# The structure–function relationship of *Pseudomonas aeruginosa* in infections and its influence on the microenvironment

Mads Lichtenberg [1], Tim Holm Jakobsen [1], Michael Kühl [2], Mette Kolpen [3], Peter Østrup Jensen [1,3], Thomas Bjarnsholt [1,3,*]

[1]Costerton Biofilm Center, Department of Immunology and Microbiology, University of Copenhagen, Blegdamsvej 3B, 2200, København, Denmark
[2]Marine Biological Section, Department of Biology, University of Copenhagen, Strandpromenaden 5, 3000 Helsingør, Denmark
[3]Department of Clinical Microbiology, Copenhagen University Hospital, Ole Maaløes vej 26, 2200, København, Denmark
*\*Corresponding author*: Costerton Biofilm Center, Department of Immunology and Microbiology, University of Copenhagen, Blegdamsvej 3B, 2200, København, Denmark. Tel: +45 20659888; E-mail: tbjarnsholt@sund.ku.dk
**One sentence summary:** We review microbe–microbe and host–microbe interactions and their influence on the bacterial microenvironment alongside alternative interventions based on the physiology of *P. aeruginosa* in infections, where the bacteria are often found as small aggregates embedded in host material and surrounded by immune cells.
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## Abstract

*Pseudomonas aeruginosa* is a human pathogen associated with both acute and chronic infections. While intensively studied, the basic mechanisms enabling the long-term survival of *P. aeruginosa* in the host, despite massive immune system attack and heavy antimicrobial treatment, remain to be identified. We argue that such infections may represent niche invasions by *P. aeruginosa* that influence the microenvironment by depleting host-derived substrate and activating the immune response. Bacteria embedded in cell aggregates establish a microenvironmental niche, where they endure the initial host response by slowing down their metabolism. This provides stable, lasting growth conditions with a constant, albeit slow supply of substrate and electron acceptors. Under such stable conditions, *P. aeruginosa* exhibits distinct adaptive traits, where its gene expression pattern reflects a life exposed to continuous attack by the host immune system and antimicrobials. Here, we review fundamental microenvironmental aspects of chronic *P. aeruginosa* infections and examine how their structural organization influences their *in vivo* microenvironment, which in turn affects the interaction of *P. aeruginosa* biofilm aggregates with the host immune system. We discuss how improving our knowledge about the microenvironmental ecology of *P. aeruginosa* in chronic infections can be used to combat persistent, hard-to-treat bacterial infections.

**Keywords:** biofilm, chronic infections, host–pathogen interactions, immune response, microenvironment, quorum sensing

## Introduction

*Pseudomonas aeruginosa* is a prominent opportunistic pathogen involved in chronic bacterial infections of, e.g. wounds and the respiratory tract, or associated with implants. Numerous studies have demonstrated the large genetic versatility and phenotypic plasticity of *P. aeruginosa* (Shen et al. 2006, Turner et al. 2015). There is also abundant literature on specific biochemical pathways or molecular characteristics of *P. aeruginosa* or the host immune response [recently reviewed in La Rosa et al. (2019) and Moser et al. (2021)]. However, the mechanisms governing the persistence of *P. aeruginosa* in chronic infections remain elusive. In this review, we assess fundamental knowledge about growth patterns of *P. aeruginosa* in chronic infections and their microenvironment, and discuss how these are affected by the host immune response. The latter is a surprisingly underexplored topic that may reveal essential insights into the long-term persistence mechanisms of chronic *P. aeruginosa* infections despite a strong immune response and antibiotic treatment. Increased understanding of the ecological niche that *P. aeruginosa* inhabits after successful colonization and consecutive infection of the human body may also identify important new targets for both diagnosis and treatment of chronic

infections. While we focus on the well-studied species of *P. aeruginosa*, we also draw parallels to other important pathogens where appropriate.

The conditions leading up to a chronic infection are not caused by the bacteria themselves but a dysfunction in the host that creates conditions promoting subsequent bacterial invasion and infection (Bjarnsholt et al. 2021). For example, in patients suffering from the hereditary condition cystic fibrosis (CF), a malfunction in the chloride channels leads to dehydrated mucus in the lower respiratory tract, causing an impaired mucociliary clearance of inhaled microbes (Høiby et al. 2010). For chronic wounds, a lowered or impaired vascularization and other impairments followed by a breach in the skin lead to abnormal healing and opportunities for persistent infection (Singer and Clark 2008). Additionally, the insertion of a foreign body and the subsequent destabilization of tissue can create niches for infection development (Jakobsen et al. 2018). While none of these conditions necessarily are the direct cause of infection, they involve formation of a matrix of abnormal host material, such as thickened mucus in the CF lung or slough in chronic wounds, wherein intruding bacteria may then gain a foothold and cause infection. Bacterial colonization can

arise from exogenous sources or from the existing microbiome of the hosts, and can involve single cells or small aggregates (Jelsbak et al. 2007, Hansen et al. 2012).

Most of our knowledge about the initial events leading up to a chronic infection is derived from patient samples. These are obtained either after the establishment of a chronic infection, typically in its late stages, or from acute infections that will not progress into the chronic state. The precise conditions that lead to chronic infections, whether bacteria- or host-specific, are therefore, still debated.

For bacteria, numerous *in vitro* studies have concluded that bacterial aggregation leads to increased tolerance toward antibiotics and the host immune defense response (Jensen et al. 2010, Goltermann and Tolker-Nielsen 2017, Ciofu and Tolker-Nielsen 2019, Moser et al. 2021). *In vivo* animal studies have demonstrated similar mechanisms (Pedersen et al. 1990, Lebeaux et al. 2013, Reizner et al. 2014, Jensen et al. 2019a). However, most animal models fail to mimic a native chronic infection, since the animal has to be manipulated into infection. Such models are also poor at emulating a persisting chronic infection, as the bacteria are usually either eradicated by the host or the animal succumbs to the infection over the experimental time interval. Besides attaining an increased tolerance toward antibiotics and host immune evasion, we know that: (i) bacteria gather in small colonies, or biofilms (Rudkjøbing et al. 2012, Bjarnsholt et al. 2013, Bay et al. 2018); (ii) the bacteria display much slower growth rates within the patients than subsequent *in vitro* growth rates (Yang et al. 2008, Kragh et al. 2014); (iii) the conditions within the host material are anoxic or hypoxic (Worlitzsch et al. 2002, Kolpen et al. 2010, James et al. 2016, Jensen et al. 2017); and (iv) the genetic diversity of bacteria is large and differs from the reference or environmental strains of the same species (Smith et al. 2006; Yang et al. 2011a,b, Jiricny et al. 2014, Vanderwoude et al. 2020, Armbruster et al. 2021; Fig. 1).

