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Abstract

In vitro studies of *P. aeruginosa* and other pathogenic bacteria in biofilm aggregates have yielded detailed insight to their potential growth modes and metabolic flexibility under exposure to gradients of substrate and electron acceptor. However, the growth pattern of *P. aeruginosa* in chronic lung infections of cystic fibrosis (CF) patients is very different from what is observed *in vitro* e.g. in biofilms grown in flow chambers. Dense *in vitro* biofilms of *P. aeruginosa* exhibit rapid O_2 depletion within <50-100 µm due to their own aerobic metabolism. In contrast, *in vivo* investigations show that *P. aeruginosa* persists in the chronically infected CF lung as relatively small cell aggregates that are surrounded by numerous PMNs, where the activity of PMN's is the major cause of O_2 depletion rendering the *P. aeruginosa* aggregates anoxic. High levels of nitrate and nitrite enable *P. aeruginosa* to persist fueled by denitrification in the PMN-surrounded biofilm aggregates. This configuration creates a potentially long-term stable ecological niche for *P. aeruginosa* in the CF lung, which is largely governed by slow growth and anaerobic metabolism and enables persistence and resilience of this pathogen even under the recurring aggressive antimicrobial treatments of CF patients. As similar slow growth of other CF pathogens has recently been observed in endobronchial secretions, there is now a clear need for better *in vitro* models that simulate such *in vivo* growth patterns and anoxic microenvironments in order to help unraveling the efficiency of existing or new antimicrobials targeting anaerobic metabolism in *P. aeruginosa* and other CF pathogens. We also advocate that host immune responses such as PMN-driven O_2 depletion play a central role in the formation of anoxic microniches governing bacterial persistence in other chronic infections such as chronic wounds.

Keywords: microenvironment, growth, chronic infection, biofilm, immune response

1. Introduction

The biofilm physiology of pathogenic bacteria has mostly been studied *in vitro* using flow-chamber setups, where a continuous flow of media has maintained the external chemical microenvironment constant (1), (2) resulting in vertically and laterally stratified distributions of nutrients and metabolites, i. e., the formation of concentration gradients, due to i) mass transfer impedance between fluid and the exopolymeric biofilm matrix, and ii) heterogeneity in biomass distribution (2-5). In presence of such gradients, the bacteria can adapt their physiology according to the actual chemical microenvironment in the biofilm resulting in distinct growth zones and modes of metabolism (2). Thus, the growth of such *in vitro* biofilms creates internal chemical and physiological gradients, which are largely governed by solute exchange with the medium and the diffusive properties and restricted bacterial mobility in the biofilm exopolymeric matrix. In biofilms associated with chronic infections CF patients, however, direct evidence of physiological gradients within *in vivo* biofilms is lacking. In fact, the finding of low and uniformly distributed growth inside biofilm aggregates of the important pathogenic bacterium *Pseudomonas aeruginosa* in the chronically infected lungs of cystic fibrosis (CF) patients (6) points to the absence of physiological differentiation inside such cell aggregates. In addition, the low *in vivo* growth rates of pathogens, the hypoxic or anoxic conditions in infected CF endobronchial mucus (7), and the accumulation of numerous polymorphonuclear leukocytes (PMNs) around bacterial biofilm aggregates (8) imply that the majority of O_2 is not consumed by the biofilm but rather by the host immune-response outside the biofilm. In particular, PMNs that accumulate around *P. aeruginosa* biofilms *in vivo* (8) can cause intense O_2 depletion during their respiratory burst (9) and the formation of nitric oxide (NO) (10) in endobronchial secretions from CF patients with chronic *P. aeruginosa* lung infection. This PMN-imposed restriction of O_2 availability for the pathogens *in vivo* is unlike most *in vitro*

biofilm studies, where normoxic media are typically supplied continuously. In this review we discuss current evidence for a new working model of chronic infections in CF patients, proposing that it mainly is the interaction between PMNs and *P. aeruginosa* biofilm aggregates that imposes physiological constraints on the *in vivo* biofilm, and modulates the biofilm microenvironment in CF lungs. We also discuss important implications of this revised view on infectious biofilms for the antibiotic treatment of chronic lung infections.

The host immune-response changes the chemical microenvironment during chronic lung infection in CF lungs

Cystic fibrosis (CF) is caused by mutations in the cystic fibrosis transmembrane conductance regulator gene affecting apical ion transport (11). The defective ion transport results in the formation of thick viscous mucus, which makes the lungs susceptible to chronic respiratory infections by preventing mucociliary clearance (12), (13) and impeding solute mass transfer and penetration of antibiotics in the mucus (14-16). Chronic lung infection is the most severe complication in CF and *P. aeruginosa* is the major bacterial pathogen causing such infection (17), (18). In the chronic lung infection, *P. aeruginosa* exists in small biofilm cell aggregates that are persistently surrounded by PMNs in the endobronchial mucus (8), (19), (20). According to O_2 measurements directly in the lungs of CF patients, the infected endobronchial mucus is subject to severe hypoxia or even anoxia (7). Besides aerobic respiration by the lung epithelium (7), the depletion of O_2 is predominantly caused by host immune cells, i.e., PMNs that inflict a strong local O_2 consumption for their production of superoxide (O_2^-) (9) and to a lesser extent for production of nitric oxide (NO) (10). The O_2 consumption by microbial aerobic respiration thus appears diminutive under such *in vivo* conditions in the CF lung (9).

