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Review

Multidrug resistance in *Pseudomonas aeruginosa*: Insights into the molecular mechanisms

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***Pseudomonas aeruginosa* is commonly found in chronic lung infections such as cystic fibrosis and others. Intrinsic antibiotic resistance of *P. aeruginosa* accompanied by its ability to acquire resistance via mutations and adapt to the heterogeneous and dynamic environment of chronically infected lungs are major threats and reasons for the ultimate failure of the current antibiotic therapies in eradicating the infection from lungs. New insights at molecular levels in the process of accumulating such beneficial mutations at faster rates, termed as hypermutation have allowed us to understand the high acquired resistance of this opportunistic pathogen. Also, these understandings will allow us to develop new therapeutic strategies to combat chronic infections.**

Key words: *Pseudomonas aeruginosa*, chronic lung infections, multi drug resistance, hypermutation.

INTRODUCTION

It is virtually impossible to completely eradicate a chronic *Pseudomonas aeruginosa* respiratory infection which is frequently observed to accompany respiratory diseases such as cystic fibrosis (Govan and Deretic, 1996; Lyczak et al., 2002; Gibson et al., 2003; Smith et al., 2006), bronchiectasis (Nicotra et al., 1995; Evans et al., 1996) and chronic obstructive pulmonary disease (COPD) (Hill et al., 2000). Dual antibiotic therapy usually with a -lactam and an aminoglycoside is given to patients to control infections and maintain lung functions (Høiby, 2002). However, years of intensive antibiotic therapy to control the negative outcomes of the chronic colonization of patients results in the development of drug resistance in the infecting bacterial population and ultimately leads to treatment failure (Fish et al., 1995; Carmeli et al., 1999; Gibson et al., 2003; Juan et al., 2005). This happens due to simultaneous or sequential selection of mutant alleles that give birth to antibiotic resistant decedents of sensitive parents and this is a critical factor in the management of chronic infections in cystic fibrosis CF) patients. Ability of *P. aeruginosa* to escape the effects of antibiotics is attributed by its capability of growing in microaerophilic environment (Hassett et al., 2002; Worlitzsch et al., 2002; Hill et al., 2005) as biofilms (Høiby, 2002; Moskowitz et al., 2004; Hill et al., 2005) both of which reduce the efficiency of many antibiotics. Resistance towards antimicrobial agents to survive in the lungs during frequent and prolonged treatments also requires great adaptive skills. Major concern however, in case of *P. aeruginosa* is combination of its inherent resistance and ability to acquire resistance via mutations to all treatments leading to increasing occurrence of multi drug resistant strains (Livermore, 2002; Aloush et al., 2006; Henrichfreise et al., 2007b). Our worst nightmare may even be more dreadful, with the occurrence of “panresistant” strains that arise due to the accumulation of multiple mechanisms of antibiotic resistance and are resistant to all antibiotics except polymyxins (Lolans et al., 2005; Bonomo and Szabo, 2006).

Chronic infections and mutator *P. aeruginosa*

Though mutation frequency in microorganisms living in primary environments, that is, in free living populations is low due to DNA damage repair systems such as methyl directed mismatch repair (MMR), mutations are one of the fastest routes for evolution of adaptive responses in them in secondary environments. It has already been shown that mutant alleles which are the genotypically

