



# *P. aeruginosa* in Cystic Fibrosis Patients Resists Host Defenses, Antibiotics

Over many generations, this pathogen produces variants that resist drugs, while adapting to host compartments and defenses

Niels Høiby

**C**ystic fibrosis (CF) patients have mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene that affect chloride channels, decreasing paraciliary fluid in the lower respiratory tract and impairing clearance there of inhaled microbes. This impairment leads to early recruitment of inflammatory defense elements, such as polymorphonuclear leukocytes (PMN) and antibodies.

CF patients suffer from chronic and recurrent respiratory tract infections, complicated by PMN inflammatory responses. In particular, persistent infections with *Pseudomonas aeruginosa* typically lead to lung failure, followed either by transplants or early death for CF patients (Fig. 1).

Adaptive mechanisms help to explain how *P.*

*aeruginosa* so effectively persists for several decades in the respiratory tracts of CF patients, overcoming host defense mechanisms as well as intensive antibiotic therapy. For example, consider one 42-year-old male CF patient with a chronic *P. aeruginosa* infection that lasted more than 28 years. This patient developed antibodies to this pathogen at the outset of the infection and was treated at regular intervals with 114 two-week courses of intravenous tobramycin and  $\beta$ -lactam antibiotics—and in the later stages, on a daily basis with nebulized colistin. Nonetheless, a mucoid *P. aeruginosa* strain persisted as a biofilm in his lungs, which were destroyed by his own activated PMNs.

## *P. aeruginosa* Survives by Adapting to Host Inflammatory Responses

*P. aeruginosa* adapts to the inflammatory defense system of CF patients by forming mucoid biofilms. For example, newly diagnosed CF infants with viral or bacterial lung infections carry significantly increased numbers of PMNs and alveolar macrophages compared to uninfected CF infants or children without CF. When PMNs phagocytose bacteria, the host cells produce highly reactive oxygen species (ROS) that kill or induce mutations in the bacteria and also damage surrounding host tissues.

We found that hydrogen peroxide by itself or activated PMNs can induce mutations in the *mucA* gene of *P. aeruginosa* strain PAO1 that confers the mucoid phenotype. Furthermore, 93% of mucoid *P. aeruginosa* from Scandinavian CF patients carry mutations in that gene, directing the bacteria to produce alginate, which is an

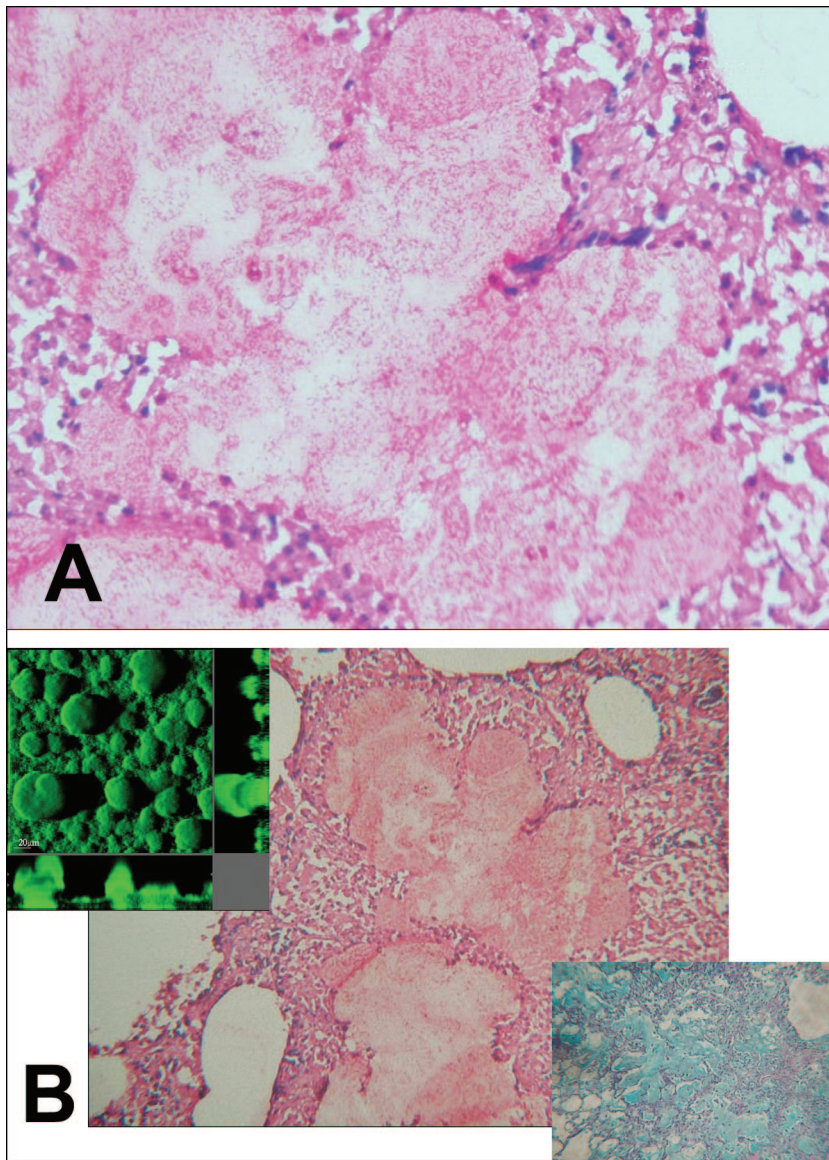
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### Summary