## The structural organization of bacteria in infections

In a range of laboratory biofilm models (flow cells, drip-flow reactors, and alike), bacteria are grown in a manner that allows for development of complex structures and many studies have shown that bacteria are capable of organizing themselves in 3D biofilm landscapes (Hall-Stoodley et al. 2004). This concept is not novel or controversial in any way, and the fossil record shows that some of the oldest known biotic structures, i.e. stromatolites, were organized as microbial biofilms communities (Garwood 2012). Such structural organization has been explained as a response to the physicochemical microenvironment surrounding the structures. For example, researchers have shown that architecture was governed by an optimal diffusive exchange of solutes in a hot-spring microbial mat, where the biomass was structured as stromatolite-like pillars (Petroff et al. 2010). However, in many cases bacterial growth is characterized by flat slabs or simple aggregates (Bridier et al. 2010).

While there is a good understanding of such structure–function relationships in many natural biofilm communities (e.g. Depetris et al., 2021, 2022), the question remains how bacteria are organized in chronic infections and whether a complex 3D structural organization and derived changes of their microenvironment confer any advantage for their persistence and resilience to the immune response or antibiotic treatment.

Surface-attached biofilms remain relevant to numerous systems such as fouling of industrial equipment (Flemming 2011) and aquatic plants (Noisette et al. 2020), stream biofilms (Besemer et al. 2012, Depetris et al. 2021), oral biofilms (Bowen et al. 2018), and implant-associated infections (Arciola et al. 2018). However, in most types of bacterial infections it is now becoming widely accepted that biofilms are not necessarily attached directly to a surface but rather suspended in an extracellular matrix (Bjarnsholt et al. 2013, Kragh et al. 2016). In the CF lung, aggregates are, thus located endobronchially, with one report showing that ∼95% of the bacteria are located more than 5 μm away from the epithelial surface (Worlitzsch et al. 2002). In wounds, bacteria aggregate in a host- or self-produced matrix (Kirketerp-Møller et al. 2008), whereby different species appear to inhabit different niches in the wound (Fazli et al. 2009). How bacteria come to be distributed in chronic wounds remains unclear, but their nonrandom distribution (Fazli et al. 2009) could be linked to differences in the microenvironment and the availability of electron acceptors for respiration between the surface and deeper parts of the wound (James et al. 2016). In acute wounds, it has been shown that bacterial aggregates form at the wound edges and in the crevices of the stratum corneum, whereas no bacteria were found in the acute wound bed (Bay et al. 2018).

It has been proposed that even multispecies infections are primarily composed of small monospecies aggregates spatially separated from each other by the host material (Burmølle et al. 2010, Rudkjøbing et al. 2012, Kvich et al. 2020). In most types of human biofilm infections, the dominating aggregate diameters are found to be 5–200 μm (Bjarnsholt et al. 2013). Here, catheter-associated biofilm patches are an exception reaching up to 1200 μm, possibly due to the large abiotic surface presenting a distinct niche for microbial colonization (Jakobsen et al. 2018). It, thus appears that there is an upper size limit of biofilms in human infections, which is significantly lower than seen for laboratory-grown surface-bound biofilms that can easily cover several square centimeters of surface. The factors that govern this apparent size limit are still not understood but may arise as a balance between phagocytosis by leucocytes and resource depletion decreasing the bacterial growth rate (Stewart 2003, Aristotelous et al. 2015, 2018).

