive' O_2 reductases. In Rbrs and/or revRbrs, the Rd site is the electron entry point, which then transfers electrons to the catalytic diiron site. When acting as peroxidases and/or O_2 reductases, Rbrs and/or revRbrs receive electrons from an external electron partner, frequently a Rd which, in turn, is reduced by NROR as it happens in the electron transfer chains of the FDPs. While the redox partners are identified in *C. acetobutylicum* (Riebe *et al*., 2009), in several clostridial genomes, the absence of Rd encoding genes indicates that alternative electron transfer partners remain to be identified. In addition, it should be noted that some Rbrs have been isolated from bacteria with mixed Fe-Zn dimetal sites (Li *et al*., 2003). So, alternative functions for these enzymes may be discovered in the future.

Physiological studies on the roles of FDPs and revRbrs in oxygen tolerance in C. difficile *and* C. acetobutylicum

The physiological role of the FDP and revRbr enzymes has mainly been studied in *C. acetobutylicum* and *C. difficile*. In *C. acetobutylicum*, indirect evidence strongly suggests a role for these enzymes in O_2 tolerance. First, the expression of genes encoding FDP, NROR, Rd and revRbr are induced upon O_2 exposure (May *et al*., 2004; Kawasaki *et al*., 2005; Riebe *et al*., 2009; Hillmann *et al*., 2009b). Second, a strain of *C. acetobutylicum* overproducing a revRbr has an increased tolerance to O_2 and H_2O_2 (Riebe *et al*., 2009). In addition, a mutant inactivated for PerR, the repressor of the H_2O_2 response (see below), has an enhanced consumption of O_2 that is correlated with an overexpression of the genes encoding the two FDPs and the revRbr (Hillmann *et al*., 2008; Hillmann *et al*., 2009a). In *C. difficile*, the two FDPs and revRbrs play a pivotal role in O_2 tolerance and their NAD(P)H-dependent O_2-reductase activities seem to be additive *in vivo* (Kint *et al*., 2020). Indeed, while single mutations have no or only a weak effect on O_2 tolerance, a quadruple mutant has a strong growth defect when exposed to 0.1% O_2 and is completely unable to grow at 0.4% O_2.

O_2 consumption through an extracellular reduction of riboflavin

F. prausnitzii, which is extremely O_2 sensitive, is mainly present in the lumen of the colon but can be weakly detected within the mucus where O_2 concentration is higher. *F. prausnitzii* A2-165 is able to grow at the air interface in a medium containing cysteine or glutathione and riboflavin (Khan *et al*., 2012b). The addition of FAD or riboflavin increases five- to 10-fold the ability of *F. prausnitzii* A2-165 to reduce an electron acceptor. A putative flavin reductase, FAEPRA-A2165_00362, may be involved in the reduction of riboflavin but a functional analysis of the protein is still lacking (Heinken *et al*., 2014). Riboflavin and thiols act as redox mediators in a process still to be clarified that would allow the transfer of electrons from NADH to O_2 (Khan *et al*., 2012a; Khan *et al*., 2012b). By consuming O_2 outside the cells, this pathway protects the O_2-sensitive *F. prausnitzii* and likely enables its growth and survival at the faecal–mucosal interface where O_2 diffuses from epithelial cells (Swidsinski *et al*., 2008). Thiols and flavins can be provided by food (Dangour *et al*., 2010). Thiols are secreted by colonocytes and may be abundant in the gut mucosa while riboflavin could be produced and excreted by microorganism of the microbiota. Providing riboflavin seems to promote *F. prausnitzii* growth in the gastrointestinal tract and reduce symptoms associated with IBD (Harmsen *et al*., 2015).