- Cystic fibrosis (CF) patients typically develop persistent *Pseudomonas aeruginosa* lung infections that lead to lung failure, attributable in part to chronic host inflammatory responses.
- *P. aeruginosa* adapts to the inflammatory defense system of CF patients and occupies specific anatomic niches, forming mucoid biofilms or microcolonies that reflect particular conditions.
- Although *P. aeruginosa* biofilms are the main reason for persistent infections in CF patients, conventional antibiotic-resistance mechanisms also play a role; the eventual appearance of mutator variants further helps to explain the high frequency of multiply antibiotic-resistant genes in strains infecting CF patients.



FIGURE 1



Cells from an autopsy of a Danish girl with CF who died due to chronic *P. aeruginosa* lung infection. She had 21 precipitating antibodies in serum against *P. aeruginosa*. (A) Hematoxylin-eosin stain; magnification, x100, showing mucoid biofilms of *P. aeruginosa* surrounded by pronounced inflammation in the lung tissue. (B) Comparison of *P. aeruginosa* growing as an in vitro biofilm in a flow cell (upper left), growing as chronic alginate biofilm in a CF mouse model (lower right; Hoffmann et al., 2005), and in a CF patient. Obvious morphological similarities are seen.

oxygen radical scavenger. The change also protects *P. aeruginosa* against phagocytosis and being cleared from host lungs. Thus, the mucoid phenotype helps to protect this pathogen against inflammatory defense mechanisms of the host.

Gram stains of mucoid *P. aeruginosa* from

sputum of CF patients show these bacteria to be biofilms (Fig. 2). When those biofilms are grown in flow-cells or animals, their resistance to PMNs and the antibiotic tobramycin depends on quorum sensing (QS)-regulated virulence factors. Thus, QS mutants or QS inhibitors (QSI) such as furanones, garlic extract, and ginseng render the biofilms susceptible to PMNs and sensitive to tobramycin.

Possible measures to prevent these effects include use of anti-inflammatory drugs or antioxidants to prevent ROS-induced mutations in the *mucA* gene, macrolides such as azithromycin to inhibit alginate synthesis, or QSIs to stop biofilms from forming. Currently, however, CF patients are treated early and aggressively in an effort to keep nonmucoid *P. aeruginosa* strains from colonizing their lungs. This approach, used in the Danish CF Centre since 1989, prevents about 80% of chronic *P. aeruginosa* lung infections in CF patients. However, once chronic biofilms are established, systemic and nebulized antibiotics are needed to maintain lung function.

### The Conductive and Respiratory Zones of Lungs

The humans lungs contain a smaller conductive zone and a larger respiratory zone (Fig. 3). The respiratory zone, which is about 3,000 ml and accounts for 95% of the lung volume, contains respiratory bronchioles and alveolar ducts and sacs. However, this part of the lungs contains no cilia and no submucosal glands. Its defense system consists of several elements, such as alveolar macrophages and defensins. All the venous blood of the body passes through the capillaries of the alveoles, forming a nearly continuous sheet of blood and only a very thin barrier between it and air.