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different forms of alleles that code for a phenotype different than that coded by the wild type alleles, get selected through selection of mutants when put under environmental stresses that act as selective pressure. High rate of spontaneous mutations in secondary environments generate more number of progenies with novel mutations and hence evolve novel mechanisms of adaptations that offer survival advantages mutants (Giraud et al., 2001). Oliver et al., had shown presence of high number of mutator *P. aeruginosa* in lungs of cystic fibrosis patients. These hypermutable or the mutator *P. aeruginosa* have high rate of spontaneous mutations. They had studied 128 *P. aeruginosa* isolates from 30 CF patients which revealed that 36% of the patients were colonized by a persistent mutator strain for years. Mutator strains were not found from any of 75 patients acutely infected with *P. aeruginosa* (Oliver et al., 2000). Ciofu et al., have shown association between oxidative stress due to lung inflamemation in cystic fibrosis patients and occurrence of mutator *P. aeruginosa*. In their study, occurrence of mutator *P. aeruginosa* was investigated directly in the sputum of 79 CF patients by exposing the isolated cells to rifampin and streptomycin and determination of rate of development of resistance after exposure. Dynamics of hypermutable strains were also studied in 141 isolates collected from the 1st and up to the 25th year of chronic lung infection from 11 patients. Mutator *P. aeruginosa* were found in 54.4% of the CF patients which were significantly more resistant to antipseudomonal antibiotics than nonmutator isolates. The level of 8-oxodG was significantly higher in mutator *P. aeruginosa* than in nonmutators and an increase in 8-oxodG was found after exposure of the reference strain PAO1 to activated polymorphonuclear leukocytes (PMNs) showing that there was DNA damage resulting in high rate of spontaneous mutation (Ciofu et al., 2005). Macia´ et al. investigated 62 *P. aeruginosa* isolates for their antibiotic susceptibilities and mutation frequencies from 30 patients with underlying non -CF chronic respiratory diseases of which, 30 (53%) of the isolates were mutator and 17 (57%) of the 30 patients were colonized by mutator strains. When complementtation assays using the cloned wild type *mutS* gene were done, strains from 11 of 17 patients were found to be defective in the MMR *mutS* gene (Macia´ et al., 2005).

Mutations and acquired resistance

Development of acquired resistance to antibiotics of different classes during antimicrobial therapy, which is rarely observed in acute infections, is a marked feature of chronic *P. aeruginosa* infections (Govan and Deretic, 1996; Lyczak et al., 2002; Gibson et al., 2003). Mutations in genes that code for topoisomerases II and IV confer fluoroquinolone resistance, derepression of the chromosomal AmpC cephalosporinase, the up-regulation of MexAB-OprM and efflux systems MexCD-OprJ, MexEF-OprN and MexXY-OprM confer fluoroquinolone resistance, some -lactams and aminoglycosides (Bonomo and Szabo, 2006; Henrichfreise et al., 2007b). The com-mon means by which *P. aeruginosa* isolates become imipenem resistant is via mutational loss of a 54-kDa protein known as OprD. Loss of OprD production is due to inactivation of the OprD gene (Ochs et al., 1999; Bonomo and Szabo, 2006; Henrichfreise et al., 2007a, b). In the lungs of cystic fibrosis patients high oxidative stress introduces lesions in the DNA of *P. aeruginosa* that lead to mutations in DNA after replication. If such a mutation results in the malfunctioning of DNA damage repair system, the rate of mutation increases giving rise to mutator strains. Consequently many genes start to accumulate mutations some of which confer properties of antibiotic resistance. Most importantly however, antibiotic therapy imposes the selective pressure for which mutants are permanently adapted and get selected. Such mutants may have similar or different mechanisms of resistance and start accumulating at the site of infection, most common of which is lungs (Juan et al., 2005; Henrichfreise et al., 2007a, b).

Hypermutation and drug resistance

In the last few years, several workers have investigated the relationship between hypermutation, which is high rate of spontaneous mutation in a bacterial cell and development of antimicrobial resistance. Based on the observations, a general hypothesis was then postulated that hypermutation is an important factor contributing to the development of antimicrobial resistance in bacteria (Oliver et al., 2000; Bla´zquez, 2003; Gutie´rrez et al., 2004; Ciofu et al., 2005; Macia´ et al., 2005; 2006; Plasencia et al., 2007; Henrichfreise et al., 2007a, b). Oliver et al. (2000) and Macia´ et al. (2005) showed a correlation between hypermutation and multiple antibiotic resistance of *P. aeruginosa* in CF patients (Oliver et al., 2000) and in other chronic conditions like bronchiectasis and COPD where multiple antibiotic resistance. In these studies multiple antibiotic resistance was documented in 42% of the mutator strains in contrast to 0% in the nonmutator strains. When resistance % of mutator and nonmutator strains were analyzed independently, it was found that not only mutator strains were much more resistant to all the antibiotics but also that most of the strains resistant to any of the antibiotics and all the strains that were resistant to multiple antibiotics were mutator (Macia´ et al., 2005). In another study, development of resistance to ceftazidime (CAZ) alone or in combination with tobramycin (TOB) or ciprofloxacin (CIP) was investigated *in vitro* and *in vivo*. Here human antibiotic regimens were followed in a mouse model of lung infection of *P. aeruginosa* PAO1 and its hypermutable derivative PAO1 *mutS* (Plasencia et al., 2007). It was found out that resistant mutants got selected from PAO1 *mutS* strain in very short period that were resistant to all 3 antibiotics tested singly or in combination of 2. Another

