Published in final edited form as:

Trends Microbiol. 2013 February ; 21(2): 73–81. doi:10.1016/j.tim.2012.10.004.

Pyocyanin effects on respiratory epithelium: relevance in *Pseudomonas aeruginosa* airway infections

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Abstract

Pseudomonas aeruginosa uses several virulence factors to establish chronic respiratory infections in bronchiectasis, chronic obstructive pulmonary disease and cystic fibrosis patients. One of its toxins, pyocyanin, is a redox-active pigment that is required for full virulence in animal models and has been detected in patients' airway secretions. Pyocyanin promotes virulence by interfering with several cellular functions in host cells including electron transport, cellular respiration, energy metabolism, gene expression and innate immune mechanisms. This review summarizes recent advances in pyocyanin biology with special attention to current views on its role in human airway infections and on its interactions with the first line of our airway defense, the respiratory epithelium.

Keywords

pyocyanin; *Pseudomonas*; respiratory; redox; epithelium

Pseudomonas aeruginosa is an opportunistic pathogen causing lung infections

Pseudomonas aeruginosa (PA) is a common Gram-negative bacterium found throughout the environment. PA rarely causes infections in healthy individuals, it mainly infects patients with burns or those that are immunocompromised and PA is one of the main causes of nosocomial infections [1]. Furthermore, this opportunistic pathogen infects the lungs of bronchiectasis, chronic obstructive pulmonary disease and cystic fibrosis (CF) patients [2, 3]. In CF PA is the most common pulmonary pathogen and it is responsible for a majority of CF disease mortalities [4, 5].

Although serious research efforts and progress have been made in the past two decades on CF, we are far from understanding why PA is the predominant pathogen in CF airways and how the bacterium could be efficiently eliminated. PA has one of the largest genomes among bacteria; its genetic adaptability is remarkable as it undergoes an entire microevolution during the years of pulmonary infection in CF [6]. At early stages, the

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planktonic bacterial forms are predominant [7]. PA uses a variety of virulence factors to attack the host and to establish infection [3]. During the later course of infection, the surface-attached virulence factors (flagellum and pilus) are downregulated to evade recognition by the immune system [8]. The bacterium switches from a non-mucoid to a mucoid phenotype, which is characterized by production of the exopolysaccharide, alginate, needed to assume the biofilm growth phase [7, 8]. Bacteria imbedded in the biofilm are protected against attacks by the immune system and maintain an inflammatory environment marked by mucus hypersecretion and aberrant neutrophil recruitment, which are major contributors to pulmonary symptoms and morbidity of CF patients [3, 8]. This review introduces pyocyanin (PYO), an important virulence factor of PA, provides a comprehensive description of details of PYO's mechanism of action, summarizes our current understanding of PYO's relevance in human infections and points to crucial directions for future research.

Pyocyanin is a redox-active virulence factor

PA possesses a wide variety of virulence factors aimed at establishing long-term lung infection. One of the most important PA virulence factors is PYO. PYO is part of the phenazines, which are heterocyclic, nitrogen-containing compounds secreted by some bacteria including *Pseudomonas* species [9]. Phenazines are synthesized from chorismic acid [9]. The two last steps of pyocyanin synthesis from its precursor, phenazine-1-carboxylic acid, are mediated by S-adenosylmethionine-dependent N-methyltransferase, PhzM, and the flavin-dependent hydroxylase, PhzS [9] (Figure 1a). PYO is a blue pigment that is produced and secreted into the medium by stationary phase cultures of PA (Figure 1b). In culture, PYO is responsible for the culture medium being a blue-greenish color because the PYO/PhzM-deficient PA strain (PA14 PhzM) fails to color the medium (Figure 1b). PYO can be easily purified from the culture medium by repeated chloroform-distilled water extraction steps resulting in a pure solution of the blue pigment (Figure 1b). The color and the characteristic absorption spectrum of PYO are pH-sensitive, as its strong blue color detected at neutral pH turns red at acidic pH (Figure 1c).[10]

