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Review Article

Anaerobiosis of *Pseudomonas aeruginosa*: Implications for Treatments of Airway Infection

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Pseudomonas aeruginosa, as an opportunistic pathogen, establishes a chronic infection in the respiratory track of patients suffering from pneumonia and bronchiectasis, including cystic fibrosis. Biofilm formation inside the oversecreted mucus layer lining the patient airway and production of virulence factors, a process controlled by quorum sensing, are considered to be the major virulence determinants in *P. aeruginosa* pathogenesis. Recently, an abnormally thickened mucus layer was proven to be anaerobic. Given the fact that currently used antibiotics are less effective under anaerobic environments, these new findings lead us to change the way we confront *P. aeruginosa* infection. This article reviews pathological features of patient airways that become susceptible to *P. aeruginosa* infection and bacterial adaptation that contributes to the prolonged survival inside the patient airway.

Key Words: *Pseudomonas aeruginosa*, Anaerobic environments, Biofilm, Quorum sensing

I. *Pseudomonas aeruginosa*

P. aeruginosa has long been considered to be a classic example of an opportunistic pathogen (1). The organism does not normally cause infections in individuals with intact immune systems, but immunocompromised patients are particularly at risk for *P. aeruginosa* infection.

P. aeruginosa, a gram-negative bacterium, is remarkably versatile in terms of the metabolism, and thus, can maximize its survival fitness in various environments including human hosts (2). The organism, however, is strictly dependent on respiration to generate energy and is often classified as a non-fermenting bacterium (3, 4).

In nature, this gram-negative bacterium is found in highly organized communities called biofilms and has been served as a model organism to explore bacterial biofilm formation (5). Biofilm is defined as a microbial "living" biomass grown on an aggregate or on a surface with distinct architecture (6, 7). Compared to its free living counterpart (i.e. planktonic cells), bacteria grown as biofilm are refractory to a variety of antimicrobial reagents including H_2O_2 (8), a range of antibiotics (9, 10), and various heavy metals (11). Moreover, bacterial biofilm is more resistant to host immune clearance (12).

P. aeruginosa has been notorious for its high level antibiotic-resistance, arguably one of the most important virulence features of clinically isolated *P. aeruginosa*. Recently, we reported that over 76% of the Korean pneumonia patients isolates showed resistance to more than one antimicrobial agent, currently employed to combat *P. aeruginosa* infection (Yoon et al., *in press*). Mechanisms by which *P. aeruginosa* acquires antibiotic-resistance have been extensively studied and reviewed in detail elsewhere (13~16).

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Bacterial virulence factors are (i) molecules produced by microbial pathogens that induce specific disease symptoms in the host and (ii) mechanisms by which pathogens deliver (or secrete) those molecules. But, in broad terms, virulence factors include any factors that contribute to the successful colonization of host tissues. As an extracellular pathogen, *P. aeruginosa* secretes an array of virulence factors, whose production is controlled by quorum sensing, a cell-density dependent gene regulatory pathway. Effectors to be secreted include elastase (17, 18), alkaline protease (19, 20), exotoxins (21, 22), phospholipase (23, 24), and pyocyanin (25). These molecules exert toxic effects on human hosts by directly degrading host tissues or eliciting oxidative stress.

II. Abnormal mucus environments in airway diseases

Under normal airway environments, invading micro-organisms are usually expelled and/or cleared by the upper airway innate immune defense system that includes the mucociliary clearance (26~28). *P. aeruginosa* being an opportunistic pathogen, however, can cause persistent infection in patients with abnormal airway mucus secretion. Patients suffering from cystic fibrosis (CF) (1, 29), bronchiectasis (30, 31), and pneumonia (32) are especially vulnerable to *P. aeruginosa* infection. Among many pathological symptoms, aforementioned airway diseases are characterized with the noticeable oversecretion of mucus on top of the airway epithelium that debilitates the mucociliary clearance activity (1, 33). As depicted in Figure 1, mucus hypersecretion is often accompanied with the depletion of the periciliary liquid layer (PLL) and subsequent loss of mucociliary clearance activity.