important observation was that 4 of the isolates that showed resistance to CAZ and CIP did not show over expression of MexAB-OprM efflux pump which is one of the well known mechanisms for development of high resistance to CAZ and CIP (Masuda et al., 2000). Thus most probable mechanism for resistance development was sequential accumulation of 2 mutations which independently conferred resistance to CAZ and CIP (Plasencia et al., 2007) . In a recent investigation, same studies were performed with meropenem and ceftazidime antibiotics and development of mutation- mediated resistance in wild-type (PAO1) and mutator *P. aeruginosa* strains were studied *in vitro* in monotherapy. In case of PAO1, mutants resistant to meropenem and ceftazidime were not detectable during first dose but upon successive addition of second and third doses of both meropenem and ceftazidime, resistant mutants got selected, started to dominate the population and completely replaced the initial susceptible population at the end of the experiment. For mutator strain, same experiment could be carried out only with meropenem where resistant mutants only partially replaced the initial population. Sequencing of oprD showed that resistant mutant of PAO1 had frameshift mutations and did not produce OprD. Resistant mutator also did not produce OprD but the underlying reason could not be found out (Henrichfreise et al., 2007a) . In an another study carried out by Henrichfreise et al., correlation between the genetic resistance determinants, the respective resistance phenotypes and its relation to resistance mechanisms of clinical multi drug resistant *P. aeruginosa* strains from CF patients and non-CF patients was investigated (Henrichfreise et al., 2007b). 11 out of 12 multi drug resistant strains from CF patients were mutator and 3 mutator strains were also found among the multi drug resistant strains isolated from non-CF patients. Acquired resistance genes were detected in only 5 multi drug resistant *P. aeruginosa* strains, all from non-CF patients. A high proportion of resistance mechanisms detected in the multi drug resistant *P. aeruginosa* strains were mutation mediated which included modifications in the genes AyrA and P*arC* (91%) coding for the quinolone target enzyme DNA gyrase and topoisomerase IV, functionally relevant modifications in genes of repressors and activators of efflux pumps with the most frequent target being repressor gene M*exZ* leading to MexXY-OprM overproduction (82%) that confers resistance to aminoglycosides and quinolones and loss of OprD due to premature stop codons or frameshifts leading to meropenem resistance (82%) (Henrichfreise et al., 2007b). Absence of acquired resistance genes in multi drug resistant strains from CF patients emphasizes the relevance of the mutator phenotype for the autarkic evolution of multi drug resistant *P. aeruginosa* strains during chronic infection of the lungs of CF patients. This study proved the hypothesis that hypermutation is a key factor in development of mutation mediated multi drug resistance in patients with chronic *P. aeruginosa* lung infections (Oliver et al., 2004; Macia´ et al., 2005; Henrichfreise et al., 2007b).

How mutator strains arise from wild type strains

Genotypic characterization and complementation studies have shown that mutator strains contain deletion and frameshift mutations in *mutS, mutL* and *mutY* genes whose products are involved in DNA recombination and mismatch repair system. These mutations increase the rate of spontaneous mutations in bacteria (Oliver et al., 2000; Macia´ et al., 2005; Henrichfreise et al., 2007a). Ciofu et al. have suggested a mechanism for occurrence of hypermutable *P. aeruginosa* in lungs of CF patients, in which chronic PMN inflammation in the CF lung promotes oxidative stress that causes DNA damage which in turn gives rise to mutator bacteria in the lungs (Ciofu et al., 2005). Though at low frequencies, mutator strains of *P. aeruginosa* have been isolated from non CF patients and from sites other than lungs such as wounds (Henrichfreise et al., 2007b), therefore mechanism suggested by Ciofu et al (2005) cannot be applied for such instances. *In vitro* studies and analytical models have suggested that in asexually reproducing organisms such as bacteria, individuals with higher mutation rate will enjoy longer life due to the savings in energy and time by not replicating their DNA accurately. Additionally these mutations can provide potential benefits under heterogeneous environments depending on the selective pressures where directional selection is favored. When adaptation has a significant role, it primarily destabilizes mutation rate and yields the emergence of strong effect mutators. However, in small populations, even if adaptations are needed, mutation rate is always blocked at the minimum attainable level, because the rate of adaptation is too slow to play a significant role. Only populations whose size is above a critical mass see their mutation rate affected by the need for adaptation (Andre´ and Godelle, 2006). These results can be correlated with the pattern of appearance of mutators in the chronic infections. In most of the cases mutators have been shown to emerge after few days or weeks of starting of an antibiotic therapy, which acts as a selective pressure. It is the heterogeneous environment of lungs that puts large demand for adaptations and has the major contribution in the emergence of mutators at very high levels from normal population (Andre´ and Godelle, 2006; Smith et al., 2006; Reinhardt et al., 2007). Recent studies have shown that certain antibiotics induce SOS response in bacteria and the changes that take place in the cellular metabolism may also be responsible for emergence of mutators. During SOS response against antibiotic ciprofloxacin, *P. aeruginosa* showed large alterations in the expression of genes related to cellular metabolism and synthesis. However induced expression of error prone polymerases was observed that have been shown to be involved in damage induced mutagenesis in *Caulobacter*