Accelerated O_2 consumption by activated PMNs has long been recognized (21) and is due to a one-electron step reduction of O_2 to O_2^- (22) by a NADPH-oxidase (23) named NOX-2 (24) that leads to a process known as the respiratory burst (25). In spite of this name, PMNs are barely engaging in aerobic respiration for acquiring ATP, and <3% of provided glucose is oxidized through the TCA in PMNs (26). The PMNs mainly produce ATP via anaerobic glycolysis (27), and inhibition of their terminal cytochrome C oxidase neither decrease O_2 consumption nor production of O_2^- in PMNs (28), (9). Thus O_2 consumption by PMNs is devoted for the production of reactive oxygen species (ROS) that are essential for the antimicrobial host response; patients with defective ROS production, such as patients with chronic granulomatous disease (29) are therefore very susceptible to bacterial and fungal infections (30).

Most infectious biofilms are characterized by a stimulation of an inflammatory response that is typically dominated by PMNs (32). Increased ROS production and thus O_2 consumption by PMNs is a stereotypical response that can be activated by both fungal intruders, Gram-positive and Gram-negative planktonic bacteria (32), (9), by bacterial biofilms (33), as well as by sterile tissue damage (34). Therefore, a variety of stimuli can strongly affect the O_2 availability for infectious microbial biofilms and consequently, we propose that O_2 depletion in infected endobronchial CF mucus is primarily due to O_2 consumption by activated PMNs. Sputum samples from CF patients with *P. aeruginosa* chronic lung infection generally contain PMNs with ongoing respiratory burst (9), (35) and NO production (10). In accordance, sputum samples from adult CF patients with *P. aeruginosa* chronic lung infection exhibit steep O_2 concentration gradients and very thin oxygenated surface zones (36). Similar O_2 gradients have also been measured in fresh sputum from pediatric CF patients with lung infections involving various bacterial species (37). During biofilm infections, activated PMNs may thus expand the O_2 depleted zones in the lung to an extent that favors

pathogenic adaptation to anaerobic physiology and such adaptation has actually been confirmed *in vivo* by several biomarkers (see below).

Activated PMNs may also decrease extracellular pH (38), (39) and secrete lactate (40), and acidic conditions of pH<6.2 have been measured in endobronchial mucus (41) and in freshly expectorated sputum from CF patients with lung infection (37). Additional host responses also affect the availability of potential alternative electron acceptors for anaerobic microbial metabolism. In CF sputum, high levels of nitrate (NO_3^-) and nitrite (NO_2^-) of~0.05-1 mM have been measured (42-44), (36), and increased levels of NO_3^- and NO_2^- in the blood have also been observed during experimental *P. aeruginosa* lung infection (45) that may be linked to the host response. Activated PMNs in infected CF sputum have thus been shown to liberate NO_3^- and NO_2^- (46) probably resulting from the degradation of peroxynitrite generated from the rapid reaction between O_2^- and NO produced by activated NOX-2 and nitric oxide synthase (47), (48).

Growth and biofilm structure: *In vitro* biofilms grown in flow cells and drip-flow reactors, exhibit formation of step chemical gradients (2), (49). These result in heterogeneous growth patterns forming a complex structural and chemical landscape (3-5), (49). In such *in vitro* biofilms. bacterial growth rate has been estimated to rapidly drop with distance from the biofilm surface reaching quasi-static growth at 40-50 μm depth (49), (50). As illustrated in Figure 1, such decline of aerobic growth can be attributed to electron acceptor limitation due to rapid *in vitro* O_2 depletion by bacterial biomass coupled with mass transfer limitation of the diffusive O_2 supply from the surrounding medium (3), (4), (51), (52).

However, we note that the presence of large surface attached biofilms with pronounced intra-biofilm gradients as seen *in vitro* remain to be demonstrated in chronic biofilm infections of CF lungs. *In vivo* biofilms in most chronic infections are typically found as small, suspended small cell aggregates that are surrounded by a high concentration of PMNs (6), (19). A meta-analysis of the size of such biofilm aggregates in various chronic infections showed a biofilm aggregate diameter range of 4 – 200 μm with a

median diameter of 50 μm in chronic CF lung infections, chronic wounds, and implant-associated and Otitis media infections (19) (Figure 2). These *in vivo* biofilm dimensions are thus in strong contrast to large area surface-attached *in vitro* biofilms typically ranging from ~50 μm to several hundred μm in thickness (53, 54).