Originally PYO was considered a metabolic waste product of PA, secreted without any other biological function [11]. Later on it was discovered that PYO is a redox-active compound capable of accepting and donating electrons. Since PYO can cross biological membranes easily, it serves as a mobile electron carrier for PA. Under aerobic conditions PYO's main electron acceptor is molecular oxygen. The colorless, reduced PYO donates one electron to O₂ thereby creating superoxide anion and turning into the blue, oxidized form [12]. This can be demonstrated in well-oxygenated stationary PA cultures that are no longer being shaken. Under vigorous shaking conditions the entire culture is green (resulting from the mix of the blue toxin and the yellowish medium) because pyocyanin is immediately oxidized throughout the entire culture (Figure 1d). Once shaking has stopped, a loss of green coloration of the medium is seen over time as oxygen tensions in the medium drop and PYO remains in its colorless reduced state (Figure 1d). Upon reoxygenation, PYO is oxidized and the culture medium turns green again (Figure 1d). Standing PA cultures have a characteristic green-yellow gradient close to the air surface because concentrations of the oxidized blue form of PYO drop in parallel with oxygen tensions with increasing distance from the air-liquid interface (Figure 1e) [13]. PYO mainly accepts electrons from NADH generated in carbon source oxidation (Figure 1e). Thus, PYO helps PA survive under oxygen-poor conditions by accepting and transporting electrons produced in respiration away from the bacterium to acceptors found at remote places. In a biofilm setting, PYO creates a biofilm electrocline, a redox potential gradient that reaches hundreds of micrometers beyond the biofilm surface [14]. The PYO-induced electrocline appears to enhance the bioavailability of iron, which is essential for biofilm formation [14]. Interestingly, CF airway epithelial cells—unlike non-CF cells—were shown to more readily release iron.[15] Recently it was shown

that PYO also serves as a signaling molecule and determines PA colony morphology [13, 16].

PYO in PA infection models

Previous studies have shown that PYO is essential for full virulence of PA in a variety of host organisms (plants, *Drosophila*, *Caenorhabditis elegans*, and mouse) [17–21]. These data have been summarized in excellent review articles [22, 23]. Other studies recently emphasized the importance of PYO by introducing the purified toxin into mouse airways over long periods and found that chronic PYO administration itself can cause a CF-like lung phenotype [24]. Chronic exposure of the mouse airways to PYO leads to Goblet cell hyperplasia and mucus overproduction, typical features of CF airways [3, 24]. Interestingly, a positive signature-tagged mutagenesis approach in a chronic murine airway infection model of PA found that inactivating mutations accumulating in PA over the course of chronic infection are associated with decreased production of PYO and other virulence factors [25]. However, these studies have their limitations and data obtained from human airway samples can help resolve these conflicting reports.

PYO in CF

The data obtained in murine models of PA infections cannot be easily extrapolated to human CF airways because there are no good mouse models of CF. Several *in vitro* studies use very high concentrations of PYO, although PYO has been detected in the low micromolar range in the proximity of PA biofilms [14]. *In vitro* treatment of human cells with PYO exposes the cells to an exaggerated oxidative stress and exhausts their antioxidant capacity. Several findings obtained under these conditions might not be relevant under the conditions found in CF airways. Despite the limitations of interpreting the vast amount of data obtained from *in vitro* cell culture and murine models and the obvious need to study PYO in human samples, surprisingly only a handful of studies to date have used CF sputum samples or clinical isolates of PA to study PYO.

Is PYO detectable in CF airways? Every article published on PYO states that PYO is important in CF because it can be found in large amounts in CF patients' airways; however, all of these reports refer to a single study published by Wilson *et al.* [26], which measured PYO concentrations in the airways of only four CF patients (Table 1). One sample was obtained directly from the large airways of one patient and contained high PYO levels of 16.5 µg/ml [26]. Sputum sol phase PYO concentrations were determined in cases of three additional patients: two samples had low PYO concentrations (0.9 µg/ml and 1.5 µg/ml) and another contained no detectable levels of PYO (Table 1.) [26]. Although these data are very important, the sampling size was limited and further studies were required surveying PYO levels in larger CF patient cohorts. A recent study by Hunter *et al.* addressed this question by measuring concentrations of PYO and phenazine-1-carboxylic acid in expectorated sputum samples of 45 CF patients [27]. The authors found that phenazine levels negatively correlate with pulmonary function (FEV1%) and rate of pulmonary function decline ($\Delta\text{FEV1\%}/\Delta t$). [27] CF sputum concentrations of PYO, phenazine-1-carboxylic acid and total phenazines all correlate with disease severity (normal > mild > moderate > severe) [27]. This study provides a long-awaited, clear confirmatory answer to the question of PYO's importance in CF airways.