The dynamics of phagocytosis by leucocytes has mainly been studied using single particles, where an increase in target size has been shown to prolong engulfment time and interestingly, nonspherical shapes also resulted in much slower engulfment than spherical particles (Paul et al. 2013). In contrast, the dynamics of phagocytosis of bacterial aggregates remain almost unexplored. A recent study demonstrated a negative correlation between the probability of phagocytosis by single polymorphonuclear neutrophils (PMNs) and the biofilm aggregate diameter (Alhede et al. 2020a), while another study showed that aggregates > 50 μm$^2$ resisted killing by human neutrophils (Pettygrove et al. 2021). It, thus appears that attaining a certain bacterial aggregate size can present a selective advantage. The main determinant for the switch from acute to chronic infections has been assumed to be correlated with bacterial aggregation (Bjarnsholt et al. 2012), but this paradigm was recently challenged. It was, thus shown that the biomass proportion of individual bacterial cells and those in biofilm aggregates were equal between acute- and chronic pulmonary infections (Kolpen et al. 2022). Rather than aggregation being the distinguishing factor of acute versus chronic infection, it was argued that metabolic activity might play a more central role, where acute infections are characterized by higher bacterial growth rates.
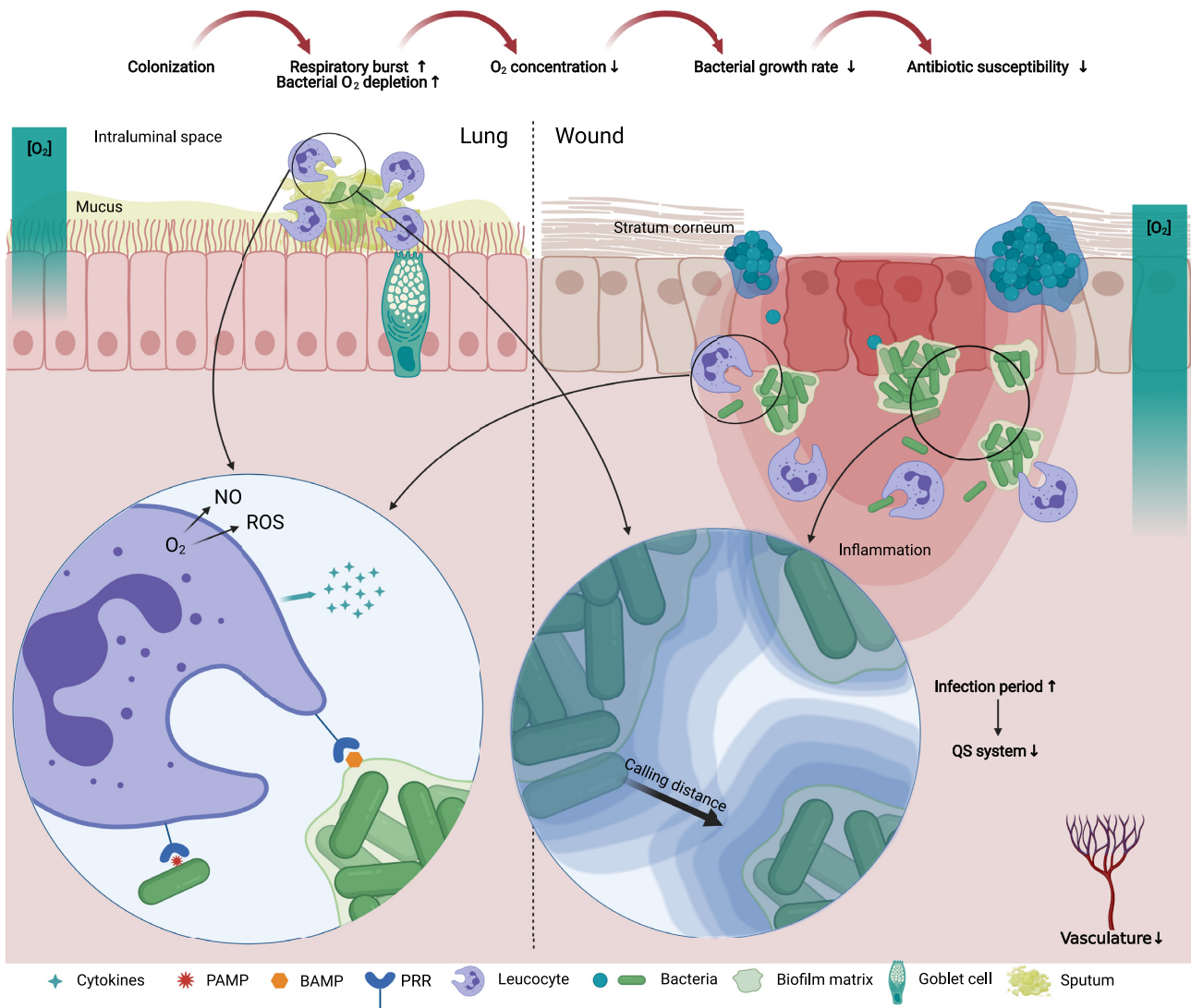
**Figure 1.** Conceptual drawing of the microenvironment of infections in the lung (left) and wound (right). Colonization by bacteria leads to innate immune activation by recognition of pathogen-associated molecular patterns (PAMP) and biofilm-associated molecular patterns (BAMP) by pattern recognition receptors (PRR) and the release of proinflammatory cytokines. Immune cell activation leads to increased $O_2$ consumption for the respiratory burst which, along with bacterial respiration, leads to lowered $O_2$ tension. In wounds, bacteria are found as monospecies aggregates separated from each other where different species appear to inhabit different zones of the wound. In lungs of CF patients, bacteria are found intraluminally embedded in thickened sputum. Bacterial interactions occur if signaling molecules reach high enough concentrations to elicit a response. The quorum sensing (QS) system has been shown to be lost or inactive in late infection stages.

## Factors shaping the microenvironment within infections

The growth limitation imposed by insufficient electron acceptor availability is influenced by the metabolic activity of the bacteria themselves, as well as human immune cells that consume $O_2$ for their respiratory burst (Jensen et al. 2017). Bacteria, thus influence their own microenvironment and larger aggregates will have less $O_2$ toward their center (Ploug et al. 1997, Kühl et al. 2007, Sønderholm et al. 2018). The minimum aggregate size necessary to deplete $O_2$ in the center can be calculated by simple diffusion–reaction models (Ploug et al. 1997, Stewart 2003). Here, we used the formulation of Stewart (2003) to explore how bacterial aggregate size varies according to the bacterial growth rate and the $O_2$ availability at the surface of the aggregate. The strong influence of $O_2$ concentration at the surface of aggregates on oxygen penetration and the subsequent growth rate of bacteria is illustrated in Fig. 2.

