Metabolism and Pathogenicity of Pseudomonas aeruginosa Infections in the Lungs of Individuals with Cystic Fibrosis

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ABSTRACT Individuals with the genetic disease cystic fibrosis (CF) accumulate mucus or sputum in their lungs. This sputum is a potent growth substrate for a range of potential pathogens, and the opportunistic bacterium Pseudomonas aeruginosa is generally most difficult of these to eradicate. As a result, P. aeruginosa infections are frequently maintained in the CF lung throughout life, and are the leading cause of death for these individuals. While great effort has been expended to better understand and treat these devastating infections, only recently have researchers begun to rigorously examine the roles played by specific nutrients in CF sputum to cue P. aeruginosa pathogenicity. This chapter summarizes the current state of knowledge regarding how P. aeruginosa metabolism in CF sputum affects initiation and maintenance of these infections. It contains an overview of CF lung disease and the mechanisms of P. aeruginosa pathogenicity. Several model systems used to study these infections are described with emphasis on the challenge of replicating the chronic infections observed in humans with CF. Nutrients present in CF sputum are surveyed, and the impacts of these nutrients on the infection are discussed. The chapter concludes by addressing the future of this line of research including the use of next-generation technologies and the potential for metabolism-based therapeutics.

INTRODUCTION

The lung is typically considered a sterile environment; however, this is rarely true in practice. Healthy individuals constantly inhale various microbes, and the innate immune system is responsible for their removal through a system of mucociliary clearance. Some individuals experience altered mucociliary clearance due to any of a number of diseases, and one of the best-studied examples of this occurs in individuals with the genetic disease cystic fibrosis (CF). A hallmark phenotype of CF is dehydration of the mucosal layer across all epithelial surfaces inside the body, resulting in a thickening of the mucus that cannot be adequately cleared despite frequent expectoration. This mucus is an excellent growth substrate for a range of bacteria, and the lung disease associated with these infections further alters the mucus, resulting in a complex mixture of host and microbe derived macromolecules called sputum that is not present in healthy lungs. The opportunistic pathogen Pseudomonas aeruginosa is generally the most difficult of these invaders to eradicate, and infections with this organism are the leading cause of morbidity and mortality for individuals with CF (¹, ²).

The goal of this chapter is to summarize our current understanding of the role CF sputum plays in establishment and maintenance of P. aeruginosa infections in the CF lung. The introduction will summarize the causes...
of CF, compare available nutrients in healthy and CF lungs, and introduce P. aeruginosa as an opportunistic pathogen. The following section will describe methods for studying P. aeruginosa infections both in vivo and in vitro, and special attention is paid to whether these models accurately reflect CF lung disease in humans. The third section will describe how specific nutritional cues, like mucins, DNA, lipids, and amino acids, that are present in CF sputum impact P. aeruginosa pathogenicity, while the final section will discuss relevant future areas of research in this field.

**CF DISEASE CAUSES AND PHENOTYPES**

In 1989, the genetic locus responsible for CF was identified on the seventh chromosome in a gene encoding a cAMP-dependent chloride ion transporter called the **Cystic Fibrosis Transmembrane Conductance Regulator** (CFTR) (3, 4). The CFTR protein broadly regulates ion flux across the epithelial surface through interactions with other ion transporters like the sodium ion (Na+) transporter ENaC (5, 6). CFTR has been localized to ciliated cells in airway epithelia, and importantly, no CFTR protein was detected in ciliated cells from individuals carrying the most common CF mutation, ΔF508 CFTR (7, 8). This is attributed to post-translational degradation of CFTR in CF cells (7, 8). Further, a study by Tucker and colleagues determined that expression of ΔF508 CFTR can spoil processing of WT CFTR in the same cells (9). This observation is significant to both heterozygous CFTR carriers as well as scientists’ attempts to cure CF using gene therapy or other strategies (10). CFTR is also an important component of the innate immune response; a study by Pier and colleagues demonstrated that the absence of CFTR diminished the ability of airway epithelial cells to internalize *P. aeruginosa*, which is a significant determinant of the host’s ability to clear this pathogen (11). Interestingly, epithelial cells lacking CFTR also possess greater levels of AMP-activated Kinase (AMPK) activity, which may be an adaptive response, because it is associated with decreased production of inflammatory effector molecules (12). Thus, the absence of CFTR both generates the thick mucus required for CF lung disease and renders the lung particularly susceptible to infection.