CF is a genetic disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene coding for Cl^- transport channel across the apical surface of secretory cells (34). In CF, hyperactivation of epithelial Na^+ channel (ENaC), an event that occurs due to the mutation in the CFTR gene (35), drives the isotonic absorption of H_2O and ions into the airway epithelium

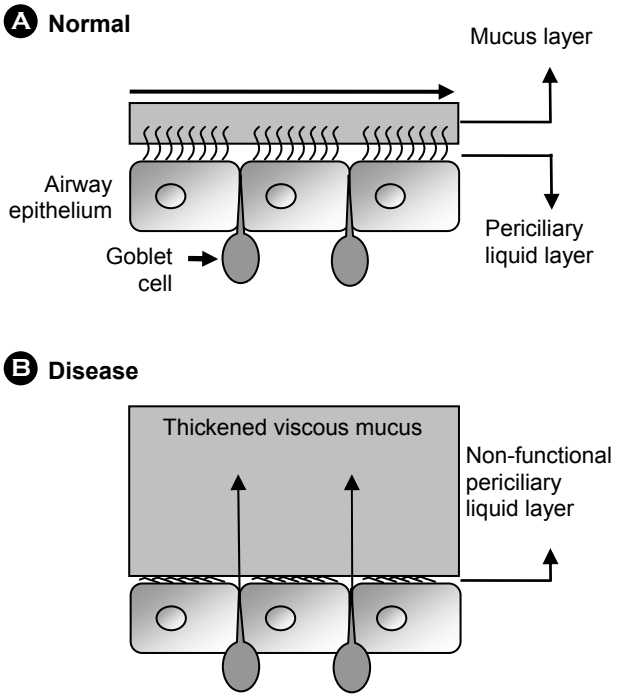

Figure 1. Schematic comparison between normal (A) and diseased (B) airway mucus environments. Maintenance of periciliary liquid layer (PLL) with constant depth and appropriate movement of the mucus layer on top of the PLL, which mediates the mucociliary clearance, is achieved in normal airways. In diseased states, however, PLL is depleted and an abnormally oversecreted (and thus, highly viscous) mucus layer is formed. This mucus layer is highly susceptible to bacterial colonization.

resulting in the dehydration of PLL and thus the formation of a stagnant mucus layer (1). Bronchiectasis (BE) is a disease state where the bronchial tree is irreversibly dilated. BE is caused by early childhood bacterial infections or pulmonary tuberculosis and patients with BE are highly susceptible to secondary infection by microbial pathogens including *P. aeruginosa*. BE is also featured with mucus hypersecretion and impaired mucociliary clearance activity (33). A recent report demonstrated that neutrophil protease present in large quantity in sputum samples from the BE patients stimulates the secretory response in tracheal submucosal glands (33).

Much evidence indicated that the oversecreted and stationary mucus layer provides a nice "habitat" for *P. aeruginosa* to colonize and proliferate (1, 36). Importantly, this abnormally altered mucus layer (Fig. 1B) also renders the host immune system ineffective against *P. aeruginosa*