Even at low growth rates observed *in vivo* in the lungs of CF patients (0.217 divisions hour$^{-1}$; range: −0.10 to 0.67; Kragh et al. 2014), only aggregates with a very small radius (0–35 μm) are fully aerobic. This modeling assumes steady-state $O_2$ concentration at the surface as well as an equal growth rate of all bacteria in the aggregate. This of course is not the case *in vivo*, where other types of electron acceptors are also present. Electron acceptors are used in succession based on their bioenergetic potential. At low $O_2$ tension, *P. aeruginosa* is known to switch to denitrification if nitrate or nitrite is available (Hasset et al. 2009, Kolpen et al. 2014b), and long-term survival of *P. aeruginosa* on pyruvate and arginine fermentation has previously been documented (Schreiber et al. 2006). However, the precise regulation of respiratory pathways is complex and is dependent on multiple factors such as sub-
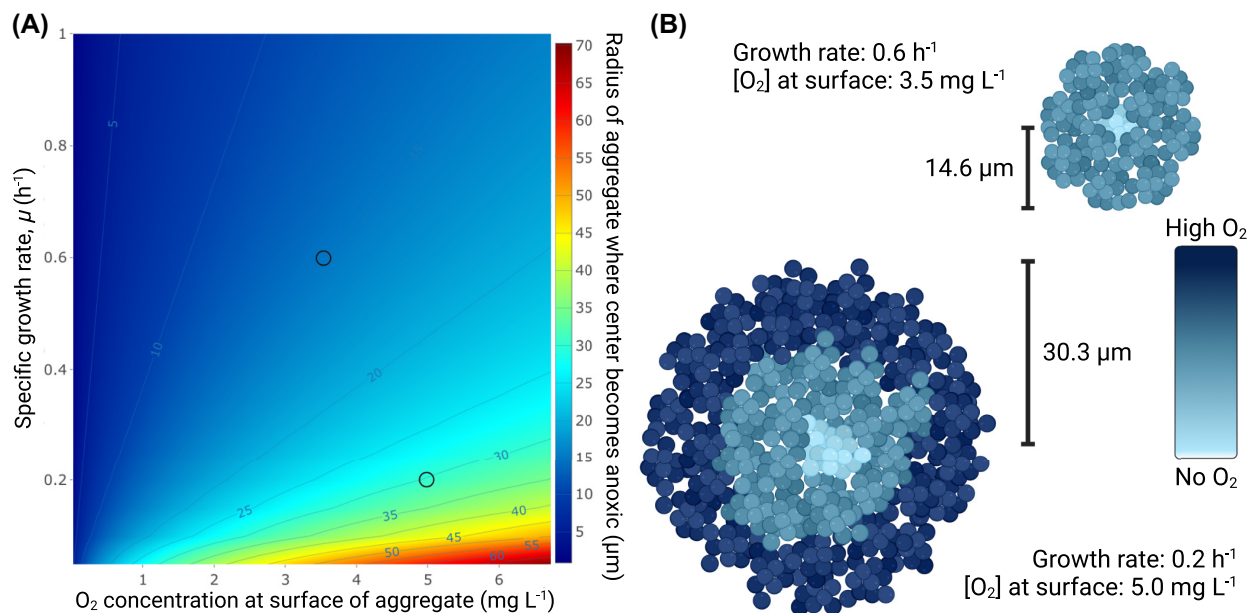
**Figure 2. (A)** Modeling of the radius of aggregates at which the $O_2$ concentration in the aggregate center goes to zero depending on the growth rate of bacteria (divisions hour$^{-1}$) and the $O_2$ concentration at the surface of the aggregate using the expressions from Stewart (2003). We used a yield coefficient of biomass on $O_2$, $Y_{xO_2} = 0.85$ mg mg$^{-1}$, a biomass density of bacteria in aggregates of $2.0 \cdot 10^5$ mg l$^{-1}$, a diffusion coefficient of $O_2$ in water, $D_{aq} = 2.0 \cdot 10^{-5}$ cm$^2$ s$^{-1}$, and an effective diffusion coefficient in the biofilm, $D_e/D_{aq} = 0.2$. **(B)** The two examples of the influence of the growth rate and surface $O_2$ concentration on the aggregate size where the center exactly becomes anoxic.

strate availability, affinities of terminal oxidases, and inhibitor molecules (Kawakami et al. 2010, Trunk et al. 2010, Lichtenberg et al. 2021). This complicates attempts to extend this type of modeling to chronic infections *in vivo*. Yet it seems, at least to some extent, that electron acceptor availability could explain the upper size limit of bacterial aggregates. At the same time, such aggregates may gain an advantage from attaining a certain size because the risk of being eliminated by phagocytosis is decreased (Alhede et al. 2020a).

The susceptibility to antibiotics is determined by the bacterial growth rate (Tuomanen et al. 1986, Evans et al. 1991), their metabolic state (Meylan et al. 2017, Lopatkin et al. 2019, Stokes et al. 2019), and the availability of $O_2$ (Brochmann et al. 2014, Dwyer et al. 2014). Thus, the uptake of $O_2$ by inflammatory cells has the potential to significantly affect the outcome of treating biofilm infections with antibiotics. It is difficult to distinguish between the $O_2$ consumption of inflammatory cells and bacterial cells in the infectious biofilm, and the resulting $O_2$ profiles in infected tissue, thus depend on the concerted action of both host cells and the bacterial biofilm (Wu et al. 2018). It should be noted, however, that in infected anaerobic endobronchial secretions from CF patients with chronic *P. aeruginosa* lung infection (Worlitzsch et al. 2002), the overall $O_2$ consumption is dominated by the host response, while the contribution of bacterial aerobic respiration to the total amount of $O_2$ consumed is apparently minimal (Kolpen et al. 2010).

The host response to biofilm infections has been thoroughly investigated in CF patients with chronic *P. aeruginosa* lung infections and from *P. aeruginosa*-specific infection models (Lorenz et al. 2016). Pathogen-associated molecular patterns (PAMPs) expressed on *P. aeruginosa* are recognized by PMNs and macrophages through pattern recognition receptors (PRRs). Further, biofilm-associated molecular patterns (BAMPs) constitute a subpopulation of PAMPs that when expressed in biofilm, induces a distinct innate im-

mune response (Moser et al. 2021). Accordingly, components of the extracellular polysaccharide matrix components in *P. aeruginosa* biofilm may qualify as BAMPs by inducing distinct responses by PMNs. In contrast, flagella failed to qualify as BAMPs due to the absence of an increased PMN response to *P. aeruginosa* biofilm with expression of flagella, even though the expression of flagella by planktonic cells increased the PMN response (Rybtke et al. 2020, Moser et al. 2021). Binding of molecular patterns to PRRs activate the innate immune response, leading to the attraction of macrophages and a multitude of PMNs (Moser et al. 2021). Further activation steps involve stimulation of the respiratory burst by the PMNs, leading to intense consumption of $O_2$ for the production of reactive oxygen species (ROS; Kolpen et al. 2010) and nitric oxide (NO) (Kolpen et al. 2014a). Additional innate responses include the PMN-mediated secretion of proteases that cause proteolytic tissue lesions (Wilgus et al. 2013) and the release of proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-$\alpha$) interleukins (IL)-1, IL-6, IL-8, and IL-12 by macrophages, which may further enhance the inflammatory response (Lavoie et al. 2011, Sweere et al. 2020).

As the adaptive immune response matures, the T-cells and the B-cells reside distantly, such as in the secondary lymphoid organs, while the plasma cells are located in the bone marrow. Activated T-cells release cytokines that reinforce the inflammation by stimulating the accumulation and activation of PMNs and production of IgG, causing further immune complex-mediated stimulation of the PMNs and activation of the classical complement pathway (Moser et al. 2017). Thus, the chronicity of biofilm infections provides the time span needed for the adaptive immune response to develop and contribute to the host response by further increasing the accumulation and activation of PMNs. This leads to the acceleration of local inflammation, resulting in collateral tissue damage but without eradicating the infectious biofilm (Jensen et al. 2010).