**NUTRIENTS PRESENT IN THE LUNG: HEALTHY VERSUS DISEASED STATE**

Healthy airway surfaces are coated in a mucus layer that is largely composed of water, salts, mucins, and surfactants. Among the ions present in sputum, iron is generally viewed as a critical nutrient for any pathogenic bacterium, and it has been observed to be present at 0.9 micromolar (μM) to 63 μM in CF sputum compared to largely undetectable levels in healthy airway secretions (13–18). Like iron, many nutrients present in CF sputum are not found in healthy lungs, and the goal of this section is to delineate these differences. We will begin by discussing differences in mucins and surfactants between healthy individuals and those with CF, while the remainder of the section will be dedicated to nutrients generally not present in healthy lungs.

Of the several gel-forming, glycoprotein mucins produced by mammals, MUC5AC, MUC5B, and to a lesser extent MUC2 have been detected in airways (19–21). Mucin-like glycoprotein was quantified from healthy individuals and asthmatic individuals with a history of sputum production, and 3- to 6-fold more glycoprotein was quantified from the asthmatic individuals (0.8 to 1.6 mg/mL versus 4.3 to 4.8 mg/mL) (19, 22). A similar study compared mucin levels in CF sputum and healthy sputum, and found that CF sputum contained significantly less MUC5AC and MUC5B (93% and 70%, respectively) (23). However, during times of heightened *P. aeruginosa* infection called exacerbations, MUC5AC and MUC5B levels increased 908% and 59%, respectively (24). Enhanced production of mucins during exacerbations may be due to the presence of *P. aeruginosa*, as several studies have indicated that the bacterium induces mucin secretion (25–29). Whether enhanced mucin production during exacerbations is a cause or effect of the exacerbations remains unclear; however, the increased presence of mucins can have a profound effect on the infecting bacteria as will become clear in future sections of this chapter. The chemical composition of mucin in individuals with CF may also be different compared to healthy mucin, as a study by Carnoy and colleagues found greater levels of glycoproteins in CF salivary mucin and those glycoproteins displayed greater sulfate and fucose content (30).

Healthy airway epithelial cells also secrete surfactants, which provide protection and facilitate gas exchange by reducing surface tension of the mucosal layer that coats alveoli. The composition of pulmonary surfactant is largely lipids (90%), of which phosphatidylcholine (PC) and its derivatives are the most common (62%) followed by phosphatidylglycerol, phosphatidinositol, phosphatidylinositol, phosphatidylethanolamine, sphingomyelin, and other lipids (28%) (31–33). The remainder of surfactant (10%) is comprised of four surfactant proteins: SP-A and SP-D are large proteins...
that are involved in protecting the surface from invasion, and SP-B and SP-C are smaller, hydrophobic proteins that are associated with lipids and interact with the surface of the epithelium (31–33). Several studies have examined the extent to which surfactant composition changes during CF disease. Significant conclusions include the fact that surfactant composition is not remarkably altered under treatment with recombinant human DNase (a common CF therapeutic which degrades sputum), and surfactant composition is largely stable until chronic infection is established, which occurs early in the lives of most individuals with CF (34, 35). Decreased PC levels and an increase in typically minor lipids in healthy individuals or those with chronic obstructive pulmonary diseases (COPD) have also been observed (34–39).