The smaller conducting zone, which is about 150 ml or 5% of the lung volume, includes the trachea, bronchi, and terminal bronchioles. This part of the lungs has cilia, goblet cells, submucal

## Høiby Focuses on Cystic Fibrosis, but Faced down Smallpox and other Scourges

With the worldwide scourge of smallpox nearly complete in 1970, Niels Høiby found himself on the second day of his medical residency caring for a student with this disease. Høiby, the rest of the hospital staff, and 500 students were quarantined for 14 days. Although the patient died, nobody else, even those who previously were not vaccinated, became sick. “This success, and the exciting experience, forever changed my life,” Høiby recalls. “I would never leave microbiology and infectious disease medicine.”

True to his word, Høiby devoted his career to microbiology and infectious diseases, focusing on *Pseudomonas aeruginosa* and other pathogens that provoke frequent and chronic lung infections in patients with cystic fibrosis (CF). His approach to aggressively treating infections with this bacterium in CF patients in Denmark has helped increase their average lifespan from age 20 in 1975 to age 50 in 2004. By comparison, CF patients in the United States typically live only into their late 30s, according to the Cystic Fibrosis Foundation.

Høiby, 65, chairs the department of clinical microbiology at Rigshospitalet and is professor of medical microbiology at the University of Copenhagen. In addition to his CF research, he runs a large diagnostic clinical microbiology lab in a 1,100-bed hospital that covers all medical specialties.

Høiby was born when German soldiers occupied Denmark during World War II. “My father, who was a stockbroker, was active in the resistance against the Germans right from the beginning,” he says. His mother, educated as a classical singer and

pianist at the Royal Danish Conservatory, gave music lessons when he was a child. He has a younger sister.

“There was no scientific tradition in my family, and I thought, when I was in high school, that I would be a stockbroker like my father,” he says. “I was performing very well in mathematics and physics in school, but I was also very interested in gymnastics and music.” He nearly followed his mother’s lead by pursuing a musical career, he adds, noting: “I played jazz trumpet for some years, and Louis Armstrong was my favorite. I have his autograph on a photo I took myself.”

However, Høiby changed his mind about a career in music during his last year of high school. “I thought that I had to work with human beings, and with some science, and therefore I decided to study medicine,” he says. Later, he received his medical degree from the University of Copenhagen.

Besides his work in microbiology and infectious diseases, Høiby writes books and articles about medical politics, a subject that can give rise to debate. He also founded and chairs the Danish Society to Assure the Doctor’s Right to Speak Freely, an organization that arose from the experience of a good friend and former professor of pediatrics, who “was forced to quit his job because he criticized the administration’s restriction on the economy of the hospital, which gave severe problems for pediatric patients,” he says.

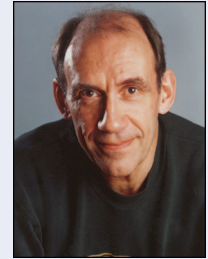
Høiby coauthored a book about an SS doctor, Carl Vaernet, who conducted medical experiments on homosexuals at the Buchenwald concentration

camp during World War II. “I have always wondered why doctors could transform from healers to killers,”

Høiby says. “Vaernet escaped to Argentina, where he died 40 years ago. His son was a famous neurosurgeon in my hospital, whom I knew very well, but the story about his father was not known by the public before we started looking in the archives in Denmark and Germany.”

Høiby visited Vaernet’s grave when he traveled to Buenos Aires to attend a CF meeting. “The book was received very well, and I was invited to give several lectures at congresses for sexuality disorders in Denmark and Scandinavia—a very unusual experience for a microbiologist,” he says.

Høiby and his wife Birgit, an art historian, have three children. Their daughter is a veterinarian, one son a physiotherapist, and their other son a painter. “My wife and I, together with all our children and grandchildren, always spend a week together in Switzerland in the skiing season,” he says. “We love this week since it brings the whole family together, [and] this tradition was started by my parents 45 years ago. We also have a summerhouse at the beach. Our children and grandchildren use this house very frequently, so we have a good family life.”