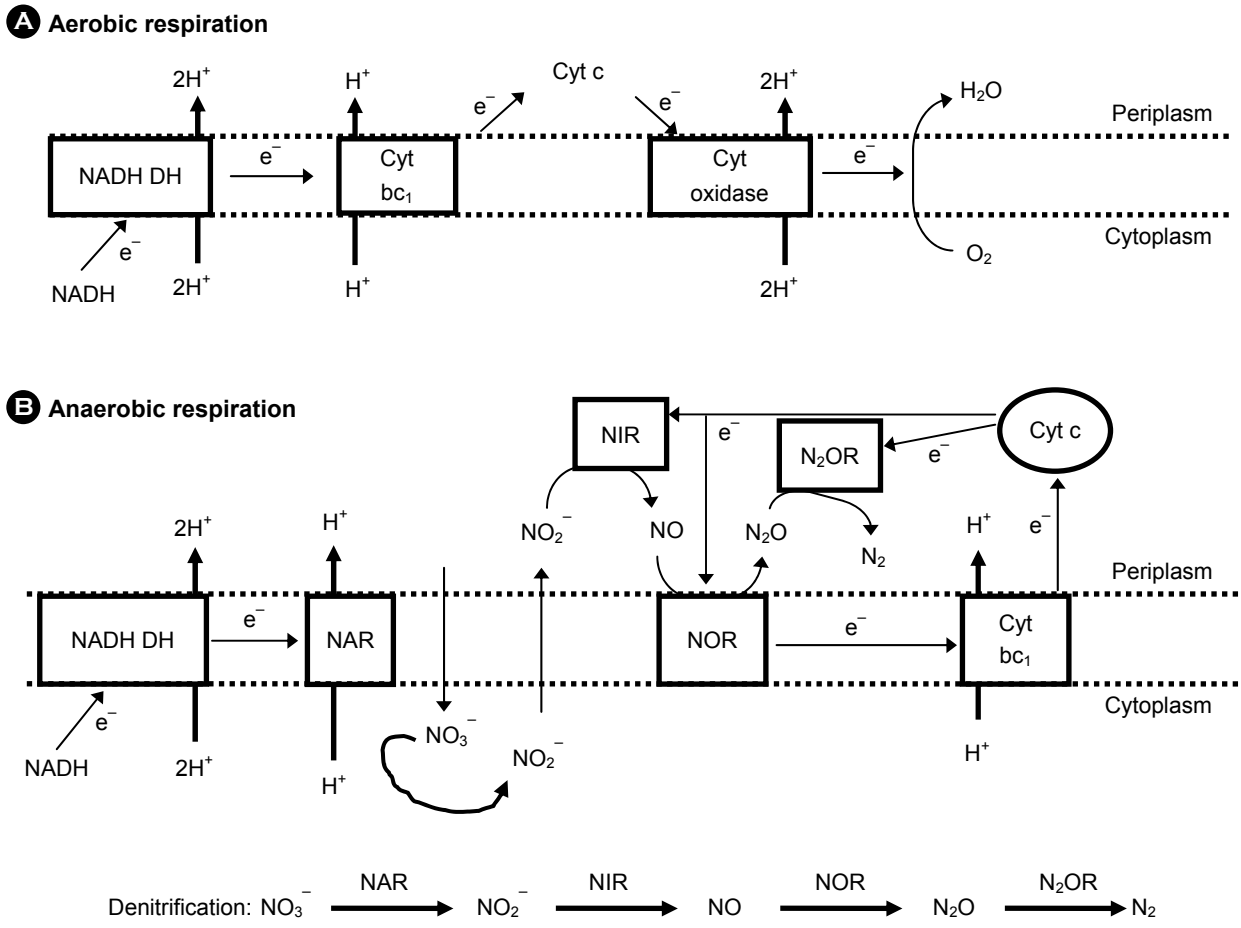

Figure 2. Aerobic (A) vs. anaerobic (B) respiratory pathways in *P. aeruginosa*. *P. aeruginosa* can use either oxygen or nitrate/nitrite as electron acceptors in the electron transport chain. NADH DH, NADH dehydrogenase; Cyt bc1, cytochrome bc1 complex; Cyt oxidase, cytochrome oxidase; NAR, nitrate (NO_3^-) reductase; NIR, nitrite (NO_2^-) reductase; NOR, nitric oxide reductase; N_2OR, nitrous oxide (N_2O) reductase.

infection. Despite a vigorous and rapid influx of neutrophils into the infected airways (37), accompanied by production of high titers of specific antibodies (38), *P. aeruginosa* infection persists and lung function progressively declines.