There are many nutrients present in CF sputum that are essentially absent in healthy lung secretions, and many of these are derived from lysed host and microbial cells. DNA is one such nutrient, and it appears that most of the DNA in CF sputum is derived from host cells that were lysed as a consequence of CF lung disease (40). Many studies have measured DNA concentrations in sputum samples that had been either expectorated or acquired using bronchoalveolar lavage (BAL), and reported concentrations range widely from 5.4 μg/mL to 17.6 mg/mL, with most reports falling into a range of 400 μg/mL to 700 μg/mL (24, 34, 41–46). The use of recombinant human DNase (rhDNase) as an inhaled therapeutic has been widespread since the 1990s, because charged DNA molecules are known to crosslink sputum, making it more difficult to clear. Many studies have examined the effect of rhDNase on DNA levels in sputum. While these studies varied in their conclusions as to whether rhDNase treatment decreased total DNA in sputum, there was a consensus that treatment changed the rheological properties of sputum, allowing for easier expectoration (24, 34, 41–46).

Several small organic acids and sugars are also present in CF sputum. Lactate appears to be an indicator of lung inflammation, and several groups have measured its concentration in CF sputum resulting in a range of 3.0 mM to 14.1 mM (15, 47, 48). Bensel and colleagues also measured lactate levels in individuals with chronic obstructive pulmonary disease and acute lung inflammation finding 1.6 mM in the former and nondetectable levels in the latter (47). Our own group has measured glucose levels in expectorated sputum and found an average concentration of 2.9 mM (15). In a separate study, we also measured levels of N-acetylglucosamine (GlcNAc), which is a component of macromolecules present in sputum, and we found it to be present at hundred micromolar levels (49). P. aeruginosa is known to rapidly consume small organic acids like lactate, pyruvate, and succinate, and can grow on glucose or GlcNAc as sole sources of carbon and energy.

Proteins and free amino acids other than the previously discussed mucins and surfactant proteins are final examples of CF sputum nutrients not typically found at high levels in the mucus of healthy individuals. For example, the sputum of individuals with CF contains high levels of proteins from immune cells and microbes, and these protein levels are correlated to exacerbations and the accompanied enhanced immune response (50–52). Both the immune and pathogenic cells present in sputum release extracellular proteases capable of cleaving proteins into free amino acids. Increased levels of free amino acids have been detected in CF sputum (5.70 mg/mL), when compared to non-CF sputum samples (2.52 mg/mL) (53). Our own measurements of sputum have indicated 4.4 to 24.7 mM free amino acids present in sputum (15). These two studies also quantified levels of individual amino acids in CF sputum, and the former found all 22 standard amino acids except tryptophan, proline, glutamate, glutamine, asparagine, cysteine, and aspartate, though some amino acids present at low levels may not have been detectable (53). In the latter study, 19 of the 22 standard amino acids were detectable, with the exceptions being asparagine and glutamine, while hydroxyproline was not measured (15). It is clear that the lungs of individuals with CF contain a remarkably different set of nutrients compared to those of healthy individuals. The inability to clear secreted mucins and surfactants combined with degradation products from lysed host and bacterial cells results in a complex, dynamic nutritional environment. As we will see in later sections, nutrients, like mucins, lipids, DNA, and amino acids, serve as sources of carbon and energy to promote P. aeruginosa growth as well as potent cues for enhanced virulence in CF sputum.

**THE OPPORTUNISTIC PATHOGEN Pseudomonas aeruginosa**

*Pseudomonas aeruginosa* is a Gram-negative opportunistic pathogen. It is commonly found in a range of diverse environments across the world including soil, fresh, and marine water. As an opportunistic pathogen, it lives a dual lifestyle as a commensal and environmental organism, but can occasionally switch to a pathogenic state, causing costly and difficult to treat...
infections. The most notable of these is in the lungs of individuals with the genetic disease cystic fibrosis, as *P. aeruginosa* colonization is associated with declining clinical outcomes, and is the leading cause of morbidity and mortality in these individuals (1, 2). It is believed that *P. aeruginosa* infections are acquired from the environment, and recent studies demonstrating a correlation between the sinus and lung microbiota suggest initial colonization of the sinus proceeds to the lower respiratory system (54–57). *P. aeruginosa* utilizes a plethora of virulence factors to colonize and maintain infections in immune-compromised hosts, and production of many of these virulence factors is regulated in a cell density-dependent manner known as quorum sensing (QS, reviewed below). *P. aeruginosa* infections are notoriously difficult to treat, because the organism is intrinsically resistant to most conventional antibiotics. This is in part due to the fact that it produces antibiotic-resistant, surface-attached communities surrounded in a polymeric matrix known as biofilms or microcolonies.