In addition to detecting PYO in CF patients' sputum samples, a few reports studied *in vitro* PYO production of CF clinical isolates of PA and found that most isolates examined secrete PYO (Table 1) [28]. PYO overproduction has been reported among isolates of the hypervirulent Liverpool epidemic CF strain of PA and was correlated with periods of pulmonary exacerbations in a set of CF patients (Table 1) [29, 30]. It is becoming accepted

that PA downregulates the expression of its virulence factors over the course of infection to establish a long-term presence in CF airways [3, 8, 31]. Such a downregulation of PYO expression in chronic infection has been suggested by two recent reports. First, levels of BPI-ANCA antibodies in CF sputum samples were found to be negatively correlated with their PYO concentrations (Table 1) [32]. Second, one of the genes found in a signature positive-tagged mutagenesis screen, PilY1, was mutated throughout a longitudinal series of CF clinical isolates of PA (Table 1) [25]. PilY1 was shown to be involved in PYO secretion, and in its absence PYO secretion is decreased [33]. The study by Hunter *et al.* also found that lung function of CF patients correlated with *in vitro* PYO production of their PA clinical isolates which seemingly contradicts the finding that *in vivo* PYO concentrations in the CF lung negatively correlate with lung function [27]. The authors also find, however, that total PA load (CFU/g sputum) strongly correlates with disease severity in CF as well [27]. Thus, *in vivo* PYO levels are not only determined by *in vitro* PYO-producing capabilities of the bacterium but also by the total PA load in sputum. Indeed, normalized PYO levels (PYO concentration $\times$ PA load, $\mu\text{M PYO} \times \text{CFY/g sputum}$) show positive correlation with CF disease severity.[27] This also questions the clinical relevance of *in vitro* PYO production data of clinical isolates alone. Finally, a negative correlation was found between PYO levels and the complexity of the microbial community in CF sputum samples [27]. This may be explained by the known antibiotic effects of PYO against other bacteria [34].

In summary, these data show that PYO is associated with disease severity and decline in lung function and it contributes to the dominance of PA in the CF lung (Table 1). However, several details of how PYO affects host cells under the conditions found in CF airways remain unanswered. Since CF airways were proposed to be hypoxic and PYO requires oxygen to cause oxidative stress, it is unclear which of the effects of PYO studied *in vitro* under normoxic conditions really take place *in vivo*. Are there any redox-independent effects of PYO in host cells? Further investigations are required to confirm these data in independent CF patient cohorts and to better understand the pathomechanisms of PYO in CF airways.

Mechanism of PYO virulence: *in vitro* studies

PYO is required for full PA virulence in animal models and most likely in human airway infections as well [23]. PYO has a wide range of effects on different host cells but the root for its diverse toxicities relies on the production of reactive oxygen species (ROS) (Figure 2a). ROS are short-lived reactive derivatives of oxygen, which are important in several cell functions, but when produced in excess they can perturb normal cell metabolism. PYO can cross host cell membranes easily and oxidize their intracellular reduced nucleotides, NADH and mainly NADPH, to produce superoxide anions and downstream ROS (Figure 2a–c) exposing the host cell to oxidative stress (Figure 2d) [22]. The increases in ROS levels and drop in reduced nucleotide concentrations evoked by PYO result in enhanced cytosolic redox potential [35]. Secondary effects of decreased NADPH levels are reduced ATP synthesis and a decreased reduced:oxidized glutathione (GSH:GSSG) ratio (Figure 2a) [35]. Although direct oxidation of reduced glutathione by PYO has been suggested previously, recently this mechanism has been questioned [36, 37]. Not only cytosolic but also mitochondrial ROS production has been proposed to take place in PYO-exposed host cells [38]. PYO-mediated oxidative stress has several consequences that depend on toxin concentrations and exposure times. Several effects of PYO in different cell types (pulmonary epithelial cells, neutrophils, macrophages, lymphocytes, and endothelial cells) have been reported over more than two decades, which are summarized in several reviews [9, 22, 23, 39]. In this review we focus on novel findings of PYO's action in a tissue that previously has been neglected, but plays a crucial role in CF airway pathogenesis: the respiratory epithelium.