Growth rates of *P.aeruginosa* within different biofilm aggregates in lung tissue from chronically infected CF patients showed significant variability among individual aggregates throughout the lungs (6). However, growth across individual biofilm aggregates, i. e., a comparison of growth rates of bacteria in the periphery and more central parts of individual aggregates, showed no significant differences (6). Thus, the heterogeneous growth patterns driven by chemical gradients in biofilms grown *in vitro* could not be demonstrated *in vivo* in biofilm aggregates characteristic of chronic CF lung infection. Instead, *in vivo* growth rate heterogeneity between individual biofilm aggregates showed a statistically significant correlation to the local concentration of PMNs surrounding the bacterial biofilm aggregates, where a higher concentration of PMNs lead to slower growth within the biofilms (6). High consumption of O_2 by the PMNs can thus have a bacteriostatic effect on cells within the biofilms as a whole. In this way, the surrounding inflammation can be viewed as a secondary matrix through which chemical gradients may build towards the periphery of the biofilm and not through the biofilm itself (6). Several studies have investigated the growth pattern of bacteria in the lungs of patients with CF. It is interesting that these studies have shown that species frequently classified as obligate aerobes such as *Staphylococcus aureus, Stenotrophomonas maltophilia* and *Achromobacter xylosoxidans* exhibit virtually zero growth, which is in line with depletion of O_2 in infected parts of the CFs lungs, whereas the facultative anaerobe *P.aeruginosa* exhibits slow growth under these conditions (6), (55-58). We conclude that PMNs apparently play a major role in modifying the chemical microenvironment thereby imposing growth restriction upon pathogens in biofilm aggregates associated with chronic lung infections of CF patients. As susceptibility to several types of antibiotics may be decrease by low availability of

O_2 (59-61) and slow growth (62-66), PMNs may also play a major role in rendering *in vivo* biofilms resistant to antibiotics (Figure 1E,F; Figure 3). In the following, we discuss how *P. aeruginosa* might operate and adapt to biofilm life in an ecological niche in CF lungs, where O_2 is largely absent due to PMN activity.

Metabolic flexibility in *P. aeruginosa*: The ability of microorganisms to exploit a wide range of electron acceptors for ATP generation provides metabolic flexibility in transient environments enabling the population of a variety of terrestrial and aquatic habitats (67). Such metabolic flexibility may also be an important trait in pathogens causing chronic infections. The opportunistic pathogen *P. aeruginosa* can grow under anoxic conditions by denitrification (68) or arginine fermentation (69), (70), while anaerobic pyruvate fermentation can support long-term survival of *P. aeruginosa*, but does not enable growth (71), (72). The intensive depletion of O_2 caused by activated PMNs in infected endobronchial secretions (9) may thus impose a necessary shift from aerobic to anaerobic life-styles of microorganisms in biofilm aggregates. Accordingly, anoxic zones in freshly expectorated sputum from CF patients with *P. aeruginosa* lung infections exhibit production of nitrous oxide (N_2O) (Figure 4) (36), (37), which is a signature of denitrification (68). This metabolic shift to anaerobic respiration may reflect adaptation as a consequence of O_2 restriction since several genes involved in denitrification in *P. aeruginosa* are upregulated by O_2 depletion as a result of O_2 sensing by Anr (73), (74). Complete bacterial denitrification is performed by the four enzymes nitrate reductase (NAR), nitrite reductase (NIR), nitric oxide reductase (NOR) and nitrous oxide reductase (N_2OR) that catalyzes the four step reduction of NO_3^- to N_2 (68):

$$NO_3^- \text{ (NAR)} \rightarrow NO_2^- \text{ (NIR)} \rightarrow NO \text{ (NOR)} \rightarrow N_2O \text{ (}N_2OR\text{)} \rightarrow N_2 \quad (1)$$

The CF pathogens *P. aeruginosa*, *A. xylosoxidans*, *B. multivorans* and *S. maltophilia* all exhibit biofilm growth associated with chronic lung infections (75-77). During anoxia, clinical isolates of

these four pathogens responded to supplemental NO_3^- by increased growth and were apparently capable of NO_3^- depletion, while only *P. aeruginosa* and *A. xylosoxidans* displayed the formation of N_2O (56). The genetic set-up for complete denitrification from NO_3^- to N_2 is found in *P. aeruginosa* (78), (79) as well as in *A. xylosoxidans* (79). However, formation of N_2 from NO_3^- via denitrification has so far only been demonstrated in cultures of *P. aeruginosa* (68) and remains to be firmly verified in cultures of *A. xylosoxidans*.

Response of *P. aeruginosa* to hypoxia:

Aerobic respiration in *P. aeruginosa* involves a four-electron reduction of O_2 to H_2O via five terminal oxidases (80-85). The cbb_3-1 oxidase, the ccb_3-2 oxidase and the aa_3 oxidase, are all cytochrome *c* oxidases, while the bo_3 oxidase and the cyanide-insensitive oxidase (CIO) are quinol oxidases. Each oxidase has a specific affinity for O_2, efficiency of proton translocation and tolerance to stress imposed by e.g. reactive nitrogen species and cyanide (86). While the cbb_3 are oxidases with high affinity for O_2 (87) the aa_3, bo_3 and CIO oxidases have low affinity to O_2 (86-88).