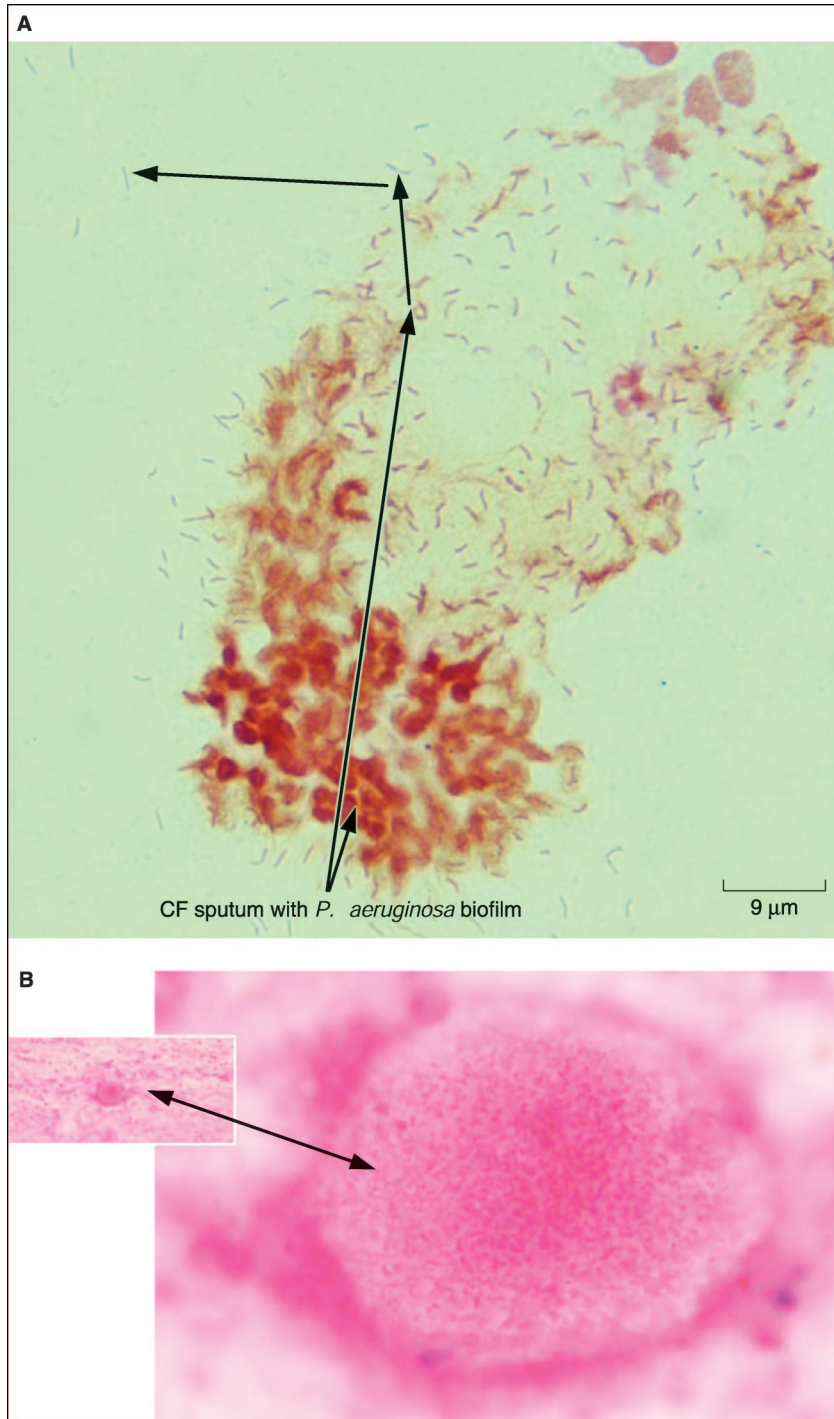


### Marlene Cimons

Marlene Cimons is a freelance writer in Bethesda, Md.



FIGURE 2



Gram-stained sputum from four CF patients. A-B. *P. aeruginosa* biofilms and PMNs are seen. C. A *P. aeruginosa* biofilm with two distinct morphologies is seen maybe representing release of nonmucoid revertant from the mucoid biofilm. D. Sputum from a CF female, 44 years old, growth of mucoid and nonmucoid *P. aeruginosa* in sputum. *P. aeruginosa* since 1970, chronic mucoid *P. aeruginosa* since 1971. She has had 35–44 precipitating antibodies in serum against *P. aeruginosa* for many years. A detached alveolus with a *P. aeruginosa* biofilm is seen (double arrow, x100 & x100).

glands, and an arterial blood supply from the aorta. The mucus is produced in the respiratory zone, and the defense system consists of elements such as the mucociliary escalator.