Interactions between PYO and the respiratory epithelium

The respiratory epithelium serves as a first line of defense against inhaled bacteria, allergens and pollutants and has a crucial role in the initiation of the immune response. Its major innate immune functions are: (i) removal of inhaled bacteria by ATP-driven, synchronized ciliary motion, (ii) mucus production to entrap pathogens, (iii) secretion of antibacterial peptides and reactive oxygen species, (iv) initiation of wound healing in the case of epithelial injury and (v) secretion of cytokines and chemokines to alert the immune system [40]. The cystic fibrosis transmembrane conductance regulator (CFTR) anion channel is expressed in the tracheobronchial epithelium in the airways [3]. Since CFTR malfunction resulting from a variety of genetic mutations leads to CF, the tracheobronchial epithelium is the primary origin of CF airway disease. Despite several existing theories, the exact mechanism of how PA colonizes the CF airways and establishes chronic infection is still not clear, although numerous observations suggest PYO plays an important role in the process by imposing oxidative stress on the respiratory epithelium. A current model on how PYO alters the function of the airway epithelium is summarized in Figure 3. Early reports showed that PYO inhibits ciliary beat frequency in ciliated airway epithelial cells and thereby could inhibit clearance of PA [41, 42]. PYO also inhibits the $\alpha 1$ protease inhibitor which can intensify neutrophil elastase-mediated tissue injury [42]. Inhibition of the vacuolar ATP-ase by PYO—most probably through decreased ATP levels—leads to altered localization of CFTR [43]. The primary product in PYO's reaction with NAD(P)H is superoxide, which dismutates quickly to hydrogen peroxide (H_2O_2) (Figure 2a). Catalase is an enzyme responsible for breaking down hydrogen peroxide produced inside of the cell and thereby protects the cell against oxidative stress. PYO inhibits catalase activity in airway epithelial cells [44] and impairs CFTR function as well [35].

Duox1 and Duox2 are NADPH oxidases releasing hydrogen peroxide into the airway surface liquid and are highly expressed in ciliated cells of the respiratory epithelium [45, 46]. The *in vivo* role of Duox enzymes in the airways remains unclear, although several functions were proposed based upon *in vitro* studies (bacterial killing, bacterial detection, mucin production, and cytokine secretion) [12, 45, 47–49]. Numerous reports have shown that the Duox-based antimicrobial system of respiratory epithelial cells is capable of killing several bacterial CF pathogens *in vitro* (PA, *Burkholderia cepacia*, and *Haemophilus influenzae*) [12, 46, 50]. According to this model, Duox-derived intraluminal H_2O_2 is used by the predominant airway peroxidase, lactoperoxidase (LPO), to oxidize its favored substrate, thiocyanate (SCN^-), into the antimicrobial hypothiocyanite ($OSCN^-$) [45]. Low concentrations of PYO inhibit Duox activation and prevent killing of PA by the Duox-based antibacterial system (Figure 3) [12]. As a counteroffensive mechanism, airway peroxidases [LPO and myeloperoxidase (MPO)] were suggested to inactivate PYO using H_2O_2 and would protect airway cells against PYO toxicity (Figure 3) [12].

In summary, PYO causes several proinflammatory changes in respiratory epithelial cells that are all characteristic of chronic PA airway infections and appear to be consequences of exposure of host cells to oxidative stress. The host epithelium has its own redox-based counteroffensive capabilities involving Duox and LPO that are directed against PA and its toxin, PYO, in the airway surface layer.

Changes in epithelial gene expressions due to PYO exposure

The above effects of PYO are manifested within the first seconds or minutes of exposure, thereby altering redox signaling and energy metabolism. Long-term consequences of PYO occurring after hours or even a few days of exposure include changes in expression of several genes in tracheobronchial cells that lead to modulation of the inflammatory response

and fate of the airway epithelium [51]. Several of the genes induced by PYO are regulated by nuclear factor- κ B (NF- κ B), and indeed NF- κ B activation by PYO has been described in airway cells [52]. ROS are known to activate this transcription factor, although ROS-independent NF- κ B activation by PYO has also been suggested. [53] Inhibition of catalase and induction of interleukin-8 (IL-8) and intercellular adhesion molecule 1 (ICAM1) expression has already been reported but recently numerous genes were shown to be induced by PYO in respiratory epithelial cells [54–56]. Besides the immediate inhibition of Duox activation, PYO was also shown to prevent induction of Duox expression by cytokines, preventing PA killing [12].