The four reductase enzymes involved in denitrification are induced by low O_2 tension and the presence of NO_3^- (89). The anaerobic regulator of arginine and nitrate reductase (Anr) (belonging to the Fnr-Crp regulator family) is on top of the regulatory network controlling the activity of the four central denitrification enzymes and thereby anaerobic energy metabolism (90-93). However, the additional transcriptional regulators Dnr and NarX-NarL are also needed for denitrification. NarX detects NO_3^- and activates NarL, that down-regulates arginine fermentation (94-96), while Dnr is highly dependent on the activation of Anr to activate the cascade of genes enconding the Nar, Nir, Nor and Nos reductases (95). In addition, Dnr responds to the presence of NO (97-99).

Furthermore, the *P. aeruginosa* quorum sensing regulator RhlR can repress the expression of the four reductase-coding genes (100) together with the quinolone signal (PQS) (101).

The Anr regulator is also a main regulatory factor controlling the five terminal oxidases involved in aerobic respiration by *P. aeruginosa* as it monitors the O_2 concentration, and at low O_2 concentrations activates the expression of the *cbb*$_3$-1 and the *ccb*$_3$-2 oxidase as well as represses the expression of CIO (86). Regulation of *aa3* and *bo3* appears to depend on nutrient and iron starvation (86).

Even though the citric acid cycle is fully operative in bacteria under denitrifying conditions (102) more energy is preserved during aerobic respiration (103). Recently, the NOx reductases has been proposed to contribute to the proton motive force by only six protons per 2 electrons from one molecule of NADH, and considering that half of the generated ATP during denitrification is available for growth, this suggests that the growth yield per oxidized NADH by denitrification is only 30 % of the growth yield during aerobic respiration (103). Accordingly, the growth of *P. aeruginosa* is expected to be lower with nitrogen oxides as electron acceptors than under aerobic conditions, and the observation of slow growth in *P. aeruginosa* biofilm in the lungs (6) along with the N_2O formation in expectorated sputum supports the hypothesis that denitrification is an important metabolic pathway for *P. aeruginos*a biofilms during lung infection in CF patients.

Bacterial response to O_2 depletion during chronic CF lung infection

First evidence for anaerobic growth of *P. aeruginosa* in CF lungs was provided by the demonstration of O_2 depletion and presence of OprF, a biomarker of denitrification in the endobronchial mucus from chronically infected CF patients (7), (104). Denitrification by *P. aeruginosa* has been further confirmed by the production of N_2O in anoxic parts of sputum samples from CF patients with chronic *P. aeruginosa* lung infection (36), (Figure 4). The absence of O_2 in

parts of the CF airways has been further confirmed by the isolation of obligate anaerobes from CF sputum and bronchoalveolar lavage fluids (105), by the demonstration of anoxic zones in CF sinuses (106), as well as by the presence of anoxic zones in CF sputum (36), (37). Several other biomarkers of *P. aeruginosa* engaged in anaerobiosis during chronic lung infection in CF have been isolated from sputum. These include; antibodies against OprF and Nar in sera (104), (107), the upregulation of the denitrification reductases in CF sputum (108) and CF isolates (109), (110), and the increased transcription of the anaerobic regulator gene *anr* and up-regulation of Anr-dependent genes (109). Additionally, after antimicrobial treatment the infected sputum content of NO_3^- increases (42) indicating a reduction in the activity of denitrifying cells.

The effect of anaerobiosis on the pathogenicity of *P. aeruginosa* may be highly relevant since production of the viscous matrix component alginate is increased when O_2 is absent (111), (7). Alginate is linked with decreased lung function (112), possibly due to the ability of alginate to provide protection against antibiotics (113), (114) and phagocytic killing (115). Additionally, components of the anaerobic respiration pathway are immunogenic as evidenced by the presence in sera of antibodies against OprF and Nar (104), (107), and the activity of the nitrite reductase is required for type III secretion resulting in prolonged survival in human monocytes (116) and enhanced virulence (117). Moreover, the N_2O production in infected CF sputum (36) indicates that NOR is active in *P. aeruginosa*, which is associated with higher tolerance against NO produced by macrophages (118) and has been shown to cause increased virulence during infection in silkworms (119).

In CF patients with chronic *P. aeruginosa* lung infection, the existence of pulmonary niches with low O_2 levels has been demonstrated directly in the bronchial mucus (7) and in sputum samples (36), (37), and evidence for growth in such niches comes from observation of the increased expression of genes involved in microaerobic respiration such as the high affinity oxidase cbb3

(109). Furthermore, *P. aeruginosa* CF PAO1 cultures kept at O_2 levels resembling the hypoxic pulmonary niches exhibit slow growth corresponding to *in vivo* pulmonary growth rates of *P. aeruginosa* reported in CF lungs (6), (88).