cresentus . Thus it may be possible that induction of SOS response increases mutation rate so that bacterial cell is able to adapt to the stress and the selective pressure. Also down regulation of most of metabolic activity, provides bacteria with more time to adapt to the environment (Cirz et al., 2006). To date only one study has reported the changes in the genome of *P. aeruginosa* that take place during chronic infections by whole genome sequencing of a 6 month old colony purified clinical isolate and its comparable 96th month old isolate, comparing them with 2 publicly available *P. aeruginosa* genome sequences that of PAO1 and PA14 as references. Single nucleotide differences within coding regions between the 2 reference strains displayed a nonsynonymous to-synonymous ratio only of 0.1 only. Mutations in regulatory and protein coding regions of many virulence factors and multidrug efflux pump genes were found with two-thirds of these predicted to effect the protein function and a ratio of nonsynonymous to synonymous mutations was as high as 1.4 which was only 0.1 between the 2 refe-rence strains, showing that most of the mutations were such that expressions and/or functions of the mutant proteins were altered. Upon sequencing of many other isolates from different patients, it was observed that most frequent mutations were in *lasR* and in *mexZ* genes which respectively control the expression of Quorum sensing and multidrug efflux pump MexXY-OprM in *P. aeruginosa.* Also in large number of isolates, nonsynonymous mutations were found in *mutS* gene which increased the frequency of mutations and resulted in generation of mutator strain (Smith et al., 2006). The observations documented in current literature (Oliver et al., 2000; Ciofu et al., 2005; Hill et al. , 2005) when compared and correlated with changes at the genetic level, it is clearly visible that *P. aeruginosa* adapts to the conditions in the lungs of chronically infected patients and major part of this adaptation comes from the mutations (Macia´ et al., 2005; Henrichfreise et al., 2007b) that take place in the genome of bacteria inside the lungs of the patients only (Smith et al., 2006). However the important role played by the selective pressure should not be neglected as it this condition which selects only those cells who have adapted to the conditions. This sequential continuous process of mutation and selection gives rise to the highly adapted strains so much so that sometimes the ability to survive in primary environment such as soil or distilled water is lost (Hogardt et al., 2007).

Conclusion

Occurrence of multidrug resistant (MDR) *P. aeruginosa* from chronically infected patients has been a major reason for ultimate failure of antibiotic treatments. Early studies had shown that it is the environment in the lungs and the virulence factors that are the major reason for the MDR nature of this opportunistic pathogen. However recent investigations have proved that it is the ability of increasing the rate of mutations that allow this organism to adapt to the heterogeneous and dynamic atmosphere of the lungs. These insights into the survival strategy of this organism will open ways to newer targets that are susceptible to new methods and allow us to tackle these infections.

REFERENCES

Aloush V, Navon-Venezia S, Seigman-Igra Y, Cabili S, Carmeli Y (2006). Multidrug-resistant *Pseudomonas aeruginosa*: risk factors and clinical impact. Antimicrob. Agents Chemother. 50(1):43-48.

Andre´ JB, Godelle B (2006). The evolution of mutation rate in finite asexual populations. Genetics 172: 611–626.

Bla´zquez J (2003). Hypermutation as a factor contributing to the acquisition of antimicrobial resistance. Clin. Infect. Dis. 37: 1201–1209.

Bonomo RA, Szabo D (2006). Mechanisms of multidrug resistance in *Acinetobacter* species and *Pseudomonas aeruginosa*. Clin. Infect. Dis. 43: 49–56.