The ability of activated PMNs to deplete $O_2$ limits bacterial aerobic respiration, which may determine bacterial growth. Accordingly, the growth of *P. aeruginosa* is inversely correlated to the amount of PMNs surrounding the biofilms in CF patients with chronic lung infection (Kragh et al. 2014). The $O_2$ consumption by the host response may also slow down the bacterial growth of pathogens other than *P. aeruginosa* (Jensen et al. 2017). Diverse methodologies, such as rRNA fluorescence *in situ* hybridization and trace incorporation of heavy water, have indicated the slow growth of *Stenotrophomonas maltophilia* (Kolpen et al. 2015), *Achromobacter xylosoxidans* (DePas et al. 2016), and *Staphylococcus aureus* (Kopf et al. 2016) in expectorated sputum from CF patients with chronic lung infections. Because of the association of slow bacterial metabolism with low susceptibility to antibiotics, the $O_2$ consumption by the PMNs may play a significant role in the recalcitrance of chronic biofilm infections to intense antibiotic treatment in CF patients (Lopatkin et al. 2019). Besides the increased tolerance imposed by low substrate-availability, antibiotic treatment may also cause low level mutations in metabolic genes conferring increased resistance by lowering basal respiration (Lopatkin et al. 2021).

The contribution of bacterial biofilms to the poor healing of chronic wounds is increasingly recognized (Bjarnsholt et al. 2008, James et al. 2008) and has been confirmed in experimental wounds infected with *P. aeruginosa* biofilms (Seth et al. 2012, Watters et al. 2013). The incidence of bacterial biofilms in chronic wounds may exceed 80% (Malone et al. 2017). The microenvironment of chronic wounds with biofilm infections may also be hypoxic as evidenced by the presence of steep $O_2$ gradients (Schreml et al. 2014), transcriptomic profiling (James et al. 2016), and the occurrence of many anaerobic bacteria (Dowd et al. 2008) and metabolites (Debats et al. 2006).

The key mechanisms of $O_2$ depletion in infected wounds remain elusive, but the accumulation of PMNs is increased in wounds with biofilm infection (Fazli et al. 2011, Trøstrup et al. 2013). The concerted activity of biofilms and the summoned PMNs may, thus cause the steep $O_2$ gradients found in chronic wounds (Wu et al. 2018). The consumption of $O_2$ by PMNs is evidenced from the relation between the extent of the respiratory burst and the bacterial load in infected wounds (Belotsky et al. 1990), but the influence of the adaptive immune response remains largely unknown (Moser et al. 2021). While the resulting lack of $O_2$ may contribute significantly to delayed wound healing (Hunt et al. 1969, Gottrup et al. 2017, Frykberg et al. 2020), the influence of hypoxia on the outcome of antibiotic treatment in chronic wounds is largely unknown. Apart from the concerted $O_2$ consumption of inflammatory cells and bacteria in wounds, the hypoxic conditions may be further exacerbated by impaired vascularization in patients suffering from conditions such as atherosclerosis and diabetes. This impairment may also lead to inadequate delivery of systemically administered antibiotics possibly resulting in sub-MIC concentrations of therapeutic drugs being delivered at the infection site (Bue et al. 2017, Jensen et al. 2019a).

## Bacterial interactions in infections

Bacterial interactions in infections are most likely important both within aggregates and between aggregates in close proximity (Azimi et al. 2020). However, a study of the distribution and diversity of bacteria in chronic venous leg ulcers showed that the diversity of bacteria in the wound could not be captured if only one biopsy was investigated (Thomsen et al. 2010), which is indicating a heterogeneous distribution of different bacteria into biofilm aggregates that are spatially separated (Burmølle et al. 2010, Kvich et al. 2020). Other studies have shown that *P. aeruginosa* and *S. aureus* colonize different depths within wounds (Fazli et al. 2009), and the overall species diversity in wounds is low and only comprises a handful of species (Thomsen et al. 2010). The majority of aggregated bacteria in infections are surrounded by PMNs (Høiby et al. 2010, Kragh et al. 2014), and thus the interaction with the host is more likely to be the predominating form of direct cell–cell interaction at the level of single aggregates.

## Inter and intraspecies signaling

Due to the obvious complications of measuring calling distances *in vivo* in humans, the scale and importance of inter and intraspecific cell–cell signaling in infections remains unknown. However, interspecies interactions and calling distances have been frequently studied in the laboratory in well-shaken cultures or in dense biofilms of both *P. aeruginosa* and other microbes (Egland et al. 2004, Weigert and Kümmerli 2017, Darch and Koley 2018). The scale of calling distance may be dependent on the surrounding environment and the specific microbe. For rhizobacteria one study e.g. reports that calling distances frequently approach 4–5 μm, while extending up to 78 μm in some cases (Gantner et al. 2006). Others studies argue that diffusible signals for interspecies interactions only function over very short distances of ∼1 μm in open systems, which means that they effectively only reach neighboring cells (Egland et al. 2004).

The redox-active phenazine pyocyanin, which is controlled by the quorum sensing (QS) system and secreted extracellularly by *P. aeruginosa*, can be a good indicator of sharing distances between cells. By using *P. aeruginosa* colonies attached to a surface, the sharing distances of the phenazine pyoverdine was reported to be at least 100 μm when the surface was soft, although they were reduced on a hard surface (Weigert and Kümmerli 2017). In a CF lung infection model, it was estimated that aggregates of ∼2000 pyocyanin-producing *P. aeruginosa* cells were unable to interact with neighboring aggregates, while clusters containing > 5000 cells could interact with others over longer distances of up to 176 μm (Darch et al. 2018). Even though impressively large calling distances relative to the size of individual bacteria have been recorded (e.g. Gantner et al. 2006), these distances are still short compared with the distribution of bacteria in infections (Thomsen et al. 2010). Furthermore, we note that even aggregates containing only 2000 bacteria are still twice as large as the aggregates observed in the CF lung (Darch et al., 2017, 2018), suggesting that the small clusters observed *in vivo* (Bjarnsholt et al. 2013) have a limited capacity for interaggregate interactions.