Regulation of the oxygen response

The σ^B regulon and its role in the control of O_2 tolerance

In most Firmicutes, a key actor of the general stress response is the sigma factor, σ^B, which provides protection to many types of stress and starvation conditions. It has been recently proposed that σ^B regulates antioxidant functions in aerobic Firmicutes (Tran and Bonilla, 2021). Not all Firmicutes have σ^B, which is absent in most of the Clostridia. However, the *sigB* gene is present in the genomes of a few Clostridia including some members of Hungateiclostridiaceae, Eubacteriaceae, Tissierellaceae and Clostridiaceae (Kint *et al*., 2019). The σ^B factor is also found in the species of the *Peptostreptococcaceae* family such as *C. difficile*, *P. sordellii* and *P. bifermentans* (Kint *et al*., 2019). Until now, the role of σ^B in Clostridia has only been studied in *C. difficile* (Kint *et al*., 2017; Kint *et al*., 2019; Kint *et al*., 2020). Besides its role in the resistance to acidification, CAMPs, toxic compounds produced by the immune system such as H_2O_2, superoxide anion or NO, σ^B plays a crucial role in O_2 tolerance in *C. difficile* (Kint *et al*., 2017). This might be also the case for the other Clostridia harbouring the σ^B factor. In agreement, the core σ^B regulon of *C. difficile* contains genes encoding proteins involved in O_2, ROS and NO detoxification (Fig. 3). This includes *fdpA*, *fdpF*, *revrbr1* and *revrbr2* encoding the four aforementioned O_2 reductases of *C. difficile*, and *CD0176*, which is an NADH-dependent oxidoreductase, part of the CO-dehydrogenase gene cluster (Kint *et al*., 2017; Boekhoud *et al*., 2020; Kint *et al*., 2020). σ^B also positively controls genes involved in DNA repair, thiol homeostasis and Fe-S cluster biogenesis (Boekhoud *et al*., 2020). These functions are important for maintenance of the metabolic
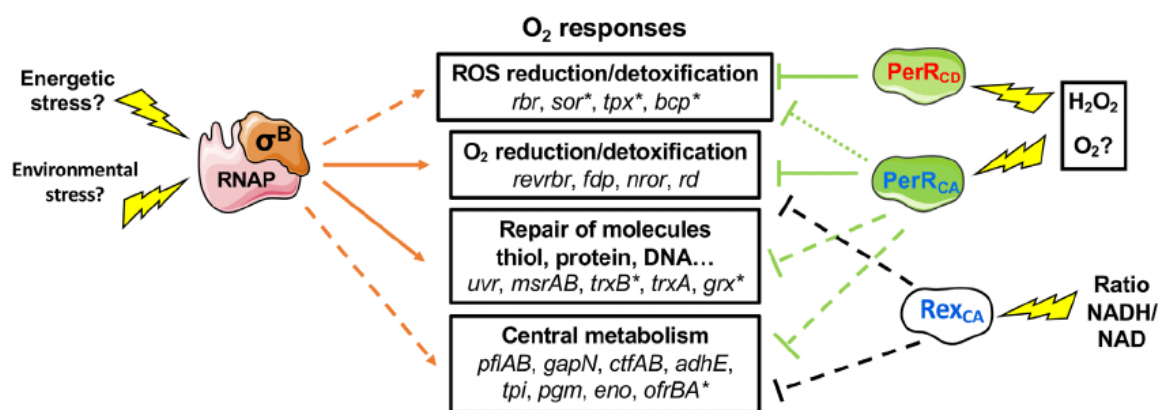
Fig 3. The mechanisms of control of O_2 adaptive responses in Clostridia. Upon stress exposure (energetic and/or environmental) indicated by the 'flash' yellow symbol, a complex signal transduction pathway triggers σ^B activation in *C. difficile* (Kint *et al*., 2019). The association of σ^B with the RNA-polymerase core enzyme leads to the transcription of its target genes. The PerR repressors of *C. acetobutylicum* (PerR$_{CA}$ in blue) and *C. difficile* (PerR$_{CD}$ in red) act as a switch to O_2 tolerance and/or ROS resistance. As in other Firmicutes, PerR might sense H_2O_2 but maybe also directly O_2 ('flash' yellow symbol). The role of Rex$_{CA}$ on central metabolism and O_2 reduction/detoxification is indicated. Direct and indirect effects are indicated by solid and dashed lines, respectively. The dotted arrows indicate that part of the genes is controlled by PerR$_{CA}$. The green arrows concern the control by PerR, the black arrows by Rex and the orange arrows by σ^B. Asterisk indicates some genes containing a PerR$_{CA}$ box in their promoter region. *fdp* (flavodiiron protein)*; rd* (Rubredoxin); *nror* (NADH:Rd-oxidoreductase), *revrbr* (reverse rubrerythrin); *sor* (desulfoferredoxin with superoxide reductase activity); *tpx* (thiol peroxidase); *bcp* (alkyl-hydroxyperoxidase); *grx* (glutaredoxin); *trxA* (thioredoxin); *trxB* (thioredoxin reductase); *sodC* (superoxide dismutase); *msrAB* (methionine sulfoxide reductase); *uvr* (excinuclease); *pfl* (pyruvate formate lyase); *gapN* (NADP-dependent glyceraldehyde-3P dehydrogenase); *adhE* (aldehyde dehydrogenase); *ctf* (butyrate-acetoacetate CoA-transferase); *ofr* (2-oxoglutarate ferredoxin oxidoreductase); *tpi/pgm/eno* (enzymes of glycolysis).

activity of anaerobes that use many Fe-S containing enzymes (Meyer, 2000) and for counteracting the effects of oxidizing compounds by repairing damaged molecules. Interestingly, one σ^B target, *iscS* encoding a cysteine desulfurase involved in Fe-S cluster biogenesis is also important for *C. difficile* growth in the presence of 1%–2% O_2 as well as for colonization in mice (Giordano *et al*., 2018b). All these regulations likely contribute to the very high level of O_2 sensitivity of the *sigB* mutant and its defect in gut colonization (Kint *et al*., 2017). Genes transcribed by σ^B that could play a key role in *C. difficile* establishment in a host remain to be clearly identified but enzymes scavenging O_2 and ROS or protecting from these toxic molecules are good candidates.

In Firmicutes, including *C. difficile*, σ^B activation is tightly controlled by a post-translational mechanism named 'partner switching', in which interaction and activation of proteins controlling σ^B activity are mediated by reversible phosphorylation (Hecker *et al*., 2007; de Been *et al*., 2011; Kint *et al*., 2019). Specific signals are detected and conveyed to phosphatase(s) that dephosphorylate(s) the anti-anti-σ factor RsbV, releasing σ^B from its anti-σ factor RsbW turning on the transcription of its target genes. The N-terminal sensory domain of phosphatases and the sensory-associated proteins vary among Firmicutes (Hecker *et al*., 2007; de Been *et al*., 2011). In *C. difficile*, a unique PP2C phosphatase, RsbZ, and a putative sensor protein, CD2684, that codes for an essential Fe-S protein, have been identified (Kint *et al*., 2019). While the RsbZ phosphatase and

the CD2684-type protein are conserved in Peptostreptococcaceae, another class of PP2C phosphatase is present in Hungateiclostridiaceae, Eubacteriaceae and Tissierellaceae families suggesting the existence of two distinct σ^B activation pathways in Clostridia (Kint *et al*., 2019). The signal(s) recognized inside the host that triggers *C. difficile* σ^B activity during colonization remains unknown. In addition, half of the σ^B core regulon is induced after air exposure (Emerson *et al*., 2008; Kint *et al*., 2017; Boekhoud *et al*., 2020) and a few σ^B-target genes are shown to be induced by H_2O_2 (Boekhoud *et al*., 2020). Further work will be required to identify signal(s) triggering σ^B activity and the transcription of the core σ^B regulon.

The role of the PerR repressor in the O_2 tolerance response in Clostridia

PerR is the transcriptional repressor involved in the H_2O_2 stress response in Firmicutes. The metalloregulator of *Bacillus subtilis*, PerR$_{BS}$ is sensitive to metal-catalysed oxidation by Fe^{2+} and H_2O_2 through oxidation of two conserved His residues (Lee and Helmann, 2006). In *B. subtilis*, the oxidation of PerR$_{BS}$ in the presence of H_2O_2 or during aerobic growth conditions modifies its capacity to bind to DNA and to repress transcription (Bsat *et al*., 1998; Lee and Helmann, 2006; Pinochet-Barros and Helmann, 2018). PerR$_{BS}$ oxidation enables a rapid induction of genes required to protect cells from a H_2O_2

stress. A homologue of PerR is present in the genome of Clostridia (Hillmann *et al*., 2008). The role of the PerR repressor has been studied in *C. acetobutylicum* where it is proposed that this regulator acts as a switch to O_2 tolerance (Hillmann *et al*., 2008). While a wild-type *C. acetobutylicum* strain loses viability after exposure to H_2O_2 or air, the inactivation of the $PerR_{CA}$ repressor leads to a better resistance to H_2O_2 as observed in other Firmicutes (Bsat *et al*., 1998; Horsburgh *et al*., 2001; Hillmann *et al*., 2008; Pinochet-Barros and Helmann, 2018) but also to an aerotolerance with a significant survival after 6 h of air exposure (Hillmann *et al*., 2008). In agreement, a more important H_2O_2 reductase activity is detected and a drastically increased consumption of O_2 allowing to re-establish anaerobiosis is observed in the *perR* mutant (Hillmann *et al*., 2008). This mutant, contrary to the wild-type strain, is able to grow slowly in the presence of air indicating that the central metabolism remains functional under these conditions. Almost 50 genes are upregulated in a *perR* mutant, half of them being induced upon 1 h of microaerophilic exposure. These include genes encoding enzymes involved in O_2 (revRbrs, FDPs, Rd) or ROS scavenging (peroxidases, superoxide reductase (SOR), thiol homeostasis (a Trx-reductase and a Grx) and energy conversion (Hillmann *et al*., 2009b). A $PerR_{CA}$ box ($ATAATN_2ATTAT$) resembling the PerR binding motif of *B. subtilis* and *Staphylococcus aureus* (Horsburgh *et al*., 2001; Pinochet-Barros and Helmann, 2018) is found upstream of 13 genes encoding proteins mainly involved in O_2 detoxification and redox balance (Fig. 3). Genes encoding FDP, Rd, NROR and revRbr contain a $PerR_{CA}$ box in their promoter region and $PerR_{CA}$ binds to the promoter region of the gene encoding the revRbr (Hillmann *et al*., 2009b). $PerR_{CA}$ acts as a key regulator of the O_2- and ROS-defence machinery and targets important genes necessary to meet the demand for the NAD(P)H required to scavenge these molecules (Fig. 3). Interestingly, the PerR regulator also plays a key role in the response to low O_2 exposure in another anaerobe, *D. vulgaris* (Wildschut *et al*., 2012).

In *C. difficile*, a recent study indicates that the 630Δ*erm* strain contains a mutation in the helix-turn-helix motif of the $PerR_{CD}$ repressor conferring a more important air resistance to this strain with a significant survival after 6 h of exposure (Troitzsch *et al*., 2021) as observed for the *perR* mutant of *C. acetobutylicum*. This indicates that $PerR_{CD}$ also contributes to the O_2 tolerance response in addition to σ^B. In addition, a short exposure to 5% O_2 or to H_2O_2 increased the O_2 tolerance of the wild-type *perR* strain suggesting the induction of an adaptive response. The *perR* gene belongs to the *rbr* operon encoding a rubrerythrin (Rbr), the PerR repressor and a desulfoferrodoxin [a 2Fe superoxide reductase

(SOR), previously known also as *rbo*]. The expression of this operon is induced by H_2O_2 in a PerR-dependent manner. $PerR_{CD}$ binds to the *rbr* promoter region (Troitzsch *et al*., 2021). It is worth noting that the two *rbr* genes are not members of the $PerR_{CA}$ regulon suggesting that the PerR regulons are different in *C. difficile* and *C. acetobutylicum* that lacks σ^B. Accordingly, the main $PerR_{CA}$ direct targets involved in O_2 detoxification in *C. acetobutylicum* (Hillmann *et al*., 2008; Hillmann *et al*., 2009a) are also key members of the σ^B regulon in *C. difficile* (Hillmann *et al*., 2008; Hillmann *et al*., 2009a; Kint *et al*., 2019; Boekhoud *et al*., 2020; Troitzsch *et al*., 2021). This highlights a diversity in the regulatory network controlling O_2 adaptive responses among Clostridia and the relative roles of both σ^B and PerR remain to be clarified.

The exact signal responsible for the inactivation of PerR and the de-repression of its target genes is still a matter of debate. In anaerobes, low levels of H_2O_2 could be recognized as a signal of aeration and detected by a PerR hypersensitive to oxidation, as proposed for the facultative anaerobe *S. aureus* (Ji *et al*., 2015). Alternatively, O_2 could directly affect the activity of PerR that would be an O_2 sensor able to detect aeration. A recent study suggests indeed that $PerR_{BS}$ could also be oxidized directly by O_2 on histidine residues *in vitro* (Sethu *et al*., 2016). What is the signal recognized by PerR in anaerobes and whether the formation of H_2O_2 from O_2 is a prerequisite necessary to trigger PerR-dependent control are interesting questions for further investigation on the role of PerR in Clostridia.

Possible role of other regulators

To reduce O_2, Clostridia require reducing equivalents whose production likely imposes a shift in the cellular redox balance. The redox-sensing regulator, Rex, which senses the $NADH/NAD^+$ ratio to control mainly genes encoding NADH consuming enzymes, might be involved. The reconstruction of the Rex regulons in Clostridia belonging to the cluster I indicates that Rex controls fermentations, hydrogen production, CO_2/CO fixation and/or NAD biosynthesis, some of these metabolic pathways being poisoned and/or differentially regulated in presence of O_2 (see above) (Zhang *et al*., 2014). In addition, in *C. acetobutylicum*, exposure to a superoxide donor increases the $NADH/NAD^+$ ratio and upregulates direct Rex targets while an opposite effect is observed after exposure to H_2O_2. Finally, a *rex* mutant is more sensitive to H_2O_2 killing, implying a role of Rex in oxidative stress response. In agreement, Rex_{CA} positively controls the expression of genes encoding revRbrs, FDPs, Rd, NROR, Rbo by an indirect and unknown mechanism (Zhang *et al*., 2014). These links between the Rex

regulator and the response to O_2 probably exist in some other Clostridia even if experimental data are lacking.

In *C. difficile* and *C. acetobutylicum*, the Fur regulator and/or iron starvation control genes whose expression is modulated upon O_2 exposure (fermentation pathways, hydrogen production, CO_2/CO fixation, Stickland metabolism, riboflavin synthesis) suggesting the existence of regulatory links between these two adaptive responses (Vasileva *et al*., 2012; Ho and Ellermeier, 2015; Berges *et al*., 2018). The *fur* mutant of *C. acetobutylicum* has a reduced viability upon exposure to O_2 and H_2O_2 compared to the wild-type strain (Vasileva *et al*., 2012). However, this increased sensitivity to oxidative stress might result from an imbalance in intracellular ion homeostasis since Fur does not control the expression of genes coding for proteins involved in O_2/ROS detoxification systems (Vasileva *et al*., 2012).

Conclusion

Obligate anaerobes such as Clostridia have developed efficient strategies to survive O_2 and air exposure and even to grow in the presence of low O_2 tensions. This O_2 tolerance involves their ability to consume O_2, eliminate its derived reactive species, to adapt their metabolism and to efficiently repair damaged molecules (proteins, thiols, DNA). The detrimental impact of O_2 exposure damaging molecules and poisoning enzymes might be direct or indirect through the production of ROS. Indeed, in *Bacteroides thetaiotaomicron*, aerated growth conditions drastically increase ROS production (H_2O_2 but mainly superoxide) (Imlay *et al*., 2019). Even if this has yet not been studied in Clostridia, a similar rise in ROS is very likely and might contribute to indirect injuries following O_2 exposure. Clostridial genomes also contain enzymes implicated in ROS detoxification such as Rbr, alkyl hydroperoxidase and SOR or dismutases. In *C. difficile*, a system of heme capture was proposed to provide ROS defence within the gut (Knippel *et al*., 2020). It is worth noting that O_2 and H_2O_2 responses interplay. Indeed, in *C. acetobutylicum*, $PerR_{CA}$ is involved in both O_2 and H_2O_2 detoxification and in *C. difficile*, σ^B directly controls the O_2 scavenging processes and indirectly H_2O_2 detoxifying pathways, (Kint *et al*., 2017; Boekhoud *et al*., 2020), $PerR_{CD}$ being the main actor for H_2O_2 detoxification (Troitzsch *et al*., 2021). Up to now, the O_2 tolerance appears to be related to the presence of two families of proteins, revRbr and FDP enzymes, which are able to reduce O_2 *in vitro* and might be important *in vivo* during O_2 exposure (Kint *et al*., 2020). In addition, revRbrs displayed peroxidase activity in a magnitude comparable to the canonical Rbrs and might be also involved in ROS detoxification. A better understanding of clostridial response to diverse oxidative stresses is

of interest both for the apprehension of pathogen infections and for biotechnological production, where the aerotolerance of industrial clostridial strains is a desirable phenotypic trait to facilitate industrial bioprocesses where strict anoxic conditions might be difficult to maintain (Tracy *et al*., 2012). For pathogens, the tolerance to O_2 might be advantageous to the colonization process in the host and O_2 likely plays a key role in the organization and structuration of multispecies microbial communities within the different environmental niches.

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