When nebulized tobramycin, colistin, or other antibiotics are used to treat *P. aeruginosa* lung infections, the drugs reach very high concentrations in the conductive zone, but remain very low in the respiratory zone. However, when antibiotics are administered systemically, they are found in very low concentrations in sputum, but in high concentrations in respiratory tissues because antibiotics move through the blood directly to alveolar capillaries before being distributed elsewhere in the body. Because both the respiratory and conductive zones of CF patients typically are infected with *P. aeruginosa*, combined systemic and nebulized antibiotic treatments are indicated.

### *P. aeruginosa* Cells Adapt To Survive in the Lung Respiratory Zone

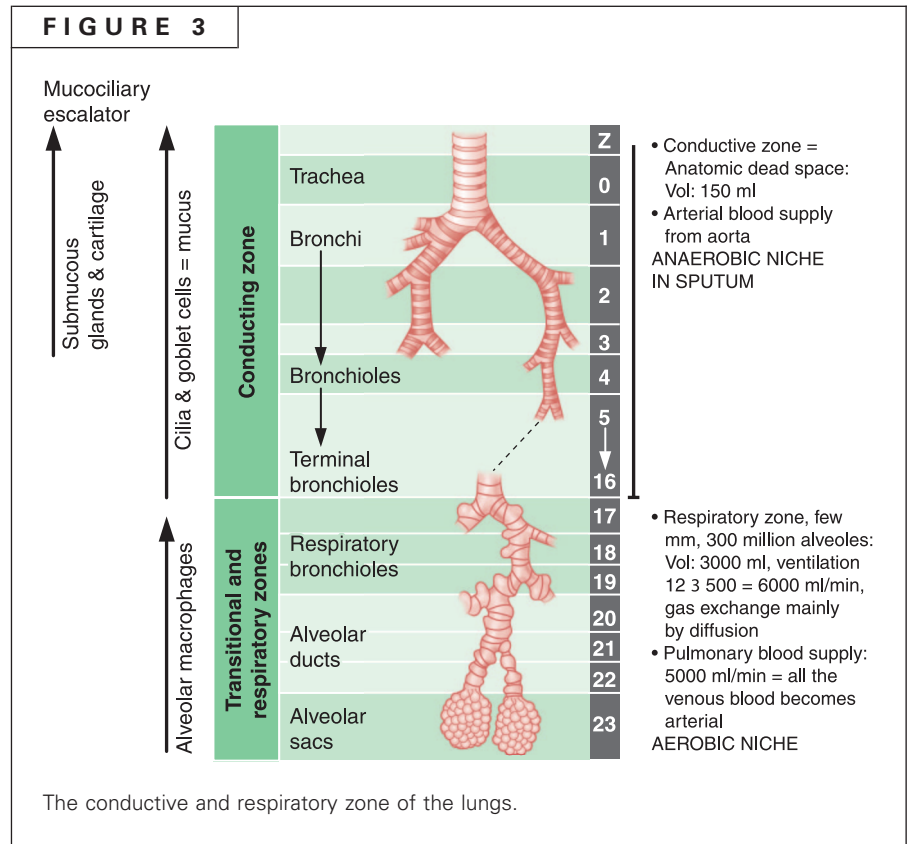
The airspace of the respiratory zone is aerobic, containing 13% O<sub>2</sub> and 5% CO<sub>2</sub>, so long as the air and blood supply remain normal. If mucus plugs the airflow, this zone can become microaerophilic, with oxygen levels dropping to 5%, and CO<sub>2</sub> rising to 6%. If abscesses form, sites within this zone become anaerobic, and then localized cells and tissues become necrotic. Although *P. aeruginosa* can grow under all these conditions, generation times are shorter when this zone is aerobic. Neither macrophages nor PNMs survive in anaerobic niches.