Effects of the chemical microenvironment on bacterial susceptibility to antimicrobials:

Most studies of antimicrobial tolerance have not focussed on the hypoxic or anoxic conditions experienced *in vivo* by *P. aeruginosa* in their biofilm micro-niche surrounded by PMNs in the chronically infected CF lung. Yet, tolerance toward antibiotics in biofilm is recognized as a major cause of therapeutic failure during chronic infection and the mechanisms of antimicrobial tolerance *in vivo* are not completely understood (120).

Physiological stratification in biofilms grown *in vitro* confers tolerance to several commonly used antibiotics due to limited O_2 availability and nutrient supply to deeper biofilm layers (120), (121). Several bactericidal antibiotics such as ciprofloxacin target aerobic respiration and induce lethal cellular damage by redox-related physiological modifications resulting in formation of ROS (60), (61), (123-125). In fact, several common types of antibiotics such as aminoglycosides, beta-lactams and quinolones target processes linked to the TCA cycle in metabolically active bacteria leading to formation of toxic ROS that contribute to the bactericidal activity of the antibiotic during aerobic respiration (60), (123). Accordingly, the bactericidal activity of ciprofloxacin and tobramycin was decreased when the availability of O_2 was reduced (59), (60). The slow growth associated with low levels of O_2 (88) may also contribute to tolerance against tobramycin and ciprofloxacin in biofilm as well as in planktonic cultures (62), (63), (65).

To overcome antibiotic tolerance in biofilms, alleviation of O_2 limitation may activate aerobic respiration and thus increase the susceptibility of pathogens to several antibiotics targeting metabolic active bacteria. As an example, hyperbaric O_2 treatment (HBOT) may significantly

enhance the efficacy of antibiotic treatment *in vitro* (126-128) and HBOT has the potential to enhance the antibiotic activity during experimental *in vivo* biofilm infections (129-132). Enhanced antibiotic activity against *in vitro* biofilm may also be achieved by supplying pure O_2 at normobarric levels (133).

In contrast, the bactericidal activity of colistin on *P. aeruginosa* does not require the formation of toxic levels of ROS from O_2 (60), and the bactericidal activity of colistin is actually enhanced in the absence of O_2 (134). The bactericidal activity of colistin mainly depends on its interaction with lipopolysaccharide (LPS) within the outer bacterial membrane (135), (136). Decreased tolerance of anaerobic biofilm against colistin may thus be due to limited ability to establish tolerance by actively modifying LPS (137-139) due to the reduced production of ATP during anaerobic respiration as discussed above. A better understanding of the effects of anoxia and re-oxygenation on the susceptibility of biofilms to various antimicrobials may facilitate optimized selection of antimicrobials against biofilm during chronic infections. There is thus a strong need for further studies focusing on relating the *in vivo* susceptibility of biofilms to antibiotics to the chemical microenvironment in chronic infections, and how it is shaped by the host immune response.

Chemical microenvironment during biofilm infection in non-CF patients:

Albeit this review focusses on how immune-responses change the growth landscape for pathogenic bacteria causing chronic lung infections in CF patients, we also advocate that similar effects could be relevant in other chronic infections. The involvement of bacterial biofilms in the poor healing of chronic wounds has lately received increased attention (140), (141), and it has been demonstrated in experimental wounds that infection with *P. aeruginosa* biofilms impairs wound closure rates (142), (143). It was also shown that steep O_2 gradients are present in the wound scab of diabetic mice with *P. aeruginosa* biofilm infection in their dorsal wound (144). Such hypoxic conditions may

contribute significantly to delayed wound healing (145-147). The source of O_2 depletion in infected wounds is far from clarified, but the finding of enhanced expression of bacterial genes associated with O_2 limitation and anaerobic growth in infected wounds of diabetic mice (144) indicate a significant consumption of O_2 outside the biofilm. Increased accumulation of PMNs in human and mouse wounds with biofilm infection (148), (149) also indicate that PMNs may dominate local O_2 consumption around bacterial aggregates in wounds similar to patterns observed in the infected CF lung, but this proposal awaits further experimental investigation.

Conclusion and outlook.

In vitro studies of *P. aeruginosa* and other pathogenic bacteria have yielded detailed insight to their potential growth modes and metabolic flexibility when switching between planktonic and biofilm habitats, and under exposure to gradients of substrate and electron acceptor. However, in chronic lung infections of CF patients the growth pattern of *P. aeruginosa* is very different from what is observed *in vitro*. Dense *in vitro* biofilms of *P. aeruginosa* exhibit rapid O_2 depletion within <50-100 µm due to their own aerobic metabolism. In contrast, *in vivo* investigations show that *P. aeruginosa* persists in the chronically infected CF lung as relatively small cell aggregates that are surrounded by many PMNs, where the activity of PMN's is the major cause of O_2 depletion rendering the *P. aeruginosa* aggregates anoxic. High levels of nitrate and nitrite enable *P. aeruginosa* to persist fueled by denitrification in the PMN-surrounded biofilm aggregates. This configuration creates a potentially long-term stable ecological niche for *P. aeruginosa* in the CF lung, which is largely governed by slow growth and anaerobic metabolism and enables persistence and resilience of this pathogen even under the recurring aggressive antimicrobial treatments of CF patients. There is now a clear need for better *in vitro* models that simulate such *in vivo* growth patterns and anoxic microenvironments and that can help unravel e.g. the efficiency of existing or