We still know very little of the potential interactions between cells over micrometer-scales in chronic infections and how different microenvironments can affect the calling distance of different molecules. Along with the complex task of untangling signal-response networks, the signaling molecules themselves are characterized by different diffusion coefficients and chemical stabilities (Yates et al. 2002), which makes the calling distance of individual molecules unique.

## Interkingdom host–bacteria signaling

Several indications of a hormonal interaction between microorganisms and their hosts exist (Singh et al. 2000). The first signs of interkingdom signaling were shown when *N*-acyl homoserine lactone (AHL) signaling molecules were found capable of modulating mammalian cell signal transduction (Telford et al. 1998), and hormones from the host were observed to modulate bacterial

gene expression (Sperandio et al. 2003). Purified AHLs have been reported to increase IL-8 in respiratory epithelial cells (DiMango et al. 1995), to inhibit lymphocyte proliferation, and to downregulate the production of TNF-$\alpha$ and IL-12 in lipopolysaccharide-stimulated macrophages (Telford et al. 1998). Phenazines from *P. aeruginosa* have been shown to bind to the aryl hydrocarbon receptor (AhR), a highly conserved ligand-dependent transcription factor in mammalian cells, affecting the expression of several host genes e.g. for production of chemokines, cytokines, and detoxifying enzymes (Moura-Alves et al. 2014). A recent study by the same group demonstrated a qualitative and quantitative interaction of QS molecules and phenazines with AhR in zebrafish, mice, and humans (Moura-Alves et al. 2019). While numerous studies have shown a multitude of indications for interkingdom signaling between bacteria and host, it remains uncertain whether such interactions are important in infections, where only a small number of pathogenic bacteria are present. Most studies use cell lines and purified test compounds such as AHL signaling molecules to show a response, which makes it difficult to extrapolate the findings directly to *P. aeruginosa* infections.

## Genetic changes in *P. aeruginosa* signaling systems during infection

For *P. aeruginosa*, it has been observed that certain genes mutate over infection periods (Diaz Caballero et al. 2015), which affect the functionality of the QS system in particular, as well as the secondary signaling system cyclic diguanylate (c-di-GMP; Jiricny et al. 2014). In CF sputum samples especially, mutations seem to develop over long infection periods (Jelsbak et al. 2007, Bjarnsholt et al. 2010, Folkesson et al. 2012, Armbruster et al. 2021). For the QS system, mutations in the *lasR* gene (Smith et al. 2006, Ciofu et al. 2010, Folkesson et al. 2012) as well as mutations in *mucA* also leading to QS repression (Ryall et al. 2014) have been reported in *P. aeruginosa* infections. In addition, transcription of the *las* QS system has been shown to be significantly lower in patient samples from different infections, compared to *in vitro P. aeruginosa* biofilms (Cornforth et al. 2018).

The observed increase in *lasR* mutants during infection has been the subject of speculation in recent decades (Feltner et al. 2016, Kostylev et al. 2019). It has been suggested that *lasR* mutants are selected for by an apparent increased metabolic advantages by upregulation of catabolic metabolism (D'Argenio et al. 2007) and a lowered probability of lytic death in stationary growth phase (Heurlier et al. 2006). Another suggestion is that *lasR* mutants can spread in populations with QS-proficient bacteria, where the QS mutants might behave as social cheaters, avoiding the costs of producing messenger molecules, which leads to a mixed population (Diggle et al. 2007). Alternatively, bacteria adjust to a mixed population over time followed by a complete loss of a functional QS system in the entire population later in the infection period. Such dynamics have previously been observed in clinical settings (Köhler et al. 2010). It has also been suggested that LasR deficient *P. aeruginosa* prevent robust neutrophil extracellular trap (NET) formation in neutrophils via transcriptional regulation of LasA protease and LasB elastase (Skopelja-Gardner et al. 2019), while another study suggested that most host-derived eDNA, *in vivo,* is not a result of NETosis (Alhede et al. 2020b) in accordance with the increased proportion of *lasR* mutants observed in infections.

A total of two well-known examples of QS-regulated compounds produced by *P. aeruginosa* are rhamnolipid, the rhamnose-containing glycolipid biosurfactant, and phenazines, which are extracellular redox-active compounds. Rhamnolipid can cause lysis of PMNs (Jensen et al. 2007) and macrophages (McClure and Schiller 1992), while pyocyanin can impose oxidative stress in human airway cells, by generating superoxide leading to the depletion of intracellular NADPH stores (Rada et al. 2008).

Functional mutations in above-mentioned systems can thus potentially change the local microenvironment surrounding the bacteria in the infection sites.

The nucleotide-based intracellular signaling molecule c-di-GMP works as a switch between a motile bacterial state and a sessile, biofilm mode of growth (Boyd and O'Toole 2012). Low intracellular concentrations of c-di-GMP favor cell motility, whereas a high concentration increases the expression of adhesion factors and extracellular matrix components, leading to cell aggregation. *Pseudomonas aeruginosa* isolated from CF patients displaying the rugose small-colony variant (RSCV) phenotype exhibit an elevated level of c-di-GMP caused by mutations in the *wsp* and *yfi* loci causing a hyperinflammatory phenotype (Starkey et al. 2009, Malone et al. 2010, Pestrak et al. 2018). This leads to high levels of c-di-GMP, which suggests a selection for the biofilm phenotype in prolonged infections (Smith et al. 2006, Blanka et al. 2015). However, it has recently been reported that aggregates and single cells can be found in equal proportions in a range of acute and chronic pulmonary diseases (Kolpen et al. 2022). C-di-GMP regulates many other cellular functions besides aggregation, so it remains unresolved whether the same mutations are found across both aggregates and single cells in long-term infections.