Mucoid *P. aeruginosa* biofilms typically concentrate in the respiratory zone of CF lungs, according to autopsy findings (Fig. 1). Such patients typically produce antibodies against alginate, and there is pronounced PMN-dominated inflammation that surrounds biofilm-containing alveoles, damaging the tissues and impairing lung functions.

Detached biofilm-containing alveoles sometimes are also found in sputum (Fig. 2D), and degradation products from elastin and collagen can be detected in urine.

Healthy lungs contain 300,000,000 alveoles, and they typically decline by 1–2% per year, or by 2,700–5,400 per day. *P. aeruginosa* biofilms are focal, and localized tissue damage can be detected by high-resolution CT scans. Gradually, however, those focal infections spread, impairing respiratory function despite intensive antibiotic therapy.

Although many PMNs are found in tissue surrounding mucoid *P. aeruginosa* biofilms, alginate apparently keeps PMNs from entering the biofilms (Fig. 1 and 2). Thus, *P. aeruginosa* adapt to the respiratory zone by forming mucoid biofilms that enable the bacteria to withstand the host inflammatory response, which, however, helps to destroy localized lung tissue.



### *P. aeruginosa* Cells also Adapt To Survive in the Lung Conductive Zone

The lung conductive zone consists of 16 sets of bronchi, extending from the trachea to the deep-set, terminal bronchioles. Only the uppermost four or five sets, or “generations,” can be directly observed by bronchoscopy, making it necessary to examine the the distal sets by lavage (or by examining lungs from transplants or at autopsy).

In the conductive zone, *P. aeruginosa* microcolonies are found mainly inside sputum, where conditions are anaerobic. Thus, few bacteria are found along the epithelial surface of the bronchi. Gram-stained smears of sputum from CF patients contain not only mucoid biofilms but also planktonic single cells. When cultured, such sputum samples typically grow mucoid and nonmucoid cells, but sometimes also grow small-colony-variant phenotypes.

Recent experiments suggest that such phenotypic variation occurs in response to differences in growth conditions that lead to mutations or other changes, such as the bacterial SOS response. For example, while growing a clinical

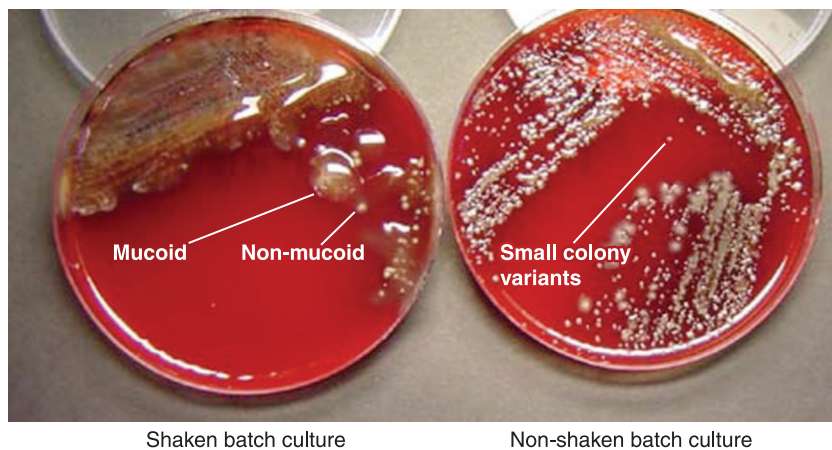
mucoid *P. aeruginosa* CF strain, we found nonmucoid cells and sometimes small-colony variants (Fig. 2 A and C, Fig. 4). The mucoid phenotype is unstable under anaerobic conditions. Meanwhile, the flagella-mediated motility of the nonmucoid phenotype is negatively regulated by the sigma factor *algT*, which positively regulates alginate biosynthesis. Thus, nonmucoid *P. aeruginosa* cells presumably move to the aerobic surface, giving them a selective advantage arising from a faster growth rate.

Mucoid *P. aeruginosa* strains from CF patients have mutation insertions in *mucA*, as do many of the nonmucoid revertants from these patients. Furthermore, many of the nonmucoid revertants are QS deficient and nonvirulent. Nonmucoid phenotypes generally do not give rise to a pronounced antibody response in the few patients who are colonized with such phenotypes, perhaps reflecting their being in sputum and away from key cells of the immune system.