329 new antimicrobials targeting anaerobic metabolism in *P. aeruginosa*. Host immune responses such
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Figure legends

Figure 1: Model of growth and activity in a surface-attached *in vitro* biofilm. (A) Cross-section of structured biofilm consisting of bacterial cells embedded in an exopolymeric matrix. (B) The chemical conditions in an *in vi*tro biofilm, going from high concentration of substrate/nutrients/O_2 in the bulk medium surrounding the biofilm and depletion with depth in the biofilm. (C) Spatial heterogeneity in growth rate as a result of chemical gradients. Cells close to the surface of the biofilm grow fast, while cell growth becomes increasingly limited with depth in the biofilm. (E) Hypothetical result of treatment with colistin. The outer layer of actively growing cells survives the treatment, while the slow-growing cells deeper in the biofilm are killed. (F) Hypothetical result of treatment with ciprofloxacin. Actively growing cells in the outer biofilm layer are killed, while the slow-growing cells in deeper biofilm layers survive ciprofloxacin treatment.

Figure 2: Fluorescence microscopy (A – B) images (x 170 magnification) of mucosal *P. aeruginosa* biofilm stained with PNA-FISH (red) and PMNs stained with DAPI (blue) in lungs from CF patient with chronical *P. aeruginosa* biofilm (Kragh et al., 2014).

Figure 3: Proposed effect of PMN accumulation on the *in vivo* susceptibility of biofilm aggregates to antibiotics in the CF lung. (A) The bronchial lumen with two non-attached biofilm aggregates surrounded by PMN-infiltrated mucus. (B) Oxygen concentration gradient in the mucus towards the biofilm aggregates. High concentration of PMNs results in stronger local O_2 depletion and thus steeper concentration gradients due to the PMN respiratory burst. (C) High concentration of PMNs around a biofilm results in local anoxia with no or very slow aerobic growth of bacteria in the biofilm aggregate, while absence or lower abundance of PMNs enables higher growth due to better

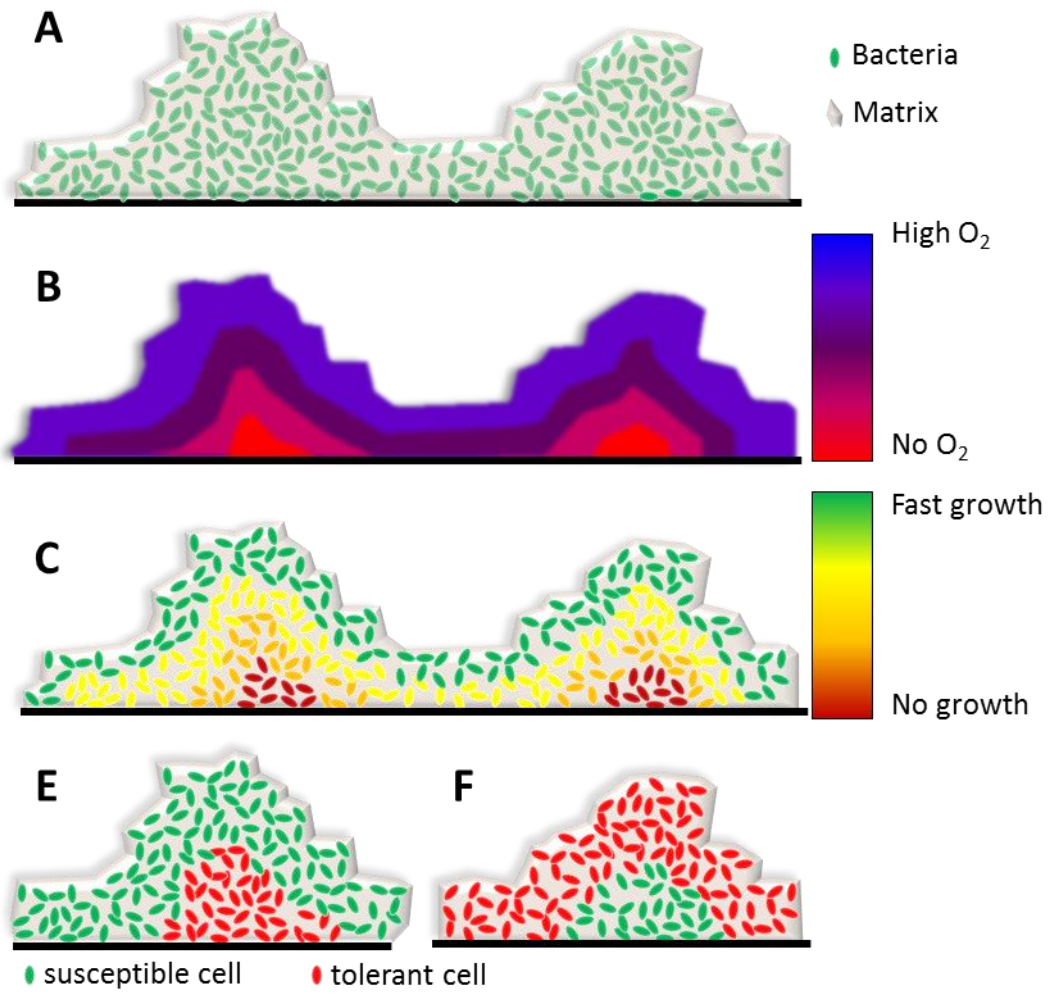
727 O_2 availability. (D) Differences in apparent growth rate of pathogenic bacteria in biofilm aggregates
728 as modulated by PMN activity could also lead to different susceptibility to antibiotic treatment.