## Treatment of biofilms based on microenvironmental characteristics

Tolerance toward antibiotics in biofilms is recognized as a major cause of therapeutic failure during chronic infection, but the mechanisms of antimicrobial tolerance *in vivo* are not completely understood (Walters et al. 2003). As part of the respiratory burst of PMNs attempting to eradicate bacteria, $O_2$ is consumed in the formation of ROS and reactive nitrogen species (RNS; *via* the inducible NO synthase; Kolpen et al., 2010, 2014a). Decreased $O_2$ tension in the biofilm environment induces reduced, hibernation-like metabolism characterized by anaerobic respiration (Kolpen et al. 2015). Consequently, the efficacy of antibiotics targeting metabolically active bacteria is reduced (Sønderholm et al. 2017, Van Acker and Coenye 2017, Crabbé et al. 2019, Jensen et al. 2019b).

Limited $O_2$-supply in bacterial biofilms has been demonstrated in several infections, such as necrotizing soft-tissue infections (NSTI; Siemens et al. 2016), cerebral abscesses, certain implant-related cerebral infections, refractory osteomyelitis, chronic ischemic ulcers, and pulmonary lung infections (Bartek et al. 2018, Moon 2019). Therefore, bacteria are subject to a hypoxic or even anoxic microenvironment affecting their sensitivity to certain types of antibiotics intended for infection control (Sønderholm et al. 2017, Jensen et al. 2019b).

Stratification of $O_2$ in biofilm aggregates grown *in vitro* confers tolerance to several commonly used antibiotics due to limited $O_2$ availability toward the center of the aggregates (Walters et al. 2003, Pamp et al. 2008). Common types of antibiotics, such as aminoglycosides, beta-lactams, and quinolones, target processes linked to the tricarboxylic acid (TCA) cycle in metabolically active bacteria, leading to formation of toxic ROS that contribute to the bactericidal activity of the antibiotic during aerobic respiration (Pakman 1971, Van Acker et al. 2013, Brochmann et al. 2014, Dwyer et al. 2014, Jensen et al. 2014, Haj et al. 2021). The bactericidal activity of quinolones and aminoglycosides decreases when
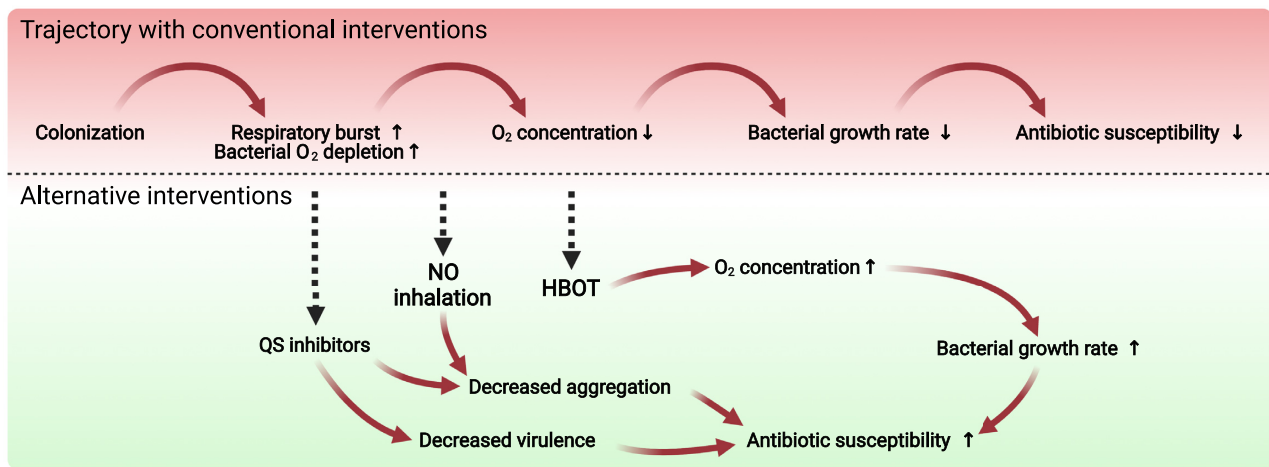
**Figure 3.** Bacterial colonization can lead to acute infections, which in healthy individuals are usually cleared by the immune response and in some cases with the aid of antibiotics. In immunocompromised patients, the infection can progress into a chronic state characterized by a continuous inflammatory response with collateral tissue damage, hypoxic conditions, and low bacterial growth rates, resulting in low antibiotic susceptibility. Alternative antipathogenic strategies include the use of QS inhibitors or quorum quenching enzymes to decrease bacterial expression of virulence factors and biofilm formation. The QS system has been shown to be lost or inactive in late infection stages so the efficacy of using QS inhibitors is most likely restricted to a certain time window. The low growth rates and high antibiotic susceptibility of bacteria in chronic infections can be reversed by treating with supplemental $O_2$ by breathing pure oxygen in either normo or hyperbaric conditions. The associated higher tissue concentrations of $O_2$ will lead to increased bacterial growth rates and higher susceptibility toward antibiotics targeting metabolically active bacteria. Alternatively, inhalation of NO can lead to upregulation of phosphodiesterases that break down the biofilm promoting molecule cyclic-di-GMP resulting in disaggregation.

the availability of $O_2$ is reduced (Borriello et al. 2004, Brochmann et al. 2014). The slow bacterial growth associated with low levels of $O_2$ (Schreiber et al. 2007) may, therefore, contribute to tolerance against both quinolones and aminoglycosides in biofilms as well as in planktonic cultures (Cozens et al. 1986, Tuomanen et al. 1986, Evans et al. 1991).