Recently, the stagnant mucus layer lining the airway of chronic CF patients was reported to be anaerobic (3, 36). The lack of oxygen potential is ascribed to (i) the limited oxygen transport into the mucus layer due to its increased viscosity and (ii) a high rate of oxygen consumption by immune-related and airway epithelial cells. This new observation provides a new insight into the establishment of *P. aeruginosa* infection under anaerobic condition.

III. Anaerobic growth of *P. aeruginosa*

Being an obligate respirer, *P. aeruginosa* is also capable of generating energy even in the absence of oxygen using NO_3^- (nitrate) or NO_2^- (nitrite) as alternative electron acceptors (1, 4). The *P. aeruginosa* genome harbors clusters of genes encoding enzymes for anaerobic respiration. Figure 2 compares the electron transport pathway between aerobic and anaerobic growth conditions. NADH, produced by the glycolysis and TCA cycle, feeds an electron to the inner-membrane bound NADH dehydrogenase (39) to initiate the electron transport pathway. During the sequential electron transports to cytochrome bc1 complex (40) and

cytochrome oxidase (41), H^+ ions are pumped out across the inner membrane generating pH gradient. Then, H^+ ions re-enter the cytoplasm via ATP synthase (42) to produce ATP.

As shown in Figure 2B, pH gradient across the inner membrane can still be generated when NO_3^- or NO_2^- is supplemented even under the anaerobic condition. Anaerobic respiration, often called denitrification (4) involves four reduction steps from NO_3^- to N_2. Each step is mediated by respiratory enzymes; nitrate reductase (NAR), nitrite reductase (NIR), nitric oxide reductase (NOR), and nitrous oxide reductase (N_2OR). It is of interest to note that NO, a toxic chemical to microorganisms (4), is produced as a byproduct during the denitrification process. This is analogous to the unavoidable production of reactive oxygen species in aerobically respiring cells. *P. aeruginosa*, however, can minimize the accumulation of the toxic NO during the anaerobic growth by the activity of NOR (4).

Importantly, NO_3^- and NO_2^- were detected in larger quantity in sputum or exhaled condensate of patients with pulmonary exacerbation of CF than those obtained from normal individuals (43, 44). This result suggests that *P. aeruginosa* proliferates well inside the anaerobic mucus layer exploiting the compounds produced by the host and may provide an insight into why *P. aeruginosa* has been such a competitive colonizer in the patient airways.

IV. *P. aeruginosa* biofilm and a new emerged concept on the enhanced biofilm formation during anaerobic respiration

Biofilm formation is often described as a process by which bacterial cells develop into a sessile community (45). Steps that can be clearly distinguished during this process include (i) initial attachment of free living bacteria to abiotic or biotic surface (46), (ii) microcolony formation with ensuing cell division (47), (iii) secretion of matrix molecules and growth of microcolonies into macrocolony (48), and (iv) differentiation into mature biofilm with 3-dimensional architecture (5).

Biofilm formation has been a major problem due to its resistance to a variety of antimicrobial treatments. Molecular basis that accounts for such a high-level resistance has been extensively studied. Recently, a role of periplasmic glucan encoded by the *ndvB* gene has been proposed to explain the antibiotic resistance of *P. aeruginosa* biofilm (49, 50). While a mutant defective in *ndvB* can form biofilms with normal structural features, the mutant exhibited enhanced sensitivity to tobramycin, an aminoglycoside-type antibiotic. It was also found that the mutant strains showed decreased binding to tobramycin, suggesting that periplasmic glucan may provide a physical barrier to prevent tobramycin from penetrating into the cytoplasm.

Recently, it was revealed that *P. aeruginosa* forms more robust biofilm during anaerobic respiration than they do when they respire aerobically (3). Since oxygen transfer to the depth of biofilm can be significantly limited (51), it has been postulated that "anaerobic" local regions may exist within mature biofilms. This result, however, shows that *P. aeruginosa* is actively responding to the anaerobic respiration in order to form more robust biofilm, a resistant mode of growth. This result further suggests that *P. aeruginosa* airway infection is clearly associated with the biofilm formation under anaerobic conditions. Understanding the molecular basis behind this anaerobiosis-induced robust biofilm formation will provide better insight into the *P. aeruginosa* pathogenic mechanisms leading us to come up with novel strategies to treat the infection.

V. *P. aeruginosa* quorum sensing and the future direction

P. aeruginosa fine-tunes its virulence by a process of inter-cellular communication known as quorum sensing (QS). In QS, *P. aeruginosa* produces, secretes, and responds to extracellular signal molecules, called autoinducers, to regulate the expression of genes involved in biofilm formation (52) and production of diverse virulence factors including exotoxin A (53), elastase (54), alkaline protease (55), rhamnolipid (54), and pyocyanin (25). Expression of genes encoding superoxide dismutase and catalase, which mediate oxidative stress responses, is also controlled by QS

(56). The role of QS in *P. aeruginosa* virulence was clearly demonstrated in studies using infection models with a range of different living hosts (57~59) and cultured host cells (60, 61).

There are three well-characterized QS systems in *P. aeruginosa*: *las*, *rhl*, and *pqs*. The *las* and *rhl* systems were initially identified to be essential for e**las**tase and **rh**amno**l**ipid production, respectively (62, 63). Each system is composed of its own transcriptional activator protein (LasR or RhlR) and cognate autoinducer synthase, LasI or RhlI, that produces *N*-(3-oxododecanoyl)-L-homoserine lactone and *N*-butyryl-L-homoserine, respectively. Each autoinducer molecule binds to its cognate transcriptional activator, LasR or RhlR, and this complex then apparently binds to RNA polymerase, which results in transcriptional activation of QS regulated genes.

Another arm of *P. aeruginosa* QS is a system where the DNA-binding affinity of MvfR (PqsR), an important virulence-associated transcriptional regulator, is enhanced upon binding with pseudomonas quinolone signal (PQS) (64, 65). Mounting evidence indicated that PQS is also a major player in the complex intertwined *P. aeruginosa* QS network and PQS-mediated QS is therefore required for the uninterrupted production of elastase (66) and rhamnolipid (67, 68).

Recently, many CF isolates were recovered that harbor mutations in *lasR* gene (69, 70). This fining is contradictory to the established knowledge that *lasR*-associated QS plays an essential role in *P. aeruginosa* virulence. Subsequent study, however, reported that mutations in *lasR* confer increased survival fitness in CF airways, where bacteria shift its energy metabolism to anaerobic respiration (71). This result further suggests that infection dynamics inside the patient airway are highly complicated and roles of QS in *P. aeruginosa* pathogenesis *in vivo* have to be re-evaluated.

Interestingly, QS mutants, in which *lasR* gene or *rhlR* gene is disrupted, lost viability during *in vitro* biofilm formation under anaerobic respiration, a phenomenon due to the overproduction of toxic nitric oxide (NO), a byproduct of anaerobic respiration (3). This suggests that QS is required to maintain viability during anaerobic biofilm formation supporting the presence of a novel function of *P. aeruginosa* QS. Further investigation is warranted to better understand the QS operation during anaerobic growth, a mode of proliferation that occurs in the patient airway.

VI. Conclusions

Although patient airways are equally exposed to diverse bacterial pathogens, *P. aeruginosa* has been a major microorganism that successfully colonizes and establishes persistent infection in the airway. *P. aeruginosa* airway infection should now be approached as an anaerobic disease of lung and this new idea necessitates further research directed on identifying new targets, inhibition of which will decrease bacterial virulence or survival under anaerobic condition. Because biofilm and QS are two major arms of virulence mechanisms of this clinically important pathogen, future therapeutic strategies for the treatment of airway infection should include molecular-level understanding of anaerobiosis-induced biofilm and QS.

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