Epithelial induction of several novel genes by PYO was reported in a recent comprehensive study (Figure 3) [51]. One group of induced genes encodes proinflammatory mediators that recruit and activate neutrophils (Figure 3) [51]. Neutrophils are the first responders to alarm signals released by airway epithelial cells, as their major function is to migrate to the site of infection and fight bacteria. The subsequent uncontrolled neutrophil infiltration and activation leads to tissue damage [57]. In CF, abnormal neutrophil recruitment and activation are hallmarks of the disease as activated neutrophils are thought to be major contributors to the respiratory clinical symptoms of these patients [58]. IL-8 is a very potent chemokine capable of recruiting neutrophils; it has been associated with CF and shown to be released by airway epithelial cells exposed to PYO [55, 59–63]. In addition, several other neutrophil chemoattractants were identified recently that are also released by tracheobronchial epithelial cells exposed to PYO (Figure 3) [51]. CXCL1, CXCL2, CXCL3 chemokines are potent recruiters of neutrophils, are all produced by airway epithelial cells and show induced expression upon PYO exposure [51]. Furthermore, CXCL1 has been associated with CF [63].

The proinflammatory cytokines tumor necrosis factor- α (TNF- α), IL-1 β , G-CSF, and GM-CSF are also induced and released from the respiratory epithelium by PYO [51]. Increased TNF- α levels have been reported in CF and its concentration in CF sputum has a negative correlation with pulmonary function in CF patients [61, 63–65]. G-CSF and GM-CSF are both expressed in the respiratory epithelium, are potent neutrophil chemoattractants and priming agents, and their gene expression is induced by PYO [51]. G-CSF levels are higher in sera of CF patients with PA infection than those without [60]. PYO also increases release of IL-1 β , a neutrophil priming cytokine associated with CF [51, 61, 63, 64]. Enhanced expression of the lipoxygenase-coding gene, ALOX5, by PYO can contribute to increased leukotriene B4 (LTB4) release and recruitment of neutrophils [51, 62, 66, 67]. In addition to these proinflammatory cytokines several other cytokines are induced in airway epithelial cells by PYO including: IL-6, IL-11, IL-19, IL-20, IL-23, and IL-24 [51]. IL-23 has been associated with CF and was reported to play an important role in PA infection in mice [68]. The robust induction of IL-24 by PYO is impressive and awaits further studies for pathophysiological correlations [51]. PYO induces fivefold increases in release of IL-6, a cytokine that has both anti- and proinflammatory properties and has been associated with CF [51, 63–66].

These novel findings indicate that gene expression changes caused by oxidative stress underlie several of the proinflammatory effects of PYO on epithelial cells although the detailed mechanisms involved await further investigation.

PYO induces mucin production

Along with its proinflammatory effects, PYO exposure of the epithelium also leads to another characteristic feature of CF lung disease: mucin hypersecretion [51]. Mucins are giant glycoproteins secreted by the airway mucosa that form a layer on top of the epithelium

and entrap inhaled bacteria and debris [69]. Pathological conditions such as CF, however, lead to uncontrolled overproduction of mucus, which is no longer beneficial for the host as it restricts normal airway clearance, obstructs the airways and promotes bacterial attachment [3]. There are 20 mucin genes in humans, 12 of which are expressed in the airway epithelium [70]. PYO significantly increases epithelial expression of five mucin genes (MUC5B, MUC20, MUC13, MUC2 and MUC5AC) [51]. The three most important mucins secreted in the human airways are MUC2, MUC5AC and MUC5B [70], and the most robust induction by PYO was observed in the case of MUC2 and MUC5AC [51]. PYO-triggered induction of both MUC2 and MUC5AC involves oxidative stress [51]. Induction of MUC5AC in the airway epithelium has already been reported by different stimuli causing oxidative stress, but its induction by PYO has never been reported [71, 72] and oxidative stress-induced increases in expression of MUC2 is entirely novel [51]. PYO-mediated induction of MUC5AC requires the EGFR signaling pathway [51]. PYO also induces MUC5B expression in cell lines and murine lung by inactivating the transcription factor FOXA2.[24]

How does PYO-triggered oxidative stress activate EGFR in the airway epithelium? Three mechanisms have been proposed. First, direct ROS-dependent activation of EGFR was already known and involves the MEK1/2 and ERK kinases [73]. Second, several proinflammatory cytokines released by PYO are known to be potent activators of EGFR and inducers of mucin secretion including: IL-1 β , IL-6, and TNF- α [69]. Third, two EGFR ligands (TGF- α and HB-EGF) appear to be induced by PYO [51]. Therefore, a complex network of autocrine and paracrine signals mediate PYO-induced mucin secretion in respiratory epithelial cells (Figure 3) [51]. This fits the current *in vivo* two-signal model of transdifferentiation of ciliated airway epithelial cells into mucin-producing Goblet cells: the first signal is activation of EGFR signaling to promote cell proliferation followed by the second signal, the Th2 cytokine IL-13, which induces differentiation into Goblet cells [74]. Pyocyanin activates EGFR signaling in airway epithelial cells promoting proliferation which—upon the secondary Th2 cytokine signal—leads to mucin production (Figure 3).

Concluding remarks

PA is an opportunistic pathogen causing infections in patients whose immune system is compromised. In CF, PA is the main respiratory pathogen establishing chronic infections, resulting in death due to pulmonary complications. Thanks to extensive basic and clinical research, the details of PA lung infections are better understood and the median predicted age of survival of CF patients has been significantly extended in recent years. However, we face several challenges in the future since the disease is not controlled and the bacterium develops resistance against most of the antibiotics used. Further research has to uncover how PA establishes infection in the CF lung. Virulence factors including PYO are crucial in this process, but their exact contribution is still unclear and needs to be studied in more detail (Box 1). Although PYO has been recognized as crucial in different animal models of PA infections, the studies model acute, rather than chronic PA infections, as seen in CF. Current data suggest that PYO is important in PA virulence to establish its presence in CF airways and PYO contributes to poor lung function. Progress in the past five years in PYO research found that PYO is not only a bacterial pigment but has signaling functions in the bacterium affecting colony morphology. Moreover, this recent progress has shown that PYO alarms innate immune cells in several, previously unknown ways. Chronic PYO instillation into mouse lungs or exposure to respiratory epithelial cells alone recapitulates most of the features of CF lung disease and presence of PYO in CF lung is associated with poor disease outcome. Future research is required to understand the mode of action of PYO to target it as a novel intervention point in PA infection as the clinical relevance of PYO is gaining broad recognition

Box 1**Outstanding questions**

- How do PYO concentrations in CF airways correlate with levels of inflammatory markers (cytokines and mucins)?
- What molecular mechanisms govern ROS-mediated gene expression changes induced by PYO?
- Are there any ROS-independent mechanisms mediating PYO's action?
- What are potential targets in the PYO biosynthetic pathway that could be exploited to develop new drugs that improve airway function of PA-infected individuals?

Acknowledgments

The authors wish to thank those who published data cited in this review contributing to a better understanding of pyocyanin immunomodulatory actions and CF disease mechanisms. Data presented in this review were generated with the support of the Intramural Research Program of the NIH, National Institute of Allergy and Infectious Diseases (ZO1-AI-000614).

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Highlights

- Pyocyanin(PYO) is a redox-active virulence factor of *Pseudomonas aeruginosa*.
- PYO promotes inflammatory responses by imposing oxidative stress on host cells.
- PYO is required for full virulence in several models of *P. aeruginosa* infection.
- PYO levels were correlated with declining lung function in cystic fibrosis.

Figure 1. Pyocyanin (PYO) is a redox-active exotoxin of *Pseudomonas aeruginosa*

(a) Depicted are chemical structures of chorismic acid, the starting point for constructing the phenazine aromatic frame, and two precursors involved in the final steps of PYO biosynthesis. (b) PYO is produced by stationary phase cultures of *P. aeruginosa* PAO1. The PhzM-deficient *P. aeruginosa* PA14 strain fails to turn the medium dark and produce pyocyanin. PYO can be purified from stationary phase *Pseudomonas* cultures by repeated chloroform–distilled water extraction cycles. (c) The absorption spectrum of PYO is pH-dependent. At low pH values PYO solutions are red, whereas at higher pH values it turns blue. (d) Well-oxygenated stationary phase cultures of PA14 are dark green due to high oxidized PYO concentrations (left). In standing cultures the medium loses its dark color as reduced PYO accumulates over time (3–20 min). Upon reoxygenation oxidized PYO turns the culture medium green again (right, 20 min). (e) Standing cultures (anaerobic conditions) show a characteristic green-yellow gradient near the air-medium surface because PYO shuffles electrons between oxygen-poor parts of the culture and the air surface.

Figure 2. Pyocyanin (PYO) exposes host cells to oxidative stress

(a) PYO directly oxidizes reduced NAD(P)H in the host cell cytoplasm and donates accepted electrons to oxygen molecules to produce superoxide anions and downstream reactive oxygen species (ROS) (GSH/GSSG: reduced:oxidized glutathione ratio). In a cell-free system NADPH consumption (b) and superoxide production (c) is enhanced by increasing concentrations (0–100 μM) of PYO. NADPH consumption detected by 260 nm absorbance changes; superoxide release detected by Diogenes luminescence. (d) Oxidative stress in bronchial epithelial (H292) cells exposed to PYO (20 μM, 60 min) is visualized by increased intracellular fluorescence of the ROS-sensitive fluorescence dye dihydrorhodamine 123.

Figure 3. Mechanistic model of the proinflammatory action of pyocyanin (PYO) in the respiratory epithelium

PYO produced by *Pseudomonas aeruginosa* enters the cytosol of airway epithelial cells and produces reactive oxygen species (ROS) by oxidizing its intracellular NADPH pool. Primary effects resulting from this are diminished reduced glutathione (GSH) and ATP levels, increased cytosolic redox potential (E_{redox}) and intracellular oxidative stress. Reduced GSH levels affect energy metabolism of the cells and inhibit ciliary beat frequency, V-ATPase activity and CFTR channel functions. Reduced NADPH levels inhibit antibacterial functions (bacterial killing) and PYO inactivation (mediated by peroxidases) due to reduced activity of epithelial NADPH oxidases, Duox1 and Duox2. Long-term effects of PYO manifest in transcriptional changes. PYO inhibits gene expression of the antioxidant and antimicrobial genes catalase and Duox. Several genes are induced in response to PYO including: oxidative stress-responsive genes, mucins, inflammatory cytokines and chemokines. The inflammatory changes evoked by PYO can be grouped into two major effects. First, PYO induces several genes mediating neutrophil recruitment to the airways. Second, PYO also induces mucin production in the airway epithelium mainly through activation of the EGFR signaling pathway. Abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator; CXCL, chemokine (C-X-C motif) ligand; Duox, dual oxidase; E_{redox} , redox potential; EGFR, epidermal growth factor receptor; IL, interleukin; LPO, lactoperoxidase; MPO, myeloperoxidase; GSH, reduced glutathione; GSSG, oxidized glutathione.

Table 1

Overview of PYO production in PA-infected human airways

Type of study	Observation ^a	Refs.
Direct measurement of PYO in airway secretions	PYO is detected in sputa of most of the bronchiectasis patients tested: 0, 0, 0.2, 0.25, 1.7, 2.5, 19.0, 27.3 µg/mL	26
	Varying PYO levels are found in 4 CF patients: 0, 0.9, 1.5 (sputum) and 16.5 µg/mL (large airway aspirate)	26
	PYO concentrations in expectorated sputum samples of 47 CF patients negatively correlate with lung function: normal (average PYO 7.7 µM), mild (10.5 µM), moderate (25.2 µM) and severe (46.8 µM)	27
<i>In vitro</i> production of PYO by clinical isolates	A majority of 12 CF clinical isolates of PA overproduce PYO <i>in vitro</i>	28
	Isolates of the hypervirulent LES PA strain overproduce PYO	30
	Most of CF clinical isolates of PA secrete PYO	27
Correlation of PYO production with CF disease markers	PYO overproduction is correlated with periods of pulmonary exacerbations in CF patients with LES PA strain	31
	High BPI-ANCA levels are associated with PYO-negative CF clinical isolates of PA	32
	PYO levels negatively correlate with complexity of microbial populations in CF sputum samples	27
	Normalized PYO concentrations (<i>in vitro</i> production by clinical isolates multiplied by total PA load in sputum) correlate with disease severity	27
Screening for changes in genes affecting PYO production and secretion	The <i>pi/YI</i> gene associated with pyocyanin secretion was shown to acquire pathoadaptive mutations in a series of longitudinal CF clinical isolates of PA	25

^a Abbreviations: BPI-ANCA, anti-neutrophil cytoplasmic antibody produced against the bactericidal/permeability increasing protein; LES, Liverpool epidemic PilY1 strain.