In general, mucoid biofilms from the aerobic respiratory zone are transported to the anaerobic sputum in the conductive zone, where the nonmucoid phenotypes split off. The anaerobic



FIGURE 4



Adaptive divergence in vitro of *Pseudomonas aeruginosa* from a CF patient (Hoffmann et al., 2005). *P. aeruginosa* CF 57388A (mucoid) after 14 days shaken (left) or nonshaken static (right) aerobic culture in a flask, notice the appearance of 3 phenotypes: mucoid, nonmucoid, and small colony variants (SCV). Shaken: Mucoid colonies dominates (after 1 week there was only mucoid colonies), Static: Nonmucoid and SCV colonies dominate (similar results after 1 week). CF 57388A (mucoid) has in *mucA*: at 170 deletion C, frame shift, stop codon 282 and at 293 insertion 105 bp. CF 57388B (nonmucoid) has additionally in *algT* at 147 insertion AGCCCAGGA, frame shift.

conditions of sputum probably account for the abundance of dead PMNs found there—along with DNA, elastase, and myeloperoxidase from dead PMNs. The *P. aeruginosa* nonmucoid revertants probably do not contribute much to

tissue damage and, therefore, do not require special therapeutic or prophylactic attention.

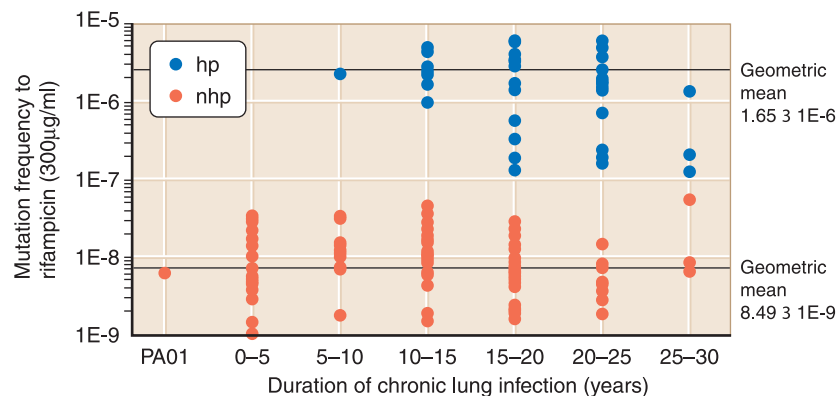
### *P. aeruginosa* Cells Also Survive by Adapting to Antibiotic Therapy

Despite being subject to intensive antibiotic therapy, chronic *P. aeruginosa* lung infections rarely, if ever, are eradicated. Although *P. aeruginosa* biofilms are the main reason for this persistence, the appearance of multiple drug-resistant isolates of *P. aeruginosa* in CF patients suggests that conventional resistance mechanisms also play a role. Drugs to which such *P. aeruginosa* strains become resistant include  $\beta$ -lactams, ciprofloxacin, tobramycin, and colistin.

Biofilms of *P. aeruginosa* respond to  $\beta$ -lactam antibiotics by producing chromosomal  $\beta$ -lactamase. Nonmucoid phenotypes are more resistant to such antibiotics and have higher oxygen radical damage than do mucoid phenotypes from the same patients. One countermeasure that improves clinical outcomes involves inducing antibodies in CF patients that are directed against chromosomal  $\beta$ -lactamases produced by *P. aeruginosa*—and thus prolonging the efficacy of this class of antibiotics.

Mutator strains help to explain the high frequency of multiply antibiotic-resistant *P. aeruginosa* strains from CF patients (Fig. 5). Mutator strains typically carry defects of their DNA repair enzymes, accounting for their much higher frequencies of mutations compared to wild-type strains. Mutator strains of *P. aeruginosa* from CF patients often have increased levels of DNA damage that may be due to encounters with activated PMNs during chronic infections. In general, we find no mutator strains during the first few years of such chronic infections. However, the numbers of such strains after 5 years increase steadily, reflecting how this pathogen survives for decades in such patients despite being exposed over many years to intensive antibiotic therapy.