729

730 Figure 4: Figure 4: A) Close up of a sputum sample from a cystic fibrosis patient with chronic *P.*
731 *aeruginosa* lung infection with an inserted microsensor. (B) Representative microprofiles of N_2O
732 and O_2 in a CF sputum sample. O_2 profiles are shown as the mean and SD of three microprofiles
733 recorded in the beginning of the experiment and did not change significantly throughout the
734 experimental period, while the N_2O profile represents the maximal N_2O levels measured about 6-7
735 h after beginning. (C) A schematic model of the involved PMN and biofilm processes in CF
736 sputum explaining the microprofiles. With permission from (36).

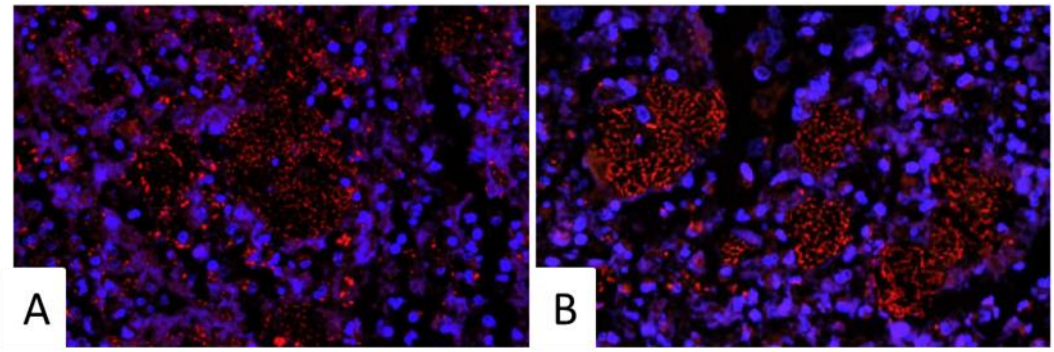
737

738 Fig 1


A
Bacteria
Matrix
B
High O_2
No O_2
C
Fast growth
No growth
E
F
susceptible cell
tolerant cell