In order to understand how CF sputum nutrients can affect the pathogenicity of *P. aeruginosa*, it is first critical to establish the infection-causing toolkit available to this organism. *P. aeruginosa* produces an arsenal of virulence factors, which are generally defined as pathogen-derived molecules that help initiate or exacerbate an infection. In the following paragraphs, we will discuss these tools that *P. aeruginosa* uses to cause infections with special emphasis on how they may affect CF sputum and the nutrients available to this organism.

**Quorum Sensing**

Quorum sensing (QS) describes a process whereby bacteria in groups coordinately regulate gene expression (58, 59). In QS, growing cells secrete small molecule signals called autoinducers, and as the number of cells increases, the concentration of the autoinducer also increases to a point where it can interact with transcriptional regulators inside the cell to alter gene expression (58, 59). *P. aeruginosa* possesses three known QS systems: the LasI/LasR system for which 3-oxododecanoyl-homoserine lactone (3OC12-HSL) is the autoinducer (60–62); the RhlI/RhlR system for which butyryl-homoserine lactone (C4-HSL) is the autoinducer (63–65); and a quinolone-based system for which 2-heptyl-3-hydroxy-4-quinoline (*Pseudomonas* quinolone signal; PQS) is the most potent autoinducer (66–68). Collectively these QS systems represent an integrated regulatory network that affects transcription of up to 10% of the *P. aeruginosa* genome (69), including genes for production of the many virulence factors that will be discussed in the following paragraphs. Additionally, several studies have indicated that CF sputum enhances *P. aeruginosa* QS, emphasizing its importance for this work (15, 70, 71).

**Secretion of Virulence Determinants and Degradative Enzymes**

In the 1960s, it was determined that *P. aeruginosa* produces several secreted factors that impact its virulence toward host cells (72–74), though some secreted factors were identified much earlier (75). The organism possesses five of the six known classes of secretion systems in Gram-negative bacteria and multiple copies of some systems exist with differential regulation and/or secreted substrates (76). The two type I secretion systems of *P. aeruginosa* are responsible for secreting an alkaline protease and a heme-binding protein that both function as virulence determinants (76–78). *P. aeruginosa* utilizes a type II secretion system to release several virulence factors beginning with Exotoxin A, which inhibits host protein synthesis in a manner similar to diphtheria toxin (79). Other protein virulence factors secreted through the type II system include several phospholipases, alkaline phosphatases, and proteases that both attack host cells and degrade macromolecules in sputum (76). *P. aeruginosa* possesses a type III secretion system in which toxins are injected directly into host cells via a needle-like structure made of proteins related to those of the flagellum (80–82). The four known effector proteins that are injected to host cells (ExoS, ExoT, ExoU, ExoY) disrupt cytoskeleton formation, signal transduction, and have phospholipase activity (80–83). *P. aeruginosa* lacks a type IV secretion system, and its type V systems are known to secrete an esterase, another protease, and a hemagglutinin-like protein (76). It also possesses three type VI secretion systems that form phage-derived nanotube structures similar to the flagellar-derived type III secretion system (76). Among the few type VI secreted effectors identified in *P. aeruginosa* are a peptidoglycanase and a phospholipase that are both likely involved in degrading neighboring bacterial cells (84, 85). Evidence that the type VI secretion system apparatus is important for virulence in the CF lung includes the fact that antibodies targeting these structures have been identified in CF sputum (76, 86). Finally, *P. aeruginosