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Review Article

Harnessing hypoxia: bacterial adaptation and chronic infection in cystic fibrosis

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Abstract

The exquisite ability of bacteria to adapt to their environment is essential for their capacity to colonize hostile niches. In the cystic fibrosis (CF) lung, hypoxia is among several environmental stresses that opportunistic pathogens must overcome to persist and chronically colonize. Although the role of hypoxia in the host has been widely reviewed, the impact of hypoxia on bacterial pathogens has not yet been studied extensively. This review considers the bacterial oxygen-sensing mechanisms in three species that effectively colonize the lungs of people with CF, namely *Pseudomonas aeruginosa*, *Burkholderia cepacia* complex, and *Mycobacterium abscessus* and draws parallels between their three proposed oxygen-sensing two-component systems: BfiSR, FixLJ, and DosRS, respectively. Moreover, each species expresses regulons that respond to hypoxia: Anr, Lxa, and DosR, and encode multiple proteins that share similar homologies and function. Many adaptations that these pathogens undergo during chronic infection, including antibiotic resistance, protease expression, or changes in motility, have parallels in the responses of the respective species to hypoxia. It is likely that exposure to hypoxia in their environmental habitats predispose these pathogens to colonization of hypoxic niches, arming them with mechanisms than enable their evasion of the immune system and establish chronic infections. Overcoming hypoxia presents a new target for therapeutic options against chronic lung infections.

Keywords: hypoxia; lung disease; chronic infection; adaptation; *Pseudomonas aeruginosa*; *Burkholderia cepacia* complex; *Mycobacterium abscessus*

Introduction

Oxygen is essential for life on Earth. Although it is potentially toxic and mutagenic (Buonocore et al. 2010), species such as cyanobacteria exploited it to their advantage. They adapted to utilize oxygen for the processes of oxygenic photosynthesis (Kopp et al. 2005) and oxidative phosphorylation ever since the Great Oxidation Event 2.5 billion years ago (Kump 2008). Oxygen and its role in aerobic respiration is ideally suited to energy generation for several reasons: it can readily diffuse across biological membranes; produces a large free energy release during electron transfer; yields 4-fold more energy per molecule of glucose than even the most efficient anaerobic respiration; and finally it can bind haem groups in proteins, such as haemoglobin and cytochromes allowing it to be transported around the body and facilitating the electron transport chain in mitochondria (Thannickal 2009).

'Hypoxia' describes a state of subphysiological oxygen levels (Span and Bussink 2015) caused by an imbalance between oxygen supply and demand (Hajdamowicz et al. 2019). In practice, it means that a tissue or organism experiences lower than normal oxygen levels. This is not exclusively pathological, for example, states of hypoxia are generated in the body by intense physical exercise or high-altitude conditions (Prefaut et al. 2000, West 2004) and physiological oxygen gradients also exist in healthy tissues. These gradients tend to be moderate and stable and are vital for processes, such as angiogenesis and immune cell homeostasis (Lenihan and Taylor 2013, Palazon et al. 2014). Examples exist in the intestinal mucosa, the renal medulla, the bone marrow, the placenta and foetus during pregnancy, the retina, and in the light zone of the germinal centre of lymph nodes (Grimm and Willmann 2012, Shah and Zúñiga-Pflücker 2014, Campbell et al. 2016, Beerman et al. 2017). Hypoxic pulmonary vasoconstriction can also contribute to the maintenance of arterial oxygenation during asphyxiation to prevent life-threatening hypoxemia (Naeije and Brimioulle 2001).

In contrast, pathological hypoxia is characterized by fluctuating severe oxygen gradients due to increased oxygen demand, coupled with a decreased blood supply and disrupted metabolic processes (Taylor and Colgan 2017). Immunological niches, both physiological and pathological, are often associated with hypoxic microenvironments (Hu et al. 2022). The impact of hypoxia on the host immune response, as well as the link between hypoxia and inflammation have been extensively reviewed elsewhere (Schaible et al. 2010, Eltzschig and Carmeliet 2011, Schaffer and Taylor 2015) and are not the focus of this review.

Localized tissue hypoxia is commonly associated with pathologies such as tumours, inflammatory conditions, and bacterial infections (Biddlestone et al. 2015, Span and Bussink 2015). In bacterial infection, increased oxygen demand caused by recruitment of

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inflammatory cells, oxygen usage by the bacteria themselves, and reduced oxygen supply due to decreased blood flow disrupts oxygen homeostasis and creates a hypoxic environment (Colgan and Taylor 2010, Schaffer and Taylor 2015, Hajdamowicz et al. 2019). While the impact of hypoxia on the host has been extensively described elsewhere (Taylor and Pouyssegur 2007, Colgan and Taylor 2010, Schaible et al. 2010, 2012, Biddlestone et al. 2015, Taylor and Colgan 2017), the impact of hypoxia on colonising pathogens has not been widely examined. Key questions such as how hypoxia can affect bacteria during chronic infections, and whether it contributes to the adaptation of bacterial pathogens remain unanswered.

The availability of oxygen is an important factor for bacterial pathogens as it determines the optimal strategy required when colonising any environment or niche (Berney et al. 2014). Bacterial responses to varying oxygen levels can be quite extreme, particularly for facultative anaerobes (Taabazuing et al. 2014). For example, *Staphylococcus aureus* metabolism is dramatically changed in hypoxic conditions in response to altered activity of the redox-responsive transcription factors AgrA, Rex, and SrrA (Christmas et al. 2019). *Pseudomonas aeruginosa* also adapts to changes in oxygen concentration by using alternate terminal electron acceptors such as nitrogen and pyocyanin (PCN) as well as multiple respiratory terminal oxidases with high affinity for oxygen (Rossi et al. 2020).

In pulmonary disease, abnormal airflow due to obstruction of airways, increased or thickened mucus on the pulmonary surface, infection, and inflammation often results in impaired gaseous exchange and poor blood oxygenation (Tuder et al. 2007). This is particularly apparent in individuals with underlying respiratory conditions such as cystic fibrosis (CF) and chronic obstructive pulmonary disorder (COPD). Both conditions result in impaired lung function and abnormal mucus clearance which facilitate the frequent bacterial infections and chronic colonization which are hallmarks of CF and COPD (Cui et al. 2014). CF is an autosomal recessive condition in which mutations in the cystic fibrosis transmembrane conductance regulator gene cause defective chloride ion (Cl^-) transport across epithelial cells (Thakur et al. 2024). People with CF experience chronic infection with cycles of exacerbations, in addition to inflammation, and mucus obstruction throughout their lives (Caverly and LiPuma 2018). Infections are typically caused by opportunistic pathogens, such as *P. aeruginosa*, *Burkholderia cepacia* complex (Bcc), *S. aureus*, *Stenotrophomonas maltophilia*, *Achromobacter xyloxidans*, nontuberculous mycobacteria (e.g. *Mycobacterium abscessus* complex), and fungi such as *Aspergillus fumigatus* (Mahenthiralingam 2014, King et al. 2016, Blanchard and Waters 2019, 2022). COPD is another complex respiratory disorder characterized by restricted airflow through the pulmonary tract due to inflammation, emphysema, and structural damage to the lung parenchyma (Barnes and Celli 2009). Individuals with COPD suffer from periods of acute exacerbations (AECOPD), which are characterized by significant deterioration in respiratory function (Leung et al. 2017). Similar to CF, localized tissue hypoxia is implicated in facilitating bacterial infections and exacerbations, however its exact role and importance are not yet well characterized (Shukla et al. 2020). Bacterial infections are an important risk factor for acute exacerbations in COPD with *Streptococcus pneumoniae*, *Moraxella catarrhalis*, *Acinetobacter baumannii*, *P. aeruginosa*, *Klebsiella pneumoniae*, *Haemophilus influenzae*, and *S. aureus* the most commonly reported bacteria (Moghoofei et al. 2020). There have also been reports of Bcc in COPD and non-CF bronchiectasis patients (Metersky et al. 2018, Ibrahim et al. 2021). Patients with chronic infection represent a subgroup of individuals with COPD. Various bacterial pathogens are implicated in low-grade chronic infections, including *H. influenzae*, *P. aeruginosa*, and *Chlamydia pneumoniae* (Sethi 2010).

In both CF and COPD, bacterial colonization by pathogenic species disrupts the natural lung microbiome and can alter oxygen availability in the lung in several ways. For example, increased oxygen consumption by immune cells recruited to fight infection as well as the oxygen required by high density bacterial populations results in a sharp rise in oxygen demand (Rossi et al. 2020). Moreover, therapeutic interventions such as antibiotic treatment can inadvertently aggravate this process by further disrupting the natural lung microbiome (Leung et al. 2017). Successful colonization is dependent on the ability of these opportunistic pathogens to adapt to these niches within the CF or COPD lung. These harsh lung environments drive the expression of certain phenotypes that supply the bacteria with the tools to not only colonize, but establish chronic infections (Cullen and McClean 2015).

The aim of this review is to summarize the state-of-the-art in pathogens' response and adaptation to hypoxic conditions during infection, with a particular focus on three challenging pathogens that employ common mechanisms of adaptation to chronically colonize the lung niche of people with CF, *P. aeruginosa*, Bcc, and *M. abscessus*, an emerging intracellular pathogen with a rapidly rising prevalence in young individuals with CF (Abidin et al. 2021). Worryingly, it has the potential to cause the most severe disease of any of the nontuberculous *Mycobacteria* group, with recent studies indicating that it is evolving towards becoming a true human pathogen (Lopeman et al. 2019). These three pathogens share common approaches to colonize low oxygen niches and consequently will be discussed to highlight some commonalities and some differences in their mechanisms of sensing and responding to low oxygen conditions.

How do bacteria sense oxygen?

As oxygen is so central to prokaryotic metabolism, it is essential that bacteria can sense oxygen levels accurately. To achieve this, they possess chemosensory systems dedicated to the sensing of environmental oxygen (Bailey-Serres and Chang 2005). These sensing mechanisms are the 'first responders', which trigger regulatory networks that actively regulate downstream targets, ranging from genes implicated in the production of virulence factors to posttranscriptional regulatory mechanisms (Green et al. 2014). There are numerous sensing systems, which enable organisms to detect different levels of oxygen tension. Bacteria rely primarily on proteins containing iron–sulphur clusters or haem domains to sense and respond to changes in oxygen concentration (Kiley and Beinert 2003, Green et al. 2009, Taabazuing et al. 2014). Once low oxygen levels have been detected, regulation systems are then activated to control gene expression. These can take the form of two-component systems (TCS) such as ArcBA in *Escherichia coli* (Alexeeva et al. 2003), the FixLJ system in Bcc, the BfiSR system in *P. aeruginosa* (which although not demonstrated yet, is proposed below), or the DosRS system in *M. abscessus* (the latter three systems are discussed in detail below). Other systems act via direct transcriptional regulation including WhiB in *Mycobacteria* (Alexeeva et al. 2003).

Two-component oxygen-sensing systems

TCS are composed of a sensor kinase, which directly phosphorylates a response regulator (RR) in response to an environmental cue. This activates the RR, which then controls the response

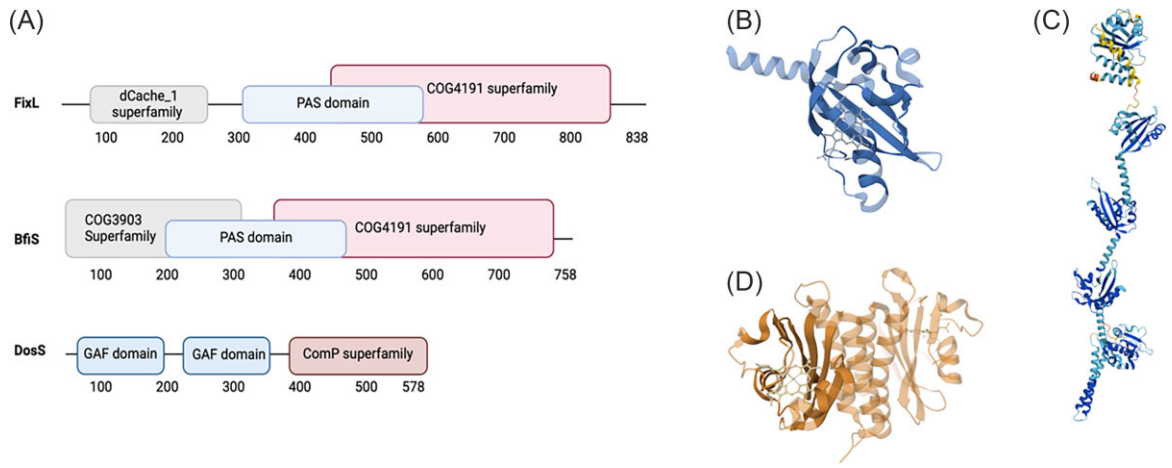

Figure 1. Comparison of the FixL, BfiS, and DosS proteins: (A) Similarities between the FixL, BfiS, and DosS proteins, showing the comparable domains. PAS domain = Per-Arnt-Sim domain, GAF domain = cGMP-specific and -regulated cyclic nucleotide phosphodiesterase, adenylyl cyclase, and *E. coli* transcription factor FhlA (Wang et al. 2023). Made using Biorender. (B) Structure of FixL showing 5 coordinate haem centre: PDB: 1XJ3. (C) Putative Structure of BfiS: generated using Alphafold 2 (Jumper et al. 2021). (D) Structure of DosS showing 5 coordinate haem centre: PDB: 2W3F.

(Chang and Stewart 1998). A well-characterized oxygen sensing system in *B. dolosa*, FixLJ, is a TCS composed of the sensor histidine kinase FixL, the RR FixJ, and the transcriptional regulator FixK (Schaefers et al. 2017, 2021). Bcc is a complex of at least 26 species with *B. multivorans*, *B. cenocepacia*, and *B. dolosa* the most prevalent Bcc species in people with CF (Cullen and McClean 2015, Velez et al. 2023). The *fixLJ* TCS of these species, *B. dolosa* (strain AU0158), *B. cenocepacia* (strains J2315 and K56-2), and *B. multivorans* (strain ATCC17616) share a high DNA sequence identity (94%–95%) (Schaefers et al. 2017). This signalling network is required for normal cellular growth under both ambient and hypoxic conditions as it has been shown to be responsible for the differential regulation of ∼11% of the genome in *B. dolosa* (Schaefers et al. 2017). Silva et al. (2016), showed that the *B. multivorans* FixL protein accumulated the highest number of mutations in the analysis of sequential chronic infection isolates taken over 20 years, highlighting its importance in adaptation to the CF lung (Silva et al. 2016, Schaefers et al. 2021). Lieberman et al. (2014) also demonstrated that FixL is under strong selective pressure in individuals with CF chronically infected with *B. dolosa* (Lieberman et al. 2014). This membrane-bound sensing component, FixL, is defined as a 'haem-sensor' that detects oxygen tension (Rodgers et al. 2008). The haem molecule is ligated to a Per-Arnt-Sim (PAS) domain in the N-terminus (Girvan and Munro 2013) (Fig. 1A). Upon binding of oxygen, the activity of the histidine kinase domain in the C-terminus is inhibited and following oxidation, the FixL haem enters an inactive oxyhaem form (Perutz et al. 1999, Ishii and Eguchi 2021). Alternatively, under hypoxic conditions oxygen dissociates from the haem-centre relieving repression of kinase activity. This five-coordinate deoxy FixL results in the phosphorylation of the RR FixJ and the downstream induction of genes that facilitate microaerobic growth (Girvan and Munro 2013) (Fig. 1B). The activation of the cognate RR FixJ results in the induction of FixK, the transcriptional regulator in this TCS. FixK acts as a positive regulator of the FixLJ system (Crosson et al. 2005).

TCSs are also potentially employed as haem-dependent oxygen sensing systems in *P. aeruginosa* (Petrova and Sauer 2009), which regulate a signalling cascade highly analogous to the FixLJ system. Biofilm initiation sensor (BfiS) is homologous to FixL, whilst the biofilm initiation regulator (BfiR) is homologous to FixJ (Schaefers et al. 2017). Interestingly, although the structure of BfiS has not been elucidated experimentally, a bioinformatic comparison revealed that both FixL and BfiS possess a PAS domain of similar length (Fig. 1A), which in FixL at least, contains a haem pocket and a putative active site. Both FixL and BfiS also contain a COG4191 superfamily domain containing a signal transduction histidine kinase (Wang et al. 2023) (Fig. 1A). In parallel to the FixLJ system, the BfiSR system could induce the activity of the transcriptional regulator, Anr (Sánchez-Jiménez et al. 2023), although a link has not yet been established. BfiS has been shown to negatively regulate rsmY and rsmZ small RNA levels, which are repressed under microaerobic conditions by Anr-dependent NarL modulation (O'Callaghan et al. 2011, Petrova and Sauer 2010). Anr is homologous to the cytoplasmic Fnr protein in *E. coli* that senses intracellular oxygen levels (Unden and Schirawski 1997). The ArcBA TCS in *E. coli* have been shown to influence cytoplasmic redox signalling impacting Fnr activity (Shalel Levanon et al. 2005). Therefore, it is possible that the BfiSR TCS, which is predicted to be expressed on the cytoplasmic membrane, could also sense intracellular oxygen levels and ultimately impact the activity of the *anr* regulon during microaerobic conditions. In *P. aeruginosa*, Anr regulates the transcription of the *anr* regulon, a regulon consisting of the 199 genes implicated in cellular growth under microaerobic conditions (Tribelli et al. 2019). A BlastP search revealed that both *P. aeruginosa* Anr and Bcc FixK belong to the CRP-FNR family of transcriptional regulators that respond to exogenous signals and share 43% sequence identity (Altschul et al. 1990) (Fig. 2). In addition, Anr binds a conserved DNA binding site in the *anr* regulon (5′-TTGATNNNNATCAA-3′) that is homologous to the proposed binding site of FixK in the low oxygen activated (Lxa) locus in *B. cenocepacia* (5′-TGATNNNNNNATCA-3′) (Winteler and Haas 1996, Trunk et al. 2010, Sass et al. 2013). The similarities in both function and sequence of these proteins suggests that both Anr and FixK act as transcriptional regulators of their respective regulons, activated in response to low oxygen conditions.

Mycobacterium abscessus also utilizes an oxygen-sensing TCS and signalling cascade, the DosRS TCS (Rustad et al. 2008, Chauhan et al. 2011, Peddireddy et al. 2017). In *Mycobacterium tuberculosis*, DosT is analogous to the activity of FixL, phosphorylating DosS (DevS) under oxygen tension (Sousa et al. 2007). Although the predicted structures are distinct (Fig. 1), DosS is comparable to FixL and BfiS in function and comprise a GAF domain,

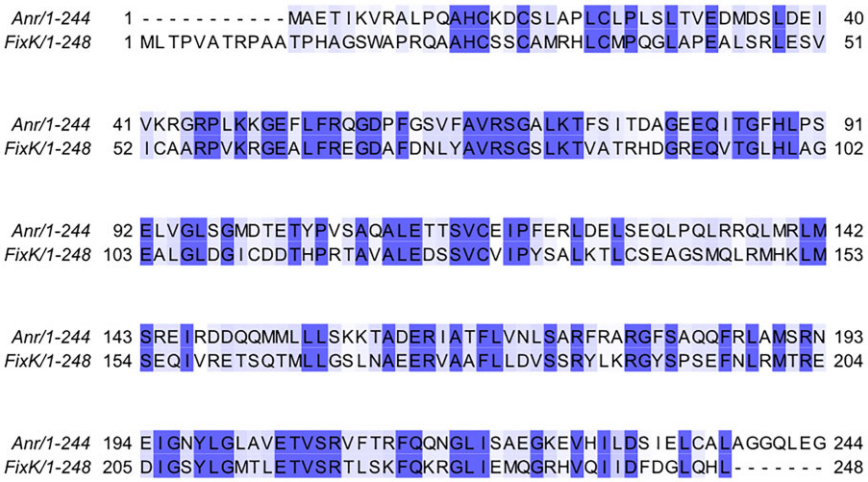

Figure 2. Amino acid sequence alignment of *B. cenocepacia* FixK and *P. aeruginosa* Anr: BlastP search revealed a 43% sequence identity indicating a high level of homology between the two transcriptional regulators. Alignment prepared by Clustal Omega Multiple Sequence Alignment (Sievers and Higgins 2020) and visualized by Jalview (v. 2.11.4.0) (Waterhouse et al. 2009)

which is similar to a PAS domain and a ComP domain which functions as a signal transduction histidine kinase (Wang et al. 2023) (Fig. 1A). DosS activates DosR the transcriptional activator of the DosRS regulon (Lobão et al. 2019). The DosRS regulon is also autoinducible (Sassi and Drancourt 2014, Simcox et al. 2023).

The Lxa locus, Anr regulon, and DosR regulon all share proteins with similar homology and function, including universal stress proteins (USPs), transcriptional regulators, oxidative stress proteins, and proteins involved in antibiotic resistance (Table 1). The DosRS regulon and Lxa locus also share proteins with roles in dormancy and dormancy resuscitation (Sass et al. 2013, Simcox et al. 2023). These overlaps beg the question as to whether these three opportunistic pathogens, two of which are Gram-negative, have evolved these similar mechanisms due to their shared capacity to colonize low oxygen environments, or common ancestral genes as a result of being colocated in the same niche.

Response to hypoxia in Bcc

In 2013, Sass et al. (2013) identified the Lxa locus in *B. cenocepacia* following a transcriptomic analysis of responses to environmental stresses. The 50 722 bp lxa locus comprises a 50-gene cluster that was dramatically upregulated under hypoxic conditions (~6% oxygen), conferring a distinct fitness advantage in low oxygen and anoxic conditions (Sass et al. 2013). Deletion of the entire locus in mutant strains revealed an impaired ability to adapt to low oxygen (Sass et al. 2013). The Lxa locus encodes six USPs together with several proteins involved in metabolism, electron transfer and regulation (Sass et al. 2013).

The FixLJ TCS allows Bcc to sense oxygen, and could possibly play a role in the activation of the Lxa locus under low oxygen conditions, increasing the expression of up to 50 genes in *B. cenocepacia* (Fig. 3). In addition to USPs, increased transcription of genes with predicted functions in ribonucleotide transport and metabolism, fatty acid and amino acid synthesis, electron transfer, transporter proteins, and transcriptional regulatory systems was observed under low oxygen tension (Sass et al. 2013). Signalling proteins, sigma factors, and toxin–antitoxin genes were also all induced in stationary phase and low oxygen conditions (Sass et al. 2013). Interestingly, a number of genes implicated in the expression of virulence factors were also highly upregulated when exposed to 6% oxygen for 2 h (Sass et al. 2013). For example, BCAS0409, a gene encoding a zinc-metalloprotease was upregulated over 32-fold (Sass et al. 2013). Furthermore, *cepI* (BCAM1870), a quorum-sensing gene responsible for the production of acyl homoserine lactone synthase was induced ~4-fold in comparison to normal oxygen levels (Sass et al. 2013). The CepIR quorum sensing system in *B. cenocepacia* is involved in positively regulating numerous virulence factors and quorum sensing signalling is maintained in sequential chronic infection isolates (McKeon et al. 2011, Suppiger et al. 2013). More recently, the elevated abundance of 20 proteins encoded by this locus, including all six USPs, α-crystallin, phosphofructokinase, and an acetyl-coA reductase, were also observed in late sequential isolates from two individuals chronically infected with *B. cenocepacia*, suggesting that this locus is also important clinically in chronic infection of the CF lung due to the hypoxic conditions typical of this environment.

Another potential player in the response to hypoxia is the second messenger cyclic-dimeric guanosine monophosphate (c-di-GMP). This second messenger has been primarily investigated as a regulator of virulence in bacteria (Gomelsky 2011, Mills et al. 2011, Romling et al. 2013, Valentini and Filloux 2019) and c-di-GMP-dependent signalling mechanisms have been elucidated in many prokaryotes (Feng et al. 2024, entini and Filloux 2016, Hu et al. 2019, Richter et al. 2019). The signal transducer is produced by diaguanylate cyclases that possess a GCDEF domain before hydrolysis by phosphodiesterases containing EAL or HD-GYP domains. c-di-GMP selectively binds to the PilZ domain-containing proteins (Tamayo et al. 2007), a large family of proteins that are implicated in signalling pathways that regulate multiple processes including virulence, motility, and biofilm formation. Eight PilZ domains have been identified in effector proteins in *P. aeruginosa* to date and increased c-di-GMP levels were directly implicated in elevated biofilm formation and reduced flagellum-driven swarming motility (Guttenplan and Kearns 2013, Baker et al. 2016). c-di-GMP targets the PilZ domains present in FlgZ and PelD, subsequently repressing motility and inducing Pel-mediated polysaccharide production, respectively (Baker et al. 2016). In Bcc, increased activity of c-di-GMP promoted the formation of wrinkly colonies, pellicles, and biofilm which have been associated with increased persistence in host environments, such as the lungs of individuals with CF, contributing to chronic infection (Fazli et al. 2011, 2017). While

Table 1. Examples of common proteins encoded by the Anr regulon, DosR regulon, and Lxa locus.

Function	Organism	Gene ID	Protein	References
Universal stress proteins	*P. aeruginosa*	PA3309	Universal stress protein	Boes et al. (2006)
		PA4352	Universal stress protein	
	M. abscessus	MAB_3904	Universal stress protein	Simcox et al. (2023)
		MAB_2489	Universal stress protein	
	B. cenocepacia	BCAM0276	Universal stress protein	Sass et al. (2013)
		BCAM0290	Universal stress protein	
		BCAM0291	Universal stress protein	
		BCAM0292	Universal stress protein	
		BCAM0294	Universal stress protein	
		BCAM0319	Universal stress protein	
Transcriptional regulators	*P. aeruginosa*	PA3341	Transcription regulator MarR/SlyA-like	Tribelli et al. (2019)
		PA0225	Probable transcriptional regulator	
		PA0797	Probable transcriptional regulator	
		PA0864	Probable transcriptional regulator	
		PA1196	Probable transcriptional regulator	
		PA1241	Probable transcriptional regulator	
		PA4902	Probable transcriptional regulator	
		PA4906	Probable transcriptional regulator	
		PA0527	Probable transcriptional regulator Dnr	
		PA0873	Probable transcriptional regulator PhhR	
	M. abscessus	MAB_4139	Transcription regulator, ArsR family	Simcox et al. (2023)
		MAB_2386	Transcriptional regulator, ArsR family	
		MAB_2602c	Transcriptional regulator, ArsR family	
		MAB_3018	Transcriptional regulator, GntR family	
		MAB_4644c	Transcriptional regulator, GntR family	
		MAB_3891c	Probable transcriptional regulator, LuxR family	
		MAB_2606c	Transcriptional regulator, TetR family	
		MAB_3883c	Transcriptional regulator, TetR family	
		MAB_2541c	Probably transcriptional regulatory protein TetR	
	B. cenocepacia	BCAM0287	CRP family regulatory protein, Anr-related	Sass et al. (2013)
		BCAM0288	Two-component regulatory system, RR	
		BCAM0322	Two-component regulatory system, RR	
Oxidative stress	*P. aeruginosa*	PA5427	Putative alcohol dehydrogenase	Tribelli et al. (2019)
		PA1500	Probable oxidoreductase	
		PA2100	Putative alcohol dehydrogenase (Zn-dependent)	
		PA2119	Putative alcohol dehydrogenase (Zn-dependent)	
		PA1991	Probable iron-containing alcohol dehydrogenase	
		PA5240	Thioredoxin	
		PA0023	Quinone oxidoreductase	
	M. abscessus	MAB_3438	Short-chain dehydrogenase/reductase	Simcox et al. (2023)
		MAB_4178c	Short-chain dehydrogenase/reductase	
		MAB_0389c	Methanol dehydrogenase transcriptional regulatory protein MoxR2	
		MAB_1874	Putative oxidoreductase	
		MAB_3438	Putative short-chain dehydrogenase/reductase	
		MAB_3133c	Putative flavohemoprotein	
		MAB_3884	Possible flavoprotein	
		MAB_0930	Putative ferredoxin/ferredoxin–NADP reductase	
	B. cenocepacia	BCAM0286	Alcohol dehydrogenase	Sass et al. (2013)
		BCAM0299	Zinc-binding alcohol dehydrogenase	
Proteases	*P. aeruginosa*	PA0459	Probable ClpA/B protease ATP binding subunit	Tribelli et al. (2019)
		PA4542	ClpB protein	
		PA2621	ClpS	
	M. abscessus	MAB_3938	Putative Clp protease subunit	Simcox et al. (2023)
	B. cenocepacia	MAB_2211c	Putative membrane protein, MmpS	Sass et al. (2013)
		BCAM0309	ATP-dependent Zn protease[67]	
Antibiotic resistance	*P. aeruginosa*	PA0458	Probable major facilitator superfamily (MFS) transporter	Tribelli et al. (2019)
		PA0246	Probable major facilitator superfamily (MFS) transporter	
		PA4595	Probable ATP-binding component of ABC transporter	
	M. abscessus	MAB_1690	ABC transporter transmembrane protein	Simcox et al. (2023)
		MAB_4910c	Putative aminoglycoside phosphotransferase	
	B. cenocepacia	BCAM0302	ABC transporter protein	
		BCAM0303	ABC transporter protein	Sass et al. (2013)
		BCAM0300	Metallo-beta-lactamase superfamily protein	

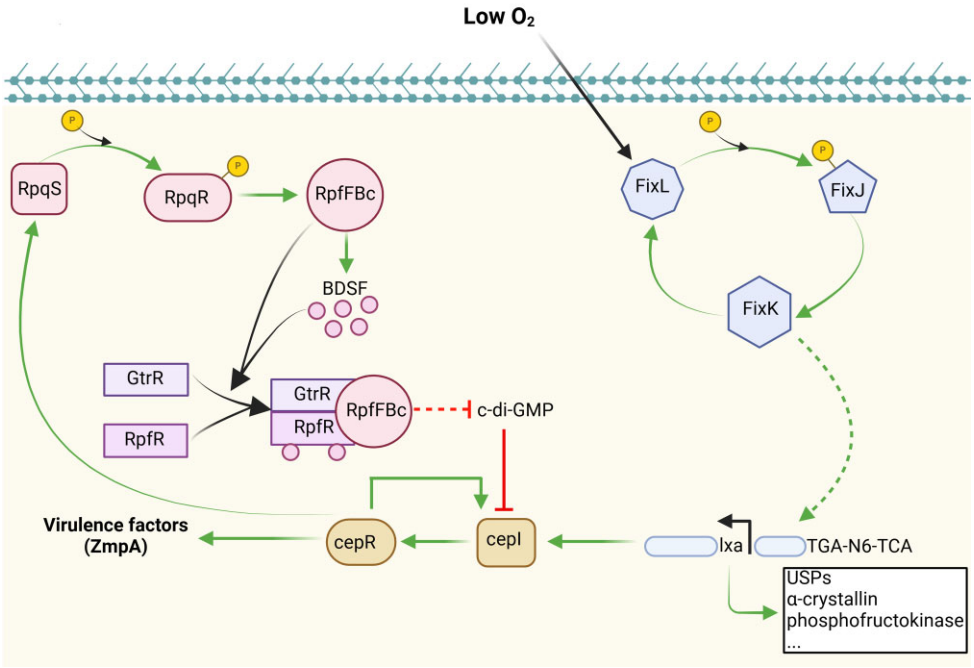

Figure 3. Proposed oxygen sensing system in *B. cenocepacia*. The histidine sensor kinase, FixL, senses the hypoxic conditions and phosphorylates FixJ, subsequently activating transcriptional activator, FixK. The transcription of genes on the Lxa locus leads to increased expression of the CepIR quorum-sensing signalling system. Besides the induction of virulence factor expression, the CepIR systems leads to increased levels of BDSF in the cell and subsequent cleaving of c-di-GMP, preventing repression of CepI. This cascade drives the transition towards a more virulent state. Predicted interactions are denoted by dotted lines.

the impact of hypoxia directly on c-di-GMP remains to be elucidated in its entirety, there is a potential interplay between oxygen sensing mechanisms and c-di-GMP levels as discussed below. In the CF lung, pathogens often transition towards a state of dormancy but both *B. cenocepacia* and *B. multivorans* have been shown to convert from a mucoidal state to a nonmucoidal state during the transition to chronic infection (Zlosnik and Speert 2010, Zlosnik et al. 2010). *B. cenocepacia* exhibits an increased expression of virulence factors such as metallo-beta-lactamases, fimbrial usher proteins, and OmpA family proteins in sequential clinical isolates (Cullen et al. 2018). This could potentially be explained by the suppression of diguanylate cyclase activity and ultimately decreased intracellular levels of c-di-GMP, under hypoxic conditions. As mentioned earlier, the acyl homoserine lactone synthase *cepI* is upregulated in response to low oxygen levels, directly linking sensing of hypoxia to increased quorum sensing activity (Sass et al. 2013). The sensing component of the novel two component system RqpSR responds to increased levels of AHL ligands as a consequence of CepIR activity (Cui et al. 2018). The phosphorylation of RqsR, in combination with activity of the bifunctional crotonase RpfFBc, results in the biosynthesis of the Burkholderia Diffusible Signal Factor (BDSF) (Wang et al. 2022). These signalling molecules bind to RpfR leading to the formation of a GtrR–RpfR complex, stimulating c-di-GMP phosphodiesterase activity (Deng et al. 2012). As a result, intracellular levels of c-di-GMP decline creating a positive feedback loop that enhances CepIR activity thus promoting the expression of virulence factors (Subsin et al. 2007) (Fig. 3). This quorum-sensing system has been implicated in virulence factor production, including regulation of *zmpA* expression that has been shown to be increased in abundance in response to low oxygen conditions (Sass et al. 2013) (O'Grady and Sokol 2011).

Response to hypoxia in *P. aeruginosa*

In *P. aeruginosa*, Anr is a global regulator of microaerobic growth with Anr activity being associated with persistence in chronic pulmonary infection models (Alvarez-Ortega and Harwood 2007, Arai 2011, Hammond et al. 2015). The core Anr regulon comprises genes encoding cytochrome peroxidases, nitrate reductases, arginine deaminases, and other proteins responsible for maintaining cellular growth under microoxic conditions (Tribelli et al. 2019). In addition, Anr activity in response to hypoxia is associated with the transition to a sessile state as it is implicated in increased biofilm formation through the regulation of acyl homoserine lactonases (AHL) ligand production (Trunk et al. 2010, Tribelli et al. 2019).

LasRI, an AHL quorum-sensing system in *P. aeruginosa*, is negatively regulated by Anr activity and implicated in virulence factor expression in addition to the repression of c-di-GMP (Hammond et al. 2015). In addition to LasRI, c-di-GMP repression is also mediated by RbdA phosphodiesterase activity (Rutherford and Bassler 2012, Xin et al. 2019). RbdA contains PAS-PAC-GGDEF-EAL multidomains and is thought to sense oxygen directly via the PAS domains whilst also cleaving c-di-GMP via the GGDEF-EAL domains (An et al. 2010). LasRI is the major hierarchical signalling system that regulates downstream systems including RhlRI and quinolone signalling systems (Lee and Zhang 2015) (Fig. 4). In previous studies, activation of LasRI QS system was implicated in the increased expression of proteases, lipases, hydrogen cyanide, iron acquisition mechanisms; the rate of virulence factor expression was inversely proportional to Anr activity (Hammond et al. 2015). Thus, increased Anr activity in response to low oxygen conditions represses LasRI activity (Fig. 4). In LasRI null mutants, the ability to establish an acute infection is impaired as a result of reduced virulence factor production suggesting that low oxygen may

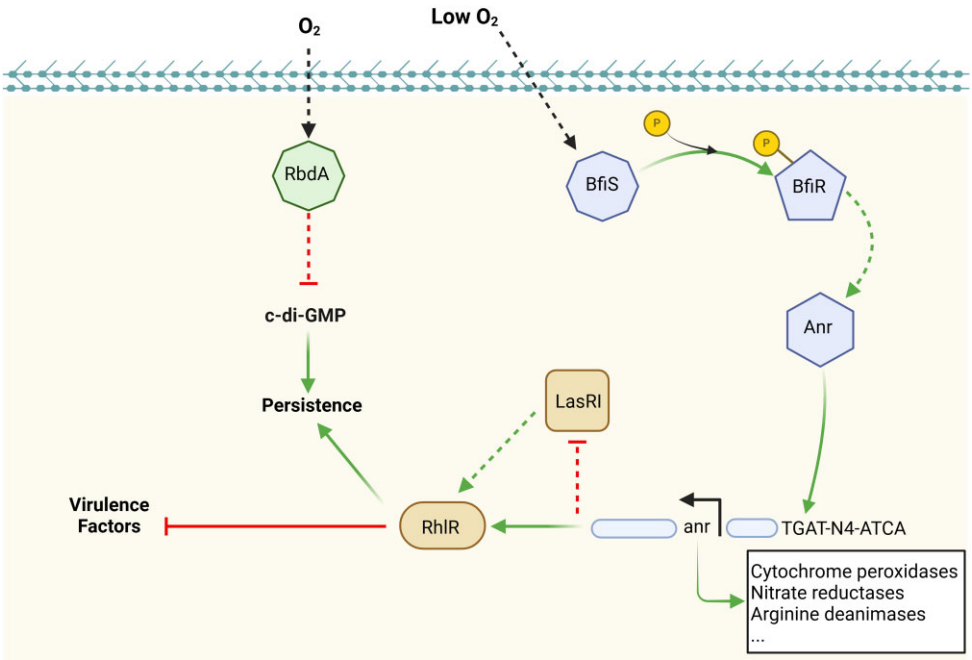

Figure 4. Proposed oxygen-sensing system in *P. aeruginosa*. The histidine sensor kinase, BfiS, senses the hypoxic conditions and phosphorylates BfiR, subsequently activating transcriptional activator of the Anr regulon. The transcription of genes on this regulon leads to reduced expression of the LasR quorum-sensing signalling system. Repression of the LasR system results in increased activity of the RhlR system; this regulatory response in tandem with increased levels of intracellular c-di-GMP furthers the transition towards a persistent state. RbdA is involved in the cleaving of c-di-GMP in the presence of oxygen. Predicted interactions are denoted by dotted lines.

result in reduced virulence in *P. aeruginosa* (Rumbaugh et al. 1999, Winstanley et al. 2016).

Clinical isolates of *P. aeruginosa* from the CF lung are often found to have loss of function mutations in genes encoding components of the LasRI quorum-sensing systems (Ciofu et al. 2010, Morin et al. 2021). In a study conducted by Smith et al. (2006), 18 out of 29 CF patients presented at least one isolate with a nonsynonymous mutation in the *lasR* gene. It is therefore possible that a strong selective pressure exists against a functional LasRI system since loss of activity confers a selective advantage and facilitates disease progression (Hoffman et al. 2009). This further indicates that adaptation to persistence within low oxygen niches in chronically infected lungs may be driven by Anr activity and downregulation of quorum-sensing systems that promote virulence.

In previous studies of chronically infected CF lungs, it has been shown that LasR-null *P. aeruginosa* isolates often feature upregulated RhIR signalling systems as a method of compensating for the absence of a hierarchical quorum sensing system (Feltner et al. 2016, Kostylev et al. 2019). The *P. aeruginosa* RhIR system is implicated in regulating factors associated with persistence and survival in a polymicrobial environment (Wang et al. 2015) (Fig. 4). In addition, factors contributing to establishment of chronic infection were Anr-dependent, for example CupA fimbriae; the *cupA* gene cluster encodes the components to assemble a putative fimbrial structure that has been shown to be important in biofilm formation and host-cell attachment (Kulasekara et al. 2005). In a study on Δ*anr* mutants, CupA fimbriae expression was significantly reduced (Vallet-Gely et al. 2007). In the absence of LasR, CupA fimbriae production was highly dependent on *anr*-mediated expression suggesting that Anr activity in response to hypoxia is a major contributor to the expression of the *cup* genes and ultimately bacterial colonization (Hammond et al. 2015).

Response to hypoxia in *M. abscessus*

The *M. abscessus* DosRS operon is autoregulated, analogous to the FixLJ TCS in Bcc (Gerasimova et al. 2011, Simcox et al. 2023) (Fig. 5). The binding motif for the DosRS regulon is an 18-bp palindrome that was first identified in *M. tuberculosis* (Chauhan et al. 2011). The DosRS regulon in *M. tuberculosis* encodes 48 genes and DosRS is deemed a dormancy survival response regulon (Sharma and Tyagi 2016). While initially considered to be quite limited in comparison to *M. tuberculosis* (Gerasimova et al. 2011), a recent study suggested that the *M. abscessus* DosRS regulon has over 127 putative DosRS regulated genes (Simcox et al. 2023). Moreover, an additional 1,063 DosRS independent hypoxia-induced genes were identified, suggesting there is complex regulatory system at play beyond DosRS regulation in this species (Simcox et al. 2023).

Deletion of the *M. tuberculosis* DosR RR resulted in defective growth under hypoxic conditions (Rustad et al. 2008). Consistent with the Anr regulon in *P. aeruginosa* and the Lxa locus in *B. cenocepacia*, these genes are upregulated in response to low oxygen levels (Rustad et al. 2008, Malhotra et al. 2009). Gene products also include USPs, heat shock proteins, diacylglycerol acyltransferase family proteins (DGATs), and nitro-reductases and ferredoxins (Hingley-Wilson et al. 2010) (Table 1), which are functionally similar to those regulated by Anr or Lxa in *P. aeruginosa* and *B. cenocepacia*, suggesting that environmental pressures in the human lung may have influenced their coevolution (Peddireddy et al. 2017). The DosRS regulated USP Rv2623 deletion mutant exhibited hypervirulence whilst overproduction of the USP led to attenuated growth in *M. tuberculosis* (Drumm et al. 2009), highlighting the importance of this USP in the maintenance of a dormant state.

Following the initial response to hypoxic conditions, there is a sustained wave of gene expression in *M. tuberculosis* known as the Enduring Hypoxic Response (EHR) (Rustad et al. 2008) (Fig. 5).

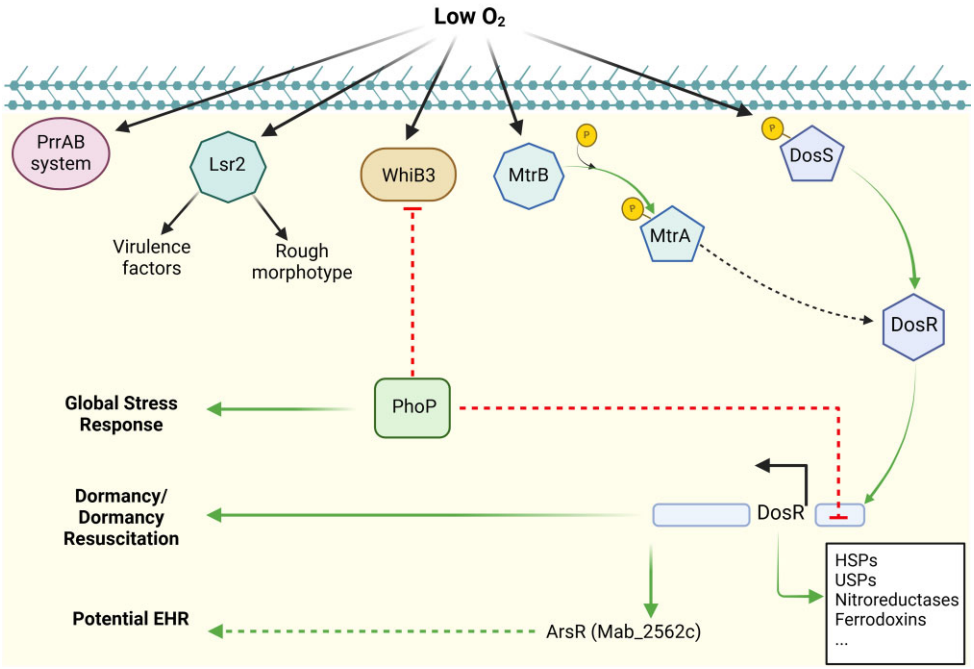

Figure 5. Proposed oxygen-sensing system in *M. abscessus*. The histidine sensor kinase DosS senses hypoxic conditions and undergoes autophosphorylation, subsequently phosphorylating and activating the RR DosR. Activated DosR promotes the expression of genes in the DosRS regulon, potentially contributing to an enduring hypoxic response (EHR)-like transcriptional program and facilitating a transition towards a dormant or stress-tolerant state. PhoP regulation of DosR transcription is circumvented by rate of response. Other potential oxygen or redox-responsive regulators include the PrrAB TCS, the nucleoid-associated protein Lsr2, the redox-sensing transcription factor WhiB3, and the MtrAB system. Predicted interactions are denoted by dotted lines.

While this EHR has been well-characterized in *M. tuberculosis*, a comparable transcriptional program has not yet been described in *M. abscessus*. However, emerging evidence suggests that *M. abscessus* may also engage distinct regulatory mechanisms during prolonged hypoxic stress. The expression of genes associated with this response are controlled by an ArsR family protein, *MAB_2562c*, which is positively regulated by DosRS in other mycobacteria including *M. tuberculosis* (Dubois et al. 2019, He et al. 2011, Sun et al. 2018). The PrrAB two component system has also been shown to be important in the response of *M. tuberculosis* to oxygen (Giacalone et al. 2022). It was reported that hypoxia increased the expression of the *M. tuberculosis* PrrAB TCS by over 2-fold relative to standard growth conditions. Interestingly, it has previously been shown that DosT can interact with a noncognate RR, PrrA, suggesting that DosT- mediated phosphorylation activity may influence PrrAB activity and thus, coordinates a global stress response (Giacalone et al. 2022, Lee et al. 2012). Although *M. abscessus* does not encode DosT, it possesses a conserved PrrAB TCS, raising the possibility that PrrAB may be similarly integrated into broader stress response pathways, potentially through interactions with other histidine kinases or stress-sensing mechanisms. It is also suggested that the DosRS regulon influences the activity of Lsr2, a member of the mycobacterial NAP family. Lsr2 has been shown to bind A-T rich DNA regions and control the expression of virulence factors in *M. abscessus*, in addition to facilitating the switch a rough colony morphology, features which are associated with late sequential chronic infection isolates following possible exposure to long-term hypoxia (Le Moigne et al. 2019).

The high number of DosRS independent hypoxia-induced genes may possibly be explained by communication between the DosRS TCS and the PhoPR TCS. PhoR encodes a histidine kinase that responds to environmental signalling cascades and modulates the activity of a cognate RR, PhoP. A DNA microarray analysis indicated that PhoP regulates DosR in *M. tuberculosis* with DosR activity downregulated in *phoP* mutants (Gonzalo-Asensio et al. 2008). It was later shown that PhoP binds the promoter region of DosR, regulating its expression and potentially coregulating the response to hypoxia (Vashist et al. 2018). Interestingly, *phoR* is the gene that most commonly acquires nonsynonymous mutations in *M. abscessus* during lung infection (Bryant et al. 2021). Since PhoR activity is influenced by environmental stressors, it is plausible that the complex interplay between PhoP and DosR could prevent dephosphorylation leading to the eventual accumulation of loss of function mutations in PhoR, facilitating the establishment of lung infection (Fig. 5).

Another TCS potentially important for the response to hypoxia in *M. abscessus* is the MtrAB system (Fig. 5). MtrAB has been identified in all mycobacterial species characterized to date and its RR, MtrA, is the only known essential RR in *M. tuberculosis* (Zahrt and Deretic 2000). It is thought to be functionally homologous to the YycFG system in Gram-positive bacteria, which regulates cell wall synthesis, cell growth and cell division (Fukushima et al. 2008, Winkler and Hoch 2008). Deletion of the *mtrB* (the sensor kinase in the system) in *M. tuberculosis* reduced its ability to survive in macrophages and to infect the lung in a murine model (Banerjee et al. 2019). The Δ*mtrB* mutant was more susceptible to hypoxic and acid stress, displayed impaired biofilm formation, and had a dramatic downregulation of genes associated with adaptation, most notably the DosRS regulon. MtrB is also suggested to be a regulator of DosR-dependant gene expression as MtrB has been shown to interact with the noncognate RR DosR (Banerjee et al. 2019), marking it as a putative part of the cellular hypoxic

Table 2. Summary of phenotypic changes identified in response to sensing of low oxygen conditions in *P. aeruginosa*, *B. cenocepacia*, and *M. abscessus*.

	P. aeruginosa	*B. cenocepacia*	*M. abscessus*	References
Colony morphology	Small colony variants and Rugose small colony variants	Small colony variant (SCV), ruffled spreader colony variant, wrinkly colony variant	Morphotype switching from smooth to rough	Howard et al. (2006), Jo¨nsson et al. (2007), Byrd et al. (2011), Malone et al. (2010), Mulcahy et al. (2008), Poltak and Cooper (2011), Malone (2015), Xu et al. (2021)
Motility	Reduced motility	Increased expression of genes associated with motility	Sliding motility reduced	Byrd and Lyons (1999), Howard et al. (2006), Smith et al. (2006), Hassett et al. (2009), Sass et al. (2013)
Biofilm formation	Enhanced biofilm formation (BfiSR)	Maintains/cocolonizes biofilm	Reduced biofilm formation	Bjarnsholt et al. (2009), Byrd and Lyons (1999), Howard et al. (2006), Francis et al. (2017), Schwab et al. (2014)
Siderophore production	Increased pyochelin, pyoverdine production	Increased ornibactin, salicylic acid, and cepabactin production	Increased myobactin production	Subsin et al. (2007), Schreuder and Parish (2014), Butt and Thomas (2017), Schalk and Perraud (2023)
Proteases	Reduced protease production	Increased protease production (including zmpA and zmpB)	Increased protease production (serine proteases)	Schaible et al. (2017), Burggraaf et al. (2019), Houben et al. (2014), Zhao et al. (2014)
Antibiotic resistance	Increased antibiotic resistance (multidrug efflux)	Increased antibiotic resistance (BCAM0300)	Increased antibiotic resistance	Schaible et al. (2013), Pessi et al. (2013), Sass et al. (2013), Liu et al. (2016), Hunt-Serracin et al. (2019), Lanni et al. (2022)
Host-cell attachment	Increased host-cell attachment (type IV pili, *cup* genes)	Increased host-cell attachment (type IV pili, cupA fimbriae, and lectins)	Increased host-cell attachment	Kulasekara et al. (2005), Sass et al. (2013), Bisht and Meena (2019)
Intracellular survival		Increased intracellular survival	Increased intracellular survival	Sajjan et al. (2008), Ganesh et al. (2020), Dubois et al. (2018), Touré et al. (2023)
Universal stress proteins		Increased expression	Increased expression	Cullen et al. (2018), O'Connor et al. (2023), Chen et al. (2016), Gröschel et al. (2016)

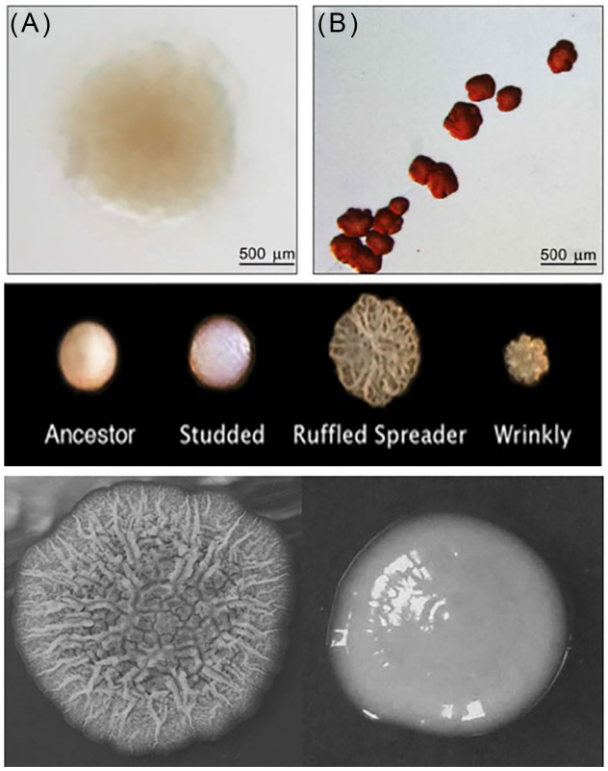

Figure 6. Examples of alternate colony morphology in *P. aeruginosa*, Bcc, and *M. abscessus*. Top: normal (A) and RSCV (B) of *P. aeruginosa* (Malone et al. 2010). Middle: studded, ruffled spreader, and wrinkly variants of *B. cenocepacia* (Poltak and Cooper 2011). Bottom: rough (left) and smooth (right) colony variants of *M. abscessus* (Rüger et al. 2014).

response cascade. Although it is not essential in *M. abscessus*, the homology between MtrA in *M. abscessus* and *M. tuberculosis* is high (91.23% identity) (Zhang et al. 2024). Functional studies on the link between hypoxia and this TCS are yet to be performed in *M. abscessus*; however, a recent preprint reported that Δ*mtrA*, Δ*mtrB* Δ*mtrAB* mutants were more susceptible to antibiotics, demonstrated defective cell division, and had decreased virulence in a murine infection model (Zhang et al. 2024), highlighting the importance of this TCS in infection.

Exploring the links between hypoxia and chronic infection phenotypes

Given that hypoxia exposure causes changes in multiple regulatory pathways, noticeable changes in phenotype are expected; however, published studies describing direct connections between responses to environmental oxygen and phenotype are limited to date. In contrast, the adaptation of bacteria to the lung environment during chronic colonization of CF patients has been widely investigated and described in detail in multiple reviews (Coutinho et al. 2011, Cullen and McClean 2015, Bolden et al. 2023, Lee et al. 2017, Pereira et al. 2020, Rossi et al. 2020, Planet 2022). These adaptations vary between different bacterial genera, species, and strains, but allow them to colonize specific niches. Although multiple environmental pressures are present in the CF lung, which may play a role in the development of adaptations associated with chronic infection, there are established links between the expression of hypoxia-related genes and phenotypes associated with chronic infection (Table 2). This suggests that hypoxia may be a key driver of these phenotypic changes involved in chronic infection. Thus, the following adaptations have been widely observed in chronic bacterial isolates the CF lung and the evidence for a potential role of hypoxia in driving these changes will be discussed. The focus will be placed on *P. aeruginosa*, *M. abscessus*, and *B. cenocepacia*, as the latter is the most well-characterized member of the Bcc.

Colony morphology

Small colony variants (SCVs) of *P. aeruginosa* have been isolated from the CF lung and are one of the many adaptations enabling persistence (Jenal and Malone 2006, Mulcahy et al. 2008, Byrd et al. 2011, Malone 2015). Recently it was shown that one of the environmental pressures causing the emergence of this phenotype is oxygen limitation (Besse et al. 2022). *Pseudomonas aeruginosa* SCVs are typically biofilm hyperproducers, usually due to higher production of exopolysaccharides (EPS), exo-proteins and eDNA release, combined with an atypical subpopulation structure (Xu et al. 2021), often referred to as a rugose small colony variant (RSCV). The SCV phenotype is also observed in Bcc, with variants such as the rugose spreader and wrinkly morphotype commonly observed, and the latter frequently isolated from the CF lung (Fig. 6) (Poltak and Cooper 2011). SCVs are not unique to the CF lung as they have been also isolated from a number of other infection sites associated with persistence of several other bacterial pathogens, including *S. aureus* and *E. coli* (Proctor et al. 2006, Anderson et al. 2007, Johns et al. 2015, Keim et al. 2023).

The switch from the normal colony variant (NCV) to SCVs in *P. aeruginosa* has been linked to increased activity of c-di-GMP signalling pathways, the GAC/Rsm pathway, and flagellar proteins (Xu et al. 2021, Besse et al. 2022). Interestingly, SCV genome analysis showed that mutations arose in two operons predominantly—*wsp* and *yfiBNR* (Besse et al. 2022). Both encode chemosensors and while the pathways they regulate have been described, their exact mechanism of action has not yet been elucidated (Malone et al. 2012). The *wsp* signalling pathway detects surface contact and regulates subsequent biofilm development (O'Neal et al. 2022). It has also been suggested that *wsp* mutations in *P. aeruginosa* and the response of the *yfiBNR* operon are a consequence of oxygen limitations (Malone et al. 2012, Tognon et al. 2017). Although members of the Bcc do not express the *yfiBNR* operon, it was confirmed that the appearance of SCVs correlate with mutations in the *wsp* operon (Cooper et al. 2014), suggesting a potential shared mechanism by which oxygen levels could cause the development of NCVs in both *P. aeruginosa* and in Bcc. SCVs have also been reported in *M. tuberculosis*, but contrary to the other two species these are quite unstable and revert quickly to a NCV (Safi et al. 2019). Mutations in *glpK* (glycerol kinase) and *orn* (oligoribonuclease) are associated with their emergence. Interestingly, *Orn* has also been shown to hydrolyze pGpG, which regulates the c-di-GMP homeostasis in *P. aeruginosa* (Orr et al. 2015, 2018). The rapid reversal to the NCV hinders the identification of *Mycobacterium* SCVs in sputum samples although they are still clinically relevant. A recent study suggests that *Mycobacterium* SCVs are associated with enhanced antimicrobial resistance and survival in hostile environments (An et al. 2010, Park et al. 2024).

Mycobacterium abscessus exhibits two alternative and distinct colony morphotypes, rough and smooth, with the rough morphotype being significantly correlated with persistent infection in CF (Jo¨nsson et al. 2007). Most environmental isolates of *M. abscessus* are smooth (Jo¨nsson et al. 2007) and spontaneous morphotype switching has been observed (Howard et al. 2006). Although the switch from smooth to rough morphotypes is known to be due

to the presence or absence of glycopeptidolipid in the cell wall (Barrow and Brennan 1982), the conversion to the rough morphotype in the CF lung may also be attributable to the hypoxic conditions. Indeed, *dosR* expression was increased in the rough variant, suggesting that response to low oxygen may facilitate a switch to the rough morphotype (Pawlik et al. 2013). An integrated transcriptomic and proteomic study identified significant overlap between genes upregulated in response to short term hypoxia and the presence of the rough morphotype, with 43% of the hypoxia response upregulated in rough morphotype colonies, including the entire DosRS regulon (Miranda-CasoLuengo et al. 2016). This strongly suggests that the rough morphotype may have a greater adaptive advantage in the CF lung due to constitutive upregulation of the DosRS regulon (Miranda-CasoLuengo et al. 2016) and highlights that the switch to the rough morphotype in chronic infection may be attributable to the hypoxic response. Interestingly, this observation was contradicted more recently when deletion of the DosRS TCS led to a switch to a rough morphotype (Simcox et al. 2023) suggesting that the switch is more complex than first thought. Ultimately, both of these studies highlight the influence of the DosRS regulon on *M. abscessus* morphotype switching but further research is needed to establish the mechanism(s) involved.

Motility

Pseudomonas aeruginosa isolates from chronically infected individuals are widely reported to have reduced motility, with a reduction in twitching motility and lower abundance of motility associated proteins (Mahenthiralingam et al. 1994, Smith et al. 2006, Cullen and McClean 2015, Cullen et al. 2015, Huus et al. 2016). Motility is dependent on quorum-sensing systems including RhlR and LasR regulators, with the undisputed involvement of c-di-GMP (Hengge 2009). The c-di-GMP phosphodiesterase RbdA increases *P. aeruginosa* swimming and swarming motility through MapZ and the activity of this c-di-GMP-binding adaptor protein is oxygen-dependent (An et al. 2010, Xin et al. 2019). Similarly in *B. cenocepacia*, reduced intracellular c-di-GMP levels in response to exogenous conditions are associated with increased motility (Kumar et al. 2018). It is interesting to note that motility of *P. aeruginosa* is repressed during microaerobic and anaerobic growth (Hassett et al. 2009), while in contrast, low oxygen appears to increase the expression of genes associated with motility in *B. cenocepacia* (Sass et al. 2013). This may attributed to the link between FixLJ activation and motility in Bcc. In *B. dolosa*, *fixLJ* mutants had significantly reduced levels of motility and invasion when compared to the WT, with motility restored following complementation (Schaefers et al. 2017). This suggests that activation of the FixLJ TCS in response to oxygen sensing may also contribute to enhanced motility.

In *M. abscessus*, the smooth morphotype is associated with enhanced sliding motility, attributed to the presence of glycopeptidolipid (GPL) in the outer layer of the mycobacterial cell wall (Byrd and Lyons 1999, Recht and Kolter 2001). In contrast, the rough morphotype, associated with increased DosR activity in response to hypoxia, is deficient in GPL and is nonmotile (Byrd and Lyons 1999, Howard et al. 2006) although it exhibits higher virulence and higher persistence clinically and in infection models (Byrd and Lyons 1999, Pawlik et al. 2013). Overall, hypoxia appears to stimulate the expression of genes associated with increased motility in *B. cenocepacia* while repressing motility in *P. aeruginosa* and *M. abscessus*.

Biofilm formation

Biofilm formation contributes significantly to the persistence of *P. aeruginosa* in the CF lung and is being targeted in new treatment development, particularly given that cells growing as a biofilm tend to have a higher antibiotic resistance (Ciofu et al. 2015, Muhammad et al. 2020, Martin et al. 2021). Biofilm formation in *P. aeruginosa* clinical isolates is increased both *in vitro* and within patient lungs, contributing to therapeutic difficulties (Bjarnsholt et al. 2009). As discussed earlier, the suggested oxygen sensing TCS BfiSR also plays a crucial role in biofilm formation and maturation via the activation of small RNA, *rsmZ* (Francis et al. 2017). Furthermore, deletion of *bfiS* impaired biofilm formation and led to structural defects (Petrova and Sauer 2009).

Interestingly, in the Bcc FixLJ has the opposite effect on biofilm formation than its *P. aeruginosa* homolog BfiSR, as deletion of *fixLJ* led to an increase of biofilm formation (Schaefers et al. 2017). In *B. cenocepacia* strain H111, the production of EPS was negatively regulated by RpfR, a quorum-sensing receptor of BDSF and upregulated through a c-di-GMP effector BerB (Steiner et al. 2022). Thus, under low oxygen conditions production of EPS is potentially increased in an oxygen-dependent manner via increases in c-di-GMP.

Nontuberculous mycobacteria including *M. abscessus* form biofilms *in vitro* (Howard et al. 2006), in the environment and in the CF lung (Qvist et al. 2015). Biofilm formation is associated with the smooth morphotype of *M. abscessus* (Recht and Kolter 2001), and as such is reduced in the chronic infection as the rough morphotype is more prevalent. However, *M. abscessus* has been found to form granulomas in the CF lung that facilitate persistence by limiting immune cell and antibiotic accessibility (Peddireddy et al. 2017). It has been shown that *M. tuberculosis* forms biofilms within granulomas and the DosRS regulon is essential for the formation of *M. tuberculosis* granulomas (Mehra et al. 2015, Hudock et al. 2017), and although this process has not been shown for *M. abscessus* to date, it is plausible that a comparable DosR-dependent mechanism may exist.

Although there are some differences in the ultimate effect, it is clear that oxygen levels are involved in the regulation of biofilm formation in each species. The different effects seen are perhaps indicative of the varying ways these bacteria adapt to hypoxia and chronic infection.

Virulence factors

Bacterial pathogens produce and excrete a vast number of secondary metabolites in response to changing environmental conditions. Phenazines are a group of such compounds that play several important roles in the bacterial virulence and adaptation in *P. aeruginosa* and are expressed in response to low oxygen. PCN is a redox-active compound and can alter expression of terminal oxidases and showed that strains deficient in PCN production are less likely to survive in anaerobic conditions (Jo et al. 2020, Wang et al. 2010). Despite this, PCN production is reduced in hypoxia and loss of phenazines is characteristic of chronic infection (Schaible et al. 2012, Vilaplana and Marco 2020). Additionally, PCN production is potentially associated with oxygen sensing through the BfiSR TCS, whereby the inactivation of the BfiS sensor protein resulted in decreased in PCN production due to inhibition of expression of PCN biosynthesis pathway proteins (Petrova and Sauer 2010). Another *P. aeruginosa* virulence factor is cyanide. *Pseudomonas aeruginosa* late CF lung isolates have demonstrated increased cyanide production relative to lab strains (Carterson et al. 2004) and it can

be detected in the exhaled breath of people chronically infected with *P. aeruginosa* (Gilchrist et al. 2015). Cyanide production in *P. aeruginosa* is under the control of Anr and quorum-sensing regulators RhlR and LasR, all of which have been implicated in the response to microaerobic conditions (Pessi and Haas 2000), indicating that the increased cyanide production in CF isolates may be a part of the hypoxic response in *P. aeruginosa*.

Siderophore production

The acquisition of iron is a critical factor in chronic infection as it is an essential micronutrient for both host and pathogen. It also plays a critical role in defining host–pathogen interactions as both opponents have devised numerous methods to scavenge and sequester iron as part of the ongoing arms race between them (Sheldon et al. 2016). In low oxygen conditions, ferric iron (Fe (III) is reduced to the more accessible ferrous Fe (II) form, suggesting that the ferrous state is readily available in high abundance within hypoxic regions of the CF lung (Sánchez et al. 2017). Levels of iron and ferritin are also increased in the mucus of individuals with CF compared with non-CF healthy controls. Moreover, there is a positive correlation between the severity of CF, the mucus iron content, and an increased ratio of Fe(II)/Fe(III), suggesting that the presence of hypoxic niches.

Pseudomonas aeruginosa uses several strategies to uptake iron from the environment, including production of siderophores, such as pyoverdin, pyochelin, and induction of haem acquisition pathways as recently reviewed (Schalk and Perraud 2023). Short-term exposure to hypoxic conditions resulted in the attenuation of pyoverdin production (Schaible et al. 2017). In contrast, the *pvdA* gene in *B. cenocepacia* that encodes synthesis of the siderophore ornibactin, is upregulated in response to hypoxic conditions, mediated by the CepIR quorum-sensing system (Subsin et al. 2007). Similar to *P. aeruginosa*, *Burkholderia* species produce a number of siderophores. In addition to ornibactin, *B. cenocepacia* produces pyochelin, while other Bcc species also produce malleobactin, cepaciachelin, and/or cepabactin (Butt and Thomas 2017). A correlation between patient mortality and Bcc pyochelin production has been reported, which was suggested to be due to the contribution of pyochelin to ROS generation (Sokol 1986, Butt and Thomas 2017). Ornibactin-deficient *B. cenocepacia* strains were cleared more easily from the lung compared to strains producing this siderophore (Sokol et al. 1999) demonstrating that siderophores have a role in the pathogenesis of both these *P. aeruginosa* and *B. cenocepacia*. In chronic infection, there is an increased abundance of iron in the sputum, mainly bound ferritin; there exists strong link between sputum iron and chronic *P. aeruginosa* infection (Reid et al. 2007).

Siderophores biosynthesis has been demonstrated in *M. tuberculosis in vivo*, however the process is poorly understood to date in *M. abscessus* (Meneghetti et al. 2016, Chao et al. 2018, Mori et al. 2023). Many *Mycobacterium* species express the mycobactin siderophore system. Bioinformatic analyses showed the presence of mycobactin components in *M. abscessus* and mycobactin synthesis genes were downregulated in *M. abscessus* biofilms (Chavadi et al. 2011, Belardinelli et al. 2021). The promoters for two mycobactin gene operons promoters were found to be active in hypoxic conditions and were dependent on DosR, suggesting a link between hypoxia and iron acquisition in *M. tuberculosis*, at least (Schreuder and Parish 2014). Deletion of the *M. tuberculosis* DosR regulator resulted in enhanced growth in an iron-limiting environment suggesting that adaptation to low oxygen conditions may limit iron acquisition and reduce growth rates in mycobacteria (Schreuder and Parish 2014).

Protease production

Late infection *P. aeruginosa* isolates isolated from patient lungs show reduced protease activity (Marvig et al. 2015, O'Brien et al. 2017, Schaible et al. 2017). Interestingly, proteomic analysis showed that protease production is downregulated in strains grown in hypoxic conditions (Schaible et al. 2017). Conversely, the proteolytic activity of *B. cenocepacia* strain H111 increased when grown in hypoxic conditions (Pessi et al. 2013). Moreover, the zinc metalloproteases genes *zmpA* and *zmpB* were among the many genes upregulated in low oxygen conditions in *B. cenocepacia* (Sass et al. 2013) and we subsequently showed increased abundance of both encoded enzymes in late infection *B. cenocepacia* isolates mirroring the hypoxia response (Cullen et al. 2018). Studies in *M. abscessus* are limited; however in *M. tuberculosis*, an increase in the serine protease, Rv3668c was seen in macrophages, which mediated increased secretion of proinflammatory cytokines and increased survival, which may be connected to hypoxia (Zhao et al. 2014).

Antibiotic resistance

It is widely accepted that CF associated-pathogens develop antibiotic resistance over the course of infection (López-Causapé et al. 2015) predominantly as a result of prolonged treatment with antibiotics. However, some data suggest that short-term exposure to hypoxia can also contribute to the emergence of resistance in *P. aeruginosa* (Schaible et al. 2012, 2013). Furthermore, biofilm and granuloma formation reduce antibiotic accessibility, facilitating increased survival of pathogens. Exposing *P. aeruginosa* to short term hypoxic stress increased expression of multidrug resistance efflux pumps of the resistance-nodulation-division family with concomitant resistance to antibiotics from the penicillin and cephalosporin groups (Schaible et al. 2013). This has also observed in *M. tuberculosis*, where almost 40% of clinical isolates incubated under hypoxic stress showed increased drug resistance (Liu et al. 2016) and in *M. abscessus*, which showed greater survival to drug treatment in hypoxic conditions (Hunt-Serracin et al. 2019, Lanni et al. 2022). *B. cenocepacia* also became more resistant to kanamycin, gentamycin, and tetracycline when it was exposed to low oxygen conditions (Pessi et al. 2013). The Lxa locus encodes a metallo beta-lactamase (BCAM0300) that contributes to resistance to beta-lactams, in addition to two ABC transporter proteins, which may contribute to this phenotype (Sass et al. 2013). Interestingly, the Anr regulon governs the expression of two probable major facilitator superfamily (MFS) transporter proteins and a probable ATP-binding component of an ABC transporter protein, suggesting Anr activity may also contribute to increased antibiotic resistance, but this has yet to be demonstrated (Tribelli et al. 2019). Likewise, the *M. abscessus* DosRS regulon encodes an ABC transport protein and a putative aminoglycoside transferase, which also may contribute to antibiotic resistance (Simcox et al. 2023).

Host–pathogen interactions and intracellular survival

The interaction between host and pathogen is a vital aspect of infection as it can determine whether an infection will be

established, how virulent it will be, and whether the host will be able to clear the pathogen effectively through the immune response. Hypoxia may play a role in the regulation of some of these mechanisms that facilitate dissemination and subversion of the host immune system.

Host cell attachment is a crucial stage in the process of colonization. *Pseudomonas aeruginosa* use multiple adhesins including fimbriae encoded by the *cup* gene clusters to attach to host epithelial cells and to facilitate biofilm formation (Kulasekara et al. 2005). Anr positively regulates CupA-encoding gene expression via a trimeric regulator (Vallet-Gely et al. 2007, McManus and Dove 2011) highlighting that this is an oxygen-sensitive process. Interestingly, the loss of the LasR quorum-sensing signalling, as observed in many CF clinical isolates, led to an increase in CupA fimbriae expression in an Anr-dependent manner; CupA1 fimbriae expression was completely absent in *anr* mutant strains (Hammond et al. 2015). Similarly in *B. cenocepacia* genes encoding putative fimbrial proteins, usher proteins, lectins, type IV pili and other proteins implicated in attachment were all shown to be significantly upregulated in response to hypoxia (Sass et al. 2013).

Burkholderia cenocepacia and *M. abscessus* have both been shown to survive and replicate intracellularly, contributing to persistence and dissemination (Sajjan et al. 2008, Valvano 2015, Dubois et al. 2018, Ganesh et al. 2020, Touré et al. 2023). This is facilitated in *B. cenocepacia*, at least in part, by the increased expression of USPs and secretion systems enabling these pathogens to persist in macrophages following phagocytosis (Sajjan et al. 2008, O'Connor et al. 2023). The intracellular survival of *M. abscessus* is central to its persistence in the host and it has been shown that the DosRS regulon also encodes proteins that have been previously linked to intracellular survival, including multiple oxidoreductases (He et al. 2017, Simcox et al. 2023). As discussed earlier, PhoR mutations may arise as a result of complex interplay between PhoP and DosR; PhoPR mutants were previously shown to be phagocytosed at lower rates and demonstrated increased survival in human macrophages when compared to their WT counterparts (Bryant et al. 2021).

Burkholderia cenocepacia clinical isolates have been shown to survive and disseminate within macrophages in zebrafish models and in human macrophages in a CepR-dependent manner (Martin and Mohr 2000, Vergunst et al. 2010). There are 10 USP genes located on chromosome 2 of *B. cenocepacia,* six of which are encoded on the Lxa-locus and were increased in expression following exposure to low oxygen levels (Sass et al. 2013). A proteomic analysis of sequential clinical CF isolates also showed an increased abundance of USPs in the late isolates from two patients, highlighting their importance in chronic infection (Cullen et al. 2018). More recently one of these USPs BCAM0276 (USP76) was implicated in the ability of *B. cenocepacia* to survive within CF macrophages (O'Connor et al. 2023). In addition, the T6SS in *B. cenocepacia* has also been shown to enhance the ability to survive and replicate within macrophages (Rosales-Reyes et al. 2012). The CepIR quorum-sensing signalling system has been shown to influence the activity of T6SS, and given that CepIR activity is oxygen dependent, it is possible that sensing of low oxygen conditions could contribute to its expression also. Additionally, the ability of FixLJ mutants to survive intracellularly is greatly reduced, suggesting another role for the sensing of low oxygen in intracellular survival (Schaefers et al. 2017).

The early secretory antigenic target (ESAT6) secretion system (ESX) in *M. tuberculosis* is a type VII secretion system that has also been implicated in survival following phagocytosis and has been reviewed in depth (Gröschel et al. 2016, Roy et al. 2020, Bar-Oz et al. 2022). In *M. abscessus*, three systems have been identified: ESX-3, ESX-4, and ESX-P (Sassi and Drancourt 2014). The expression of the ESX secretion system is initiated by the WhiB proteins, which regulate the response to oxygen levels as part of a complex global regulatory system that involves the DosRS regulon in *M. marinum*, another nontuberculous mycobacteria (Chen et al. 2016). These systems have been shown to induce a proinflammatory response leading to elevated rate of phagocytosis thus emphasizing the role of low oxygen in the intracellular lifestyle of *M. abscessus*. ESX-4 in particular has been implicated in limiting phagosomal acidification and mediating membrane damage (Ferrell et al. 2022). This suggests that ESX systems, in tandem with DosR-dependent regulatory pathways, may be enhance the ability of the *M. abscessus* to survive intracellularly in macrophages.

There is growing evidence to suggest that *P. aeruginosa* can persist within epithelial cells (Balakrishnan et al. 2018, Crabbé 2024, Weimann et al. 2024). Additionally, a recent report showed survival of CF-adapted *P. aeruginosa* clones for up to 4 h within CF-macrophages (Malet et al. 2024, Resko et al. 2024, Swart et al. 2024, Weimann et al. 2024). It is possible that adaptation to hypoxia is driving enhanced tolerance to oxidative stress through increased expression of oxidative stress tolerance genes, such as *katA* encoded by the Anr regulon.

Hypoxia's hidden arsenal: preadaptive traits fuelling chronic infections

Despite being exposed to a vast abundance of other opportunistic pathogens, the CF lung is frequently colonized by a consistent subset of bacterial species. As discussed earlier, the microbiome of the CF lung is remarkably consistent across CF patients globally (Price et al. 2013). Therefore, the prevalence of this specific subset of species in people with CF are likely due to the shared ability of these pathogens to grow and thrive in microaerobic environments. The natural habitats of these species frequently necessitate their survival in hypoxic conditions, with *P. aeruginosa*, Bcc, and *M. abscessus* all surviving ubiquitously in the environment, living in soil, water, and intracellularly in amoeba (Gharbi et al. 2021).

As discussed earlier, all three pathogens possess analogous mechanisms that respond to oxygen tension. The homologies that exist between the individual components of these systems suggest convergent evolution via retention of the most efficient system in the face of identical pressures. These opportunistic pathogens also exhibit similar pathogenic traits, with each showing upregulation of functionally homologous proteins in response to hypoxic conditions. In light of this, we posit that environmental adaptation to hypoxia may be driving the ability of opportunistic pathogens to effectively colonize individuals with chronic lung disease. Surviving the crucible of environmental hypoxia may preadapt these species, arming them with powerful artillery to efficiently colonize the CF lung. This may be a spandrel, which is likely the result of a shared evolutionary past with devastating consequences for disease progression. It is probable that these intrinsic mechanisms are shared across CF-related pathogens due to their common niche. A study published by Brown et al. (2012), discussed the preadaptation of opportunistic pathogens to a given environment. These pathogens are readily equipped with mechanisms that aid efficient adaptation to a host environment by the expression of virulence factors. The environment facilitates the development of mechanisms that enable the 'virulent exploitation' of a host. In this case, it is possible that the microaerobic soil habitat of *P. aeruginosa,* Bcc and *M. abscessus* promoted the

evolution of highly similar mechanisms that assist the survival in a hypoxic environment (Brown et al. 2012).

Soil is a dynamic, low oxygen environment where microorganisms are regularly exposed to reactive oxygen species (ROS) generated by various biotic and abiotic processes (Berrios and Rentsch 2022) (Fig. 7). Given that soil is one of the major environmental habitats for these CF pathogens, exposure to ROS is likely to contribute to the tolerance of CF pathogens. Bcc, *P. aeruginosa* and *M. abscessus* are all renowned for their ability to tolerate oxidative stress, which contributes to the persistence of all three pathogens within macrophages is likely due to the hostile environment they are exposed to in the soil (Yu and Kuzyakov 2021). *Pseudomonas aeruginosa* has been observed to form persister cells that survive in macrophages during chronic infection, *B. cenocepacia* and *M. abscessus* have been shown to survive and replicate intracellularly in individuals with CF (Hastings et al. 2023, Martin and Mohr 2000, Roux et al. 2016, Ribeiro et al. 2017). This is not surprising, as an increase in mutations associated with tolerance to oxidative stress is a hallmark of chronic Bcc infection (Hassan et al. 2020). Adaptation to the soil environment includes the production of antioxidant enzymes, such as superoxide dismutase (SOD), catalase, and peroxidases, which neutralize ROS and mitigate their damaging effects on cellular components (Guo et al. 2023). The presence of these enzymes not only aids in survival in the soil but also enhances the pathogens' ability to withstand oxidative stress encountered during infection in the human host. Under hypoxic conditions, oxygen availability is limited, but it is still present in small quantities. These ROS are by-products of microbial respiration and are more likely to accumulate under low oxygen conditions due to the inefficiency of electron transport (Aguirre et al. 2005). Soil also contains various minerals, such as iron and manganese, which can participate in redox reactions. Under hypoxic conditions, the reduced forms of these minerals (e.g. Fe^{2+} and Mn^{2+}) can react with the small amounts of available oxygen, leading to the production of ROS (Yu and Kuzyakov 2021). For example, the Fenton reaction, involving Fe^{2+} and H_2O_2, produces hydroxyl radicals ($^{\bullet}OH$), one of the most reactive forms of ROS (Yu and Kuzyakov 2021) (Fig. 6). This reaction is particularly relevant in soils with fluctuating oxygen levels, where alternating between aerobic and anaerobic conditions promotes the cycling of these metal ions (Yu and Kuzyakov 2021). Additionally, certain soil microbes possess enzymes like NADPH oxidases and peroxidases, which can produce ROS as part of their metabolic activities. Macrophages in particular produce large amounts of H_2O_2 (Goddu et al. 2018). Following bacterial engulfment by macrophage, at the point of phagolysosome formation, the macrophage undergoes a respiratory burst leading to the production of the same ROS that are commonly found in the soil (Slauch 2011). NADPH oxidase, an enzyme complex located in the phagosomal membrane, is activated during the respiratory burst. NADPH oxidase catalyses the transfer of electrons from NADPH to molecular oxygen (O_2), producing superoxide anion (O_2^-), the precursor to various ROS (Borisov et al. 2021). SOD converts superoxide anion into H_2O_2. In the presence of ferrous iron (Fe^{2+}), hydrogen peroxide can be converted into hydroxyl radicals through the Fenton reaction (Borisov et al. 2021). As a result, it is tempting to hypothesize that the tolerance to ROS, as a result of growth in a hypoxic soil environment has preadapted Bcc, *P. aeruginosa* and *M. abscessus* to survive in the hostile environment of the human lung, especially within macrophages, where oxidative stress is a primary defence mechanism.

In this review, we have considered the potential impact of hypoxia as a driver of adaptation. The prolonged exposure to this environmental pressure undoubtedly changes the characteristics and phenotypes of the bacteria, facilitating the transition to a pathogen capable of chronically colonising susceptible hosts. The stable changes that arise from this response can be used to investigate the progression of disease within the lung of an individual with CF. Following colonization, *P. aeruginosa*, for example, rapidly adapts to the CF lung aided by its inherent genetic plasticity (Jurado-Martín et al. 2021) and as discussed, a myriad of genes are upregulated in response to hypoxia. These factors facilitate a transition to a persistent state, while the expression of siderophore and other effectors allow *P. aeruginosa* to dominate the CF lung microbiome (Bhagirath et al. 2016, Jurado-Martín et al. 2021). The exceptional ability of *P. aeruginosa* to dominate the airways and proliferate leads to the development of chronic lung infection, often with fatal outcomes (Wood et al. 2023). Similarly, Bcc also adapts to the CF lung environment. From studies of sequential clinical isolates, late *B. cenocepacia* isolates exhibited increased rates of host cell attachment, protease activity, intracellular survival in addition to the multitude of virulence factors mentioned earlier (Cullen et al. 2018). This is in contrast to *P. aeruginosa*, which shows reduced expression of virulence factors over time of infection, favouring a transition towards persistence (Jurado-Martín et al. 2021). As discussed, *B. cenocepacia* can survive and replicate within macrophages due to a range of factors upregulated in response to exposure to hypoxic conditions. The ability to thrive intracellularly facilitates the dissemination of *B. cenocepacia* leading to the increased rates of morbidity and mortality associated with chronic infection (Vergunst et al. 2010). Similarly, the *M. abscessus* switch to a rough morphotype, which is accompanied by an increase in DosR expression and may possibly be in response to exposure to hypoxia enhances its ability to persist in the CF lung and aids colonization by resisting immune-cell and antibiotic based intervention (Byrd and Lyons 1999, Hunt-Serracin et al. 2019, Lanni et al. 2022, Simcox et al. 2023). In addition, the increased expression of *mmpl*4 aids adhesion to host cells (Parmar and Tocheva 2023) and siderophores such as mycobactin enhance the ability of *M. tuberculosis* to colonize the airways by helping it resist host-antimicrobial factors and antibiotic therapies (Rodriguez et al. 2022).

Overall, it seems very likely that low oxygen levels play a role in the development of chronic lung infections in individuals with CF, which presents us with a unique opportunity to develop a novel therapeutic approach. The preexisting shared strategies in these environmental bacteria that facilitate colonization of the CF lung could also be potentially targeted to prevent colonization. These pathogens detect, respond, and react to hypoxia with highly analogous systems. The changes in gene expression in response to sensing oxygen tension could be used to prevent the transition to a chronic infection and prevent persistent colonization. This could potentially reduce our reliance on antibiotic intervention and may improve the lives of individuals with CF. Increased oxygen tension has already been shown to have positive effects. In 2017, Kolpen et al. (2017), showed that hyperbaric oxygen treatment, enhanced the efficacy of ciprofloxacin over clinically relevant time periods. The increased bactericidal effect of ciprofloxacin was observed in tandem with indicators of aerobic respiration restoration in *P. aeruginosa*, endogenous lethal oxidative stress, and increased bacterial growth, highlighting that increased oxygen presence in the CF lung reduces the tolerance of *P. aeruginosa* to therapeutics and immune cell function (Kolpen et al. 2017).

Bacterial transcriptional regulators are ideal targets for a novel therapeutic approach as they are absent in humans and can be essential to bacterial cell function. Inhibitory drugs could target

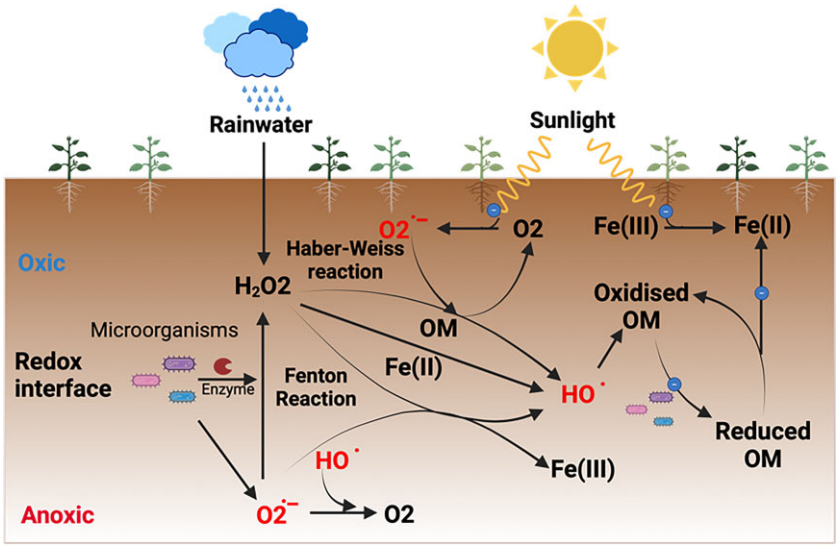

Figure 7. The Fenton reaction and generation of ROS in the soil environment OM = organic matter, SRO minerals = short range ordered minerals. Modified from Yu and Kuzyakov (2021) .

a diverse range of functions of the transcriptional regulators; signal perception, protein–protein interactions, and DNA-binding capabilities. Homologous transcriptional regulators, are expressed in all three pathogens, activating the responses to hypoxia and downstream virulence factors and in both *B. cenocepacia* and *M. abscessus*, this response may be essential for their ability to persist. Thus, targeting the regulators of the response to hypoxia or global stress responses is an enticing notion. Mansour et al. (2023) explored the impact of a series of small molecules on the activation of the FixLJ TCS with promising results (Mansour et al. 2023). This suggests that inhibition of FixK activity via repression of the FixLJ TCS may also pose as a promising therapeutic target.

There have been previous attempts to block the function of the mycobacterial transcriptional regulators of the DosRS and PhoPR systems (Kaur et al. 2014, Johnson et al. 2015, Wang et al. 2016, Zheng et al. 2019). Zheng et al. (2019), repressed expression of the regulon by inhibiting DosR DNA-binding with the inhibitor, HC104A, which directly bound to the haem binding region of DosT, preventing induction of the DosRS regulon and also reducing *M. tuberculosis* cell viability (Zheng et al. 2019). Due to the similarities across the three sensor histidine kinases (FixL, BfiS, and DosS) it is tempting to speculate that this may offer a novel therapeutic approach to target Bcc, *P. aeruginosa* and *M. abscessus*, preventing the transition to chronic infection and reducing the likelihood of the development of resistance to the therapy. Alternatively, Kaur et al. (2014), used phage-derived peptides to bind DosS and block kinase activity thus preventing activation of the DosRS regulon. Under hypoxic conditions, treatment of *M. tuberculosis* with the 'DevRS' peptides lead to a complete inhibition of cell survival (determined by CFU count) (Kaur et al. 2014).

Alternative approaches to target AHL quorum-sensing systems to impede hypoxia-driven adaptations in *P. aeruginosa* and *B. cenocepacia* could also be considered as they are also not present in humans. A novel phenolic derivative, GM-50, inhibited the transcription of las- and rhl-regulated genes, repressing virulence factor expression in clinical isolates and reducing mortality in *Galleria mellonella*, without affecting cell viability (Bernabè et al. 2022). Strategies like these may offer new approaches to adjunct treatments in synergy with existing therapies; by preventing the development of chronic infection, these pathogens may be easier to target and treat.

In conclusion, this review outlined the processes by which pathogens sense, respond and adapt to hypoxic conditions during infection, with a particular focus on common pathogens, which chronically colonize people with CF. The common mechanisms, which sense and respond to low oxygen conditions were highlighted, with a focus on downstream effects and their contribution to the development of chronic infection. It is likely that these opportunistic pathogens may be preadapted to the lung as a consequence of their environmental soil habitats, which may explain the similarities in these mechanisms. Overall, these pathways could be a potential target for the development of novel therapeutics.

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