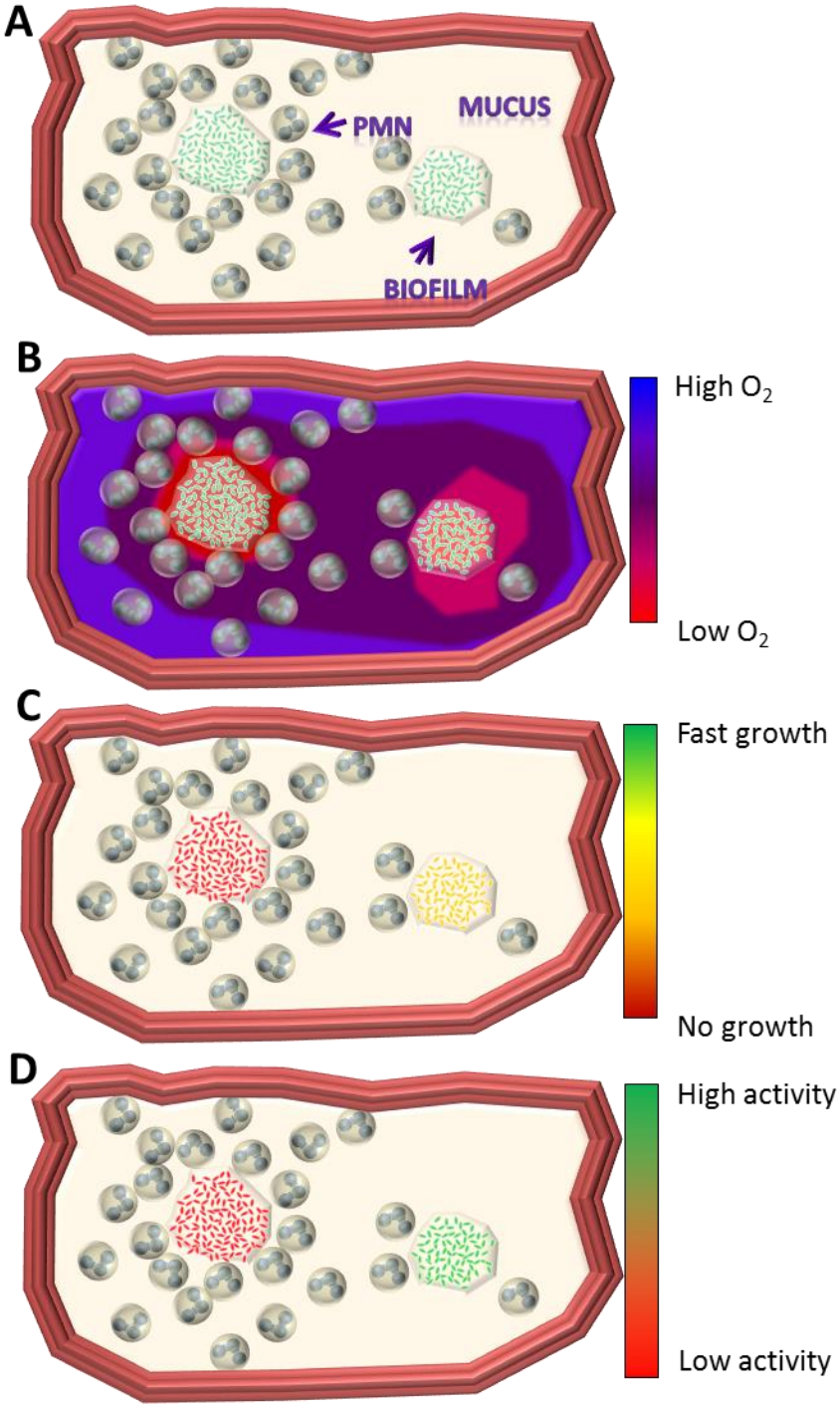
739

740 Fig 2



741

742 Fig 3



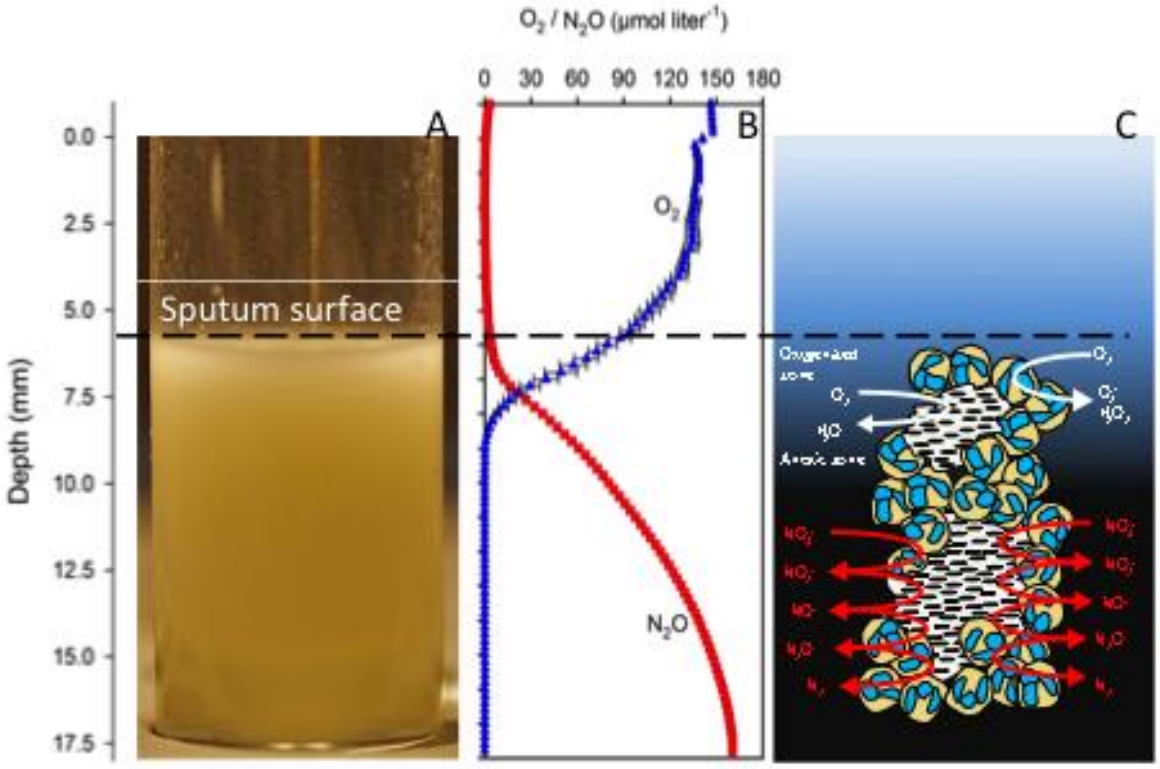
743

744

745

746

747 Fig 4


O_2 / N_2O (µmol liter^{-1})
0 30 60 90 120 150 180
A
B
C
Depth (mm)
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
Sputum surface
O_2
N_2O

748