Carmeli Y, Troillet N, Eliopoulos GM, Samore MH (1999). Emergence of antibiotic-resistant *Pseudomonas aeruginosa*: comparison of risks associated with different antipseudomonal agents. Antimicrob. Agents Chemother. 43: 1379-1382.

Ciofu O, Riis B, Pressler T, Poulsen HE and Høiby N (2005). Occurrence of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis patients is associated with the oxidative stress caused by chronic lung inflammation. Antimicrob. Agents Chemother. 49: 2276–2282.

Cirz RT, O'Neill BM, Hammond JA, Head SR, Romesberg FE (2006). Defining the *Pseudomonas aeruginosa* SOS response and its role in the global response to the antibiotic ciprofloxacin. J. Bacteriol. 188: 7101-7110.

Evans SA, Turner SM, Bosch BJ, Hardy CC, Woodhead MA (1996). Lung function in bronchiectasis: the influence of *Pseudomonas aeruginosa*. Eur. Respir. J. 9: 1601-1604.

Fish DN, Piscitelli SC, Danziger LH (1995). Development of resistance during antimicrobial therapy: a review of antibiotic classes and patient characteristics in 173 studies. Pharmacother. 15: 279-291.

Gibson RL, Burns JL, Ramsey BW (2003). Pathophysiology and management of pulmonary infections in cystic fibrosis. Am. J. Respir. Crit. Care Med.168: 18–951.

Giraud A, Matic I, Tenaillon O, Clara A, Radman M, Fons M, Taddei F (2001). Costs and benefits of high mutation rates: adaptive evolution of bacteria in the mouse gut. Science, 291: 2606-2608.

Govan JRW, Deretic V (1996). Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. Microbiol. Rev. 60: 539–574.

Gutiérrez O, Juan C, Pérez JL, Oliver A (2004). Lack of association between hypermutation and antibiotic resistance development in *Pseudomonas aeruginosa* isolates from intensive care unit patients. Antimicrob. Agents Chemother. 48: 3573-3575.

Hall LMC, Henderson-Begg SK (2006). Hypermutable bacteria isolated from humans – a critical analysis. Microbiology, 152: 2505–2514.

Hassett DJ, Cuppoletti J, Trapnell B, Lymar SV, Rowe JJ, Yoon SS, Hilliard GM, Parvatiyar K, Kaman MC, Wozniak DJ, Hwanga SH, McDermottg TR, Ochsnerh UA (2002). Anaerobic metabolism and quorum sensing by *Pseudomonas aeruginosa* biofilms in chronically infected cystic fibrosis airways: rethinking antibiotic treatment strategies and drug targets. Adv. Drug Deliv. Rev. 54: 1425-1443.

Henrichfreise B, Wiegand I, Luhmer-Becker I, Wiedemann B (2007a). Development of resistance in wild-type and hypermutable *Pseudomonas aeruginosa* strains exposed to clinical pharmacokinetic profiles of meropenem and ceftazidime simulated *in vitro*. Antimicrob. Agents Chemother. 51: 3642-3649

Henrichfreise B, Wiegand I, Pfister W, Wiedemann B (2007b). Resistance mechanisms of multiresistant *Pseudomonas aeruginosa* strains from Germany and correlation with hypermutation. Antimicrob. Agents Chemother. 51: 4062–4070.

Hill AT, Campbell EJ, Hill SL, Bayley DL, Stockley RA (2000). Association between airway bacterial load and markers of airway

inflammation in patients with stable chronic bronchitis. Am. J. Med. 109: 288-295.

Hill D, Rose B, Pajkos A, Robinson M, Bye P, Bell S, Elkins M, Thompson B, Macleod C, Aaron SD, Harbour C (2005). Antibiotic susceptabilities of *Pseudomonas aeruginosa* isolates derived from patients with cystic fibrosis under aerobic, anaerobic, and biofilm conditions. J. Clin. Microbiol. 43: 5085-5090.

Hogardt M, Hoboth C, Schmoldt S, Henke C, Bader L, Heesemann J (2007). Stage-specific adaptation of hypermutable *Pseudomonas aeruginosa* isolates during chronic pulmonary infection in patients with cystic fibrosis. J. Infect. Dis. 195: 70-80.

Høiby N (2002). New antimicrobials in the management of cystic fibrosis. J. Antimicrob. Chemother. 49: 235-238.