FIGURE 5



Mutation frequency after exposure to rifampicin of 141 *P. aeruginosa* isolates collected in five periods of chronic lung infection (0–5, 5–10, 10–15, 15–20, and 20–25 years) from 11 patients with CF (Ciofu et al. 2005). The results for hypermutable (hp: filled triangles) and nonhypermutable (open circles) *P. aeruginosa* isolates are presented. The mutation frequency after exposure to rifampicin of reference strains PA01 is shown.

## Evolutionary Implications of the Adaptability of *P. aeruginosa*

The genome of the *P. aeruginosa* PAO1 strain contains 6.2 million base pairs and 5,570 genes, including a high proportion of regulatory, catabolic, transport, efflux, and chemotaxis genes, that help to explain its adaptability. Strains from CF patients with chronic lung infections show some obvious differences when compared to the PAO1 strain. For instance, the strains from such patients typically are alginate producers, grow in biofilms, split off nonmucoid and small colony variant phenotypes, and often are mutators

with many accumulated mutations that make them auxotrophs.

Søren Molin from Danish Technical University, other collaborators, and I find that clinical CF strains grow much slower than PAO1, with a mean generation time of 4 hours. After surviving in CF lungs for 30 years, such strains typically undergo 65,000 divisions, enough time to enable them to adapt in many ways to life in CF lungs but perhaps no longer to survive elsewhere. Genomic analysis of such isolates could help to explain more precisely the extent of this adaptive process.

### SUGGESTED READING

- Bagge, N., M. Hentzer, J. B. Andersen, O. Ciofu, M. Givskov, and N. Høiby. 2004. Dynamics and spatial distribution of beta-lactamase expression in *Pseudomonas aeruginosa* biofilms. *Antimicrob. Agents Chemother.* 48:1168–1174.
- Bjarnsholt, T., P.-Ø. Jensen, M. Burmølle, M. Hentzer, J. A. J. Haagensen, H. P. Hougen, H. Calum, K. G. Madsen, C. Moser, S. Molin, N. Høiby, and M. Givskov. 2005. *Pseudomonas aeruginosa* tolerance to tobramycin, hydrogen peroxide and polymorphonuclear leukocytes is quorum-sensing dependent. *Microbiology* 151:373–383.
- Bjarnsholt, T., P.-Ø. Jensen, T. B. Rasmussen, L. Christophersen, H. Calum, M. Hentzer, H.-P. Hougen, J. Rygaard, L. Eberl, N. Høiby, and M. Givskov. 2005. Garlic blocks quorum sensing and promotes rapid clearing of pulmonary *Pseudomonas aeruginosa* infections. *Microbiology* 151:3873–3880.
- Ciofu, O., B. Riis, T. Pressler, H. E. Poulsen, and N. Høiby. 2005. Occurrence of hypermutable *P. aeruginosa* in cystic fibrosis patients is associated with the oxidative stress caused by chronic lung inflammation. *Antimicrob. Agents Chemother.* 49:2276–2282.
- Hoffmann, N., T. B. Rasmussen, P. Ø. Jensen, C. Stub, M. Hentzer, S. Molin, O. Ciofu, M. Givskov, H. K. Johansen, and N. Høiby. 2005. Novel mouse model of chronic *Pseudomonas aeruginosa* lung infection mimicking cystic fibrosis. *Infect. Immun.* 73:2504–2514.
- Høiby, N. 2002. Understanding bacterial biofilms in patients with cystic fibrosis: current and innovative approaches to potential therapies. *J. Cystic Fibrosis* 1:249–254.
- Lee, B., J. A. J. Haagensen, O. Ciofu, J. B. Andersen, N. Høiby, and S. Molin. 2005. Heterogeneity of biofilms formed by non-mucoid *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. *J. Clin. Microbiol.* 43:5247–5255.
- Mathee, K., Ciofu, O., Sternberg, C., Lindum, P.W., Campbell, J.I.A., Jensen, et al. 1999. Mucoïd conversion of *Pseudomonas aeruginosa* by hydrogen peroxide: a mechanism for virulence activation in the cystic fibrosis lung. *Microbiology*: 145:1349–1357.
- Song, Z. J., H. Wu, O. Ciofu, K. F. Kong, N. Høiby, J. Rygaard, et al. 2003. *Pseudomonas aeruginosa* alginate is refractory to Th1 immune response and impedes host immune clearance in a mouse model of acute lung infection. *J. Med. Microbiol.* 52:731–740.