## Hyperbaric oxygen treatment

To overcome antibiotic tolerance in biofilms, introducing more $O_2$ may activate aerobic respiration and, thus increase the susceptibility of pathogens to several antibiotics that target metabolically active bacteria (Fig. 3). The addition of extra $O_2$ by hyperbaric oxygen treatment (HBOT) can significantly enhance the efficacy of antibiotic treatment *in vitro* (Mader et al. 1980, Lima et al. 2015, Kolpen et al. 2016) and has been shown to enhance antibiotic activity during experimental *in vivo* biofilm infections (Stewart et al. 1999, Kolpen et al. 2016, Özkan et al. 2016). Biofilm infections that may become susceptible to antibiotics through the use of oxygenation include endocarditis (Özkan et al. 2016, Lerche et al. 2017), osteomyelitis (Yu et al. 2011), brain abscesses (Bartek et al. 2016, Kutlay et al. 2005), and device-related infections (Bartek et al. 2018). However, the clinical effects of HBOT treatment on infections are mainly available from pro and retrospective case-control studies (Thom 2011), whereas randomized, controlled trials are still lacking. Traditionally, the rationale for the use of HBOT, especially for necrotizing soft tissue infections, is based on retro and prospective clinical and preclinical data showing a bacteriostatic effect on anaerobic bacterial growth and reduction in the production of bacterial toxins (Moon 2019). However, recent preclinical data suggest that it is the combination of HBOT with certain types of antibiotics that contributes to infection control, as bacteria are subject to metabolic adaptations to the biofilm environment in which $O_2$ is involved (Sønderholm et al. 2017, Jensen et al. 2019b).

The amount of dissolved $O_2$ is proportional to its partial pressure at a specific temperature, according to Henry's law (Trayhurn 2019). Therefore, the standard therapy of HBOT exploits this phenomenon by increasing the pressure and reducing the volume of gas-filled spaces according to Boyle's law (Thom 2011). The state of hyperoxia obtained using HBOT is a treatment modality, in which patients breathe 100% $O_2$ at increased atmospheric pressure (ATA) of up to 2.0–2.8 bar to enhance the amount of $O_2$ dissolved in the body tissues. During HBOT, arterial $O_2$ tension typically exceeds 2000 mmHg, and levels of 200–400 mmHg occur in tissues (Thom 1989, Choudhury 2018).

Reoxygenation by HBOT in an agarose *P. aeruginosa* biofilm model with slow-growing bacterial subpopulations in $O_2$-free zones leads to increased susceptibility to antibiotics (Kolpen et al., 2016, 2017, Møller et al. 2019). In combination with tobramycin treatment, reoxygenation with HBOT enhanced the killing of clinical *P. aeruginosa* isolates from CF patients grown as biofilm more than a million times (Møller et al. 2019), while a combination HBOT and ciprofloxacin treatment enhanced the eradication of *P. aeruginosa* biofilm more than 100 times (Kolpen et al., 2016, 2017). HBOT also reduced the amount of tobramycin needed to achieve the clinically relevant biofilm bactericidal concentration (BBC) by more than 50% (Møller et al. 2019).

## NO treatment

Another potential treatment of infections involves NO, which is an effective dispersal agent of bacterial biofilms that can lead to increased susceptibility to antimicrobials (Barraud et al. 2006). Here, NO acts as a signaling molecule leading to upregulation of phosphodiesterases that break down the biofilm promoting molecule cyclic-di-GMP (Barraud et al. 2009). In a randomized clinical trial, adjunctive NO was shown to decrease *P. aeruginosa* aggregate sizes in lungs of CF patients and to induce biofilm dispersal and decreased tolerance toward tobramycin and ceftazidime *ex vivo* (Howlin et al. 2017). Furthermore, NO is a potential CF therapeutic due to its mucolytic and bactericidal properties (Reighard et al. 2017, Ahonen et al. 2019), where NO can e.g. be released by polymers or nanoparticles with superior bactericidal and mu-

colytic action (Yepuri et al. 2013, Barraud et al. 2015, Rouillard et al. 2020).

## QS inhibition

Novel antipathogenic strategies beyond the use of antibiotics have gained considerable attention over the past few decades as alternative methods alleviating the increasing challenge from antibiotic resistance and tolerance in bacterial infections. Degradation of signal molecules to change the functionality of the QS system using enzymes (quorum quenching) and chemical compounds for inhibiting the functionality of the system (QS inhibitors, or QSIs) are two ways of targeting bacterial virulence (Fig. 3). Several studies have identified potent QSIs with highly diverse molecular structures originating both from natural sources (Jakobsen et al. 2012a,b, Chatterjee et al. 2017, Cheng et al. 2020) and synthetic compound libraries (Borlee et al. 2010, de Lima Pimenta et al. 2013, Starkey et al. 2014). The change from QS-proficient to QS-deficient *P. aeruginosa* isolates due to increasing *lasR* mutants during infection (Jiricny et al. 2014, Cornforth et al. 2018) raises questions about targeting the QS system for the treatment of chronic infections in particular. However, the loss of a functional Las system supports the Rhl and *Pseudomonas* quinolone signal (PQS) parts of the QS system as a focus for treatment, maybe especially in the early infection state. A range of other possible limitations in the use of QSIs have been identified. For example, low selectivity of quorum quenching substances could possibly lead to disturbance of the commensal microbiome and opposing effects on virulence have been reported, where some species showed increased aggregation (see Krzyżek 2019 for a recent review).

## Conclusion

In summary, the structural organization of bacteria in chronic infections and derived microenvironmental consequences for the pathogens are still not completely resolved, and the involved bacteria are not necessarily organized solely as aggregates but also as single cells (Kolpen et al. 2022). Bacterial biofilm aggregates are typically small and surrounded by host immune cells (Bjarnsholt et al., 2009, 2013, Jensen et al. 2017), and individual aggregates in multispecies infections are mainly composed of single species (Burmølle et al. 2010, Kvich et al. 2020). The growth rates of bacteria in infections are slow due to substrate limitation (Kragh et al. 2014), hypoxic zones are often present (Worlitzsch et al. 2002, James et al. 2016), and high doses of antibiotics are not able to eradicate all bacteria in such cases (Jensen et al. 2019a).

It is of paramount importance to improve our understanding of the infectious microenvironment, which is highly dynamic as the infection progresses and exhibits distinct changes in both physico-chemical properties as well as the gene expression profiles of both host and microbe. We argue that such information should be put into context, depending on the scientific question asked, and adapted for relevant *in vitro* models. New tools are being developed to validate *in vitro* models against the transcriptome of both bacteria and host cells in infections (Cornforth et al. 2020). The use of alternative interventions for biofilm eradication is still in its infancy compared to conventional antibacterial therapies and clinical trials are missing to get a better understanding of their efficacy. Further, we suggest that a better simulation of the infectious microenvironment, combined with relevant *in vitro* testing of clinical isolates, is needed for the development of optimized treatment strategies.

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