Juan C, Gutiérrez O, Oliver A, Ayestarán JI, Borrell N, Pérez JL (2005). Contribution of clonal dissemination and selection of mutants during therapy to *Pseudomonas aeruginosa* antimicrobial resistance in an intensive care unit setting. Clin. Microbiol. Infect. 11: 887-892

Livermore DM (2002). Multiple mechanisms of antimicrobial resistance in *Pseudomonas aeruginosa*: our worst nightmare? Clin. Infect. Dis. 34: 634–640.

Lolans K, Queenan AM, Bush K, Sahud A, Quinn JP (2005). First nosocomial outbreak of *Pseudomonas aeruginosa* producing an integron-borne metallo-beta-lactamase (VIM-2) in the United States. Antimicrob. Agents Chemother. 49: 3538-3540

Lyczak JB, Cannon CL, Pier GB (2002). Lung infections associated with cystic fibrosis. Clin. Microbiol. Rev. 15:194–222.

Macia´ MD, Blanquer D, Togores B, Sauleda J, Pe´rez J, Oliver A (2005). Hypermutation is a key factor in development of multiple-antimicrobial resistance in *Pseudomonas aeruginosa* strains causing chronic lung infections. Antimicrob. Agents Chemother. 49: 3382–3386.

Maciá MD, Borrell N, Segura M, Gómez C, Pérez JL, Oliver A (2006). Efficacy and potential for resistance selection of antipseudomonal treatments in a mouse model of lung infection by hypermutable *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 50: 975-983.

Masuda N, Sakagawa E, Ohya S, Gotoh N, Tsujimoto H, Nishino T (2000). Substrate specificities of MexAB-O172:611–626.prM,

MexCD-OprJ, and MexXY-oprM efflux pumps in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 44: 3322-3327.

Moskowitz SM, Foster JM, Emerson J, Burns JL (2004). Clinically feasible biofilm susceptibility assay for isolates of *Pseudomonas aeruginosa* from patients with cystic fibrosis. J. Clin. Microbiol. 42: 1915-1922.

Nicotra MB, Rivera M, Dale AM, Shepherd R, Carter R (1996). Clinical, pathophysiologic, and microbiologic characterization of bronchiectasis in an aging cohort. Chest 108: 955-961.

Ochs MM, McCusker MP, Bains M, Hancock RE (1999). Negative regulation of the *Ps*172:611–626.*eudomonas aeruginosa* outer membrane porin OprD selective for imipenem and basic amino acids. Antimicrob. Agents Chemother. 43:1085-1090.

Oliver A, Cantón R, Campo P, Baquero F, Blázquez J (2000). High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. Science 288(5469): 1251-1254.

Oliver A, Levin BR, Juan C, Baquero F, Blázquez J (2004). Hypermutation and the preexistence of antibiotic-resistant *Pseudomonas aeruginosa* mutants: implications for susceptibility testing and treatment of chronic infections. Antimicrob. Agents Chemother. 48: 4226-4233.

Plasencia V, Borrell N, Maciá MD, Moya B, Pérez JL, Oliver A (2007). Influence of high mutation rates on the mechanisms and dynamics of *in vitro* and *in vivo* resistance development to single or combined antipseudomonal agents. Antimicrob. Agents Chemother. 51: 2574–2581.

Reinhardt A, Köhler T, Wood P, Rohner P, Dumas JL, Ricou B, van Delden C (2007). Development and persistence of antimicrobial resistance in *Pseudomonas aeruginosa*: a longitudinal observation in mechanically ventilated patients. Antimicrob. Agents Chemother. 51: 1341-1350.

Smith EE, Buckley DG, Wu Z, Saenphimmachak C, Hoffman LR, D'Argenio DA, Miller SI, Ramsey BW, Speert DP, Moskowitz SM, Burns JL, Kaul R, Olson MV (2006). Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. Proc Natl Acad Sci U S A. 103: 8487-8492.

Worlitzsch D, Tarran R, Ulrich M, Schwab U, Cekici A, Meyer KC, Birrer P, Bellon G, Berger J, Weiss T, Botzenhart K, Yankaskas JR, Randell S, Boucher RC, Döring G (2002). Effects of reduced mucus oxygen concentration in airway *Pseudomonas* infections of cystic fibrosis patients. J. Clin. Invest. 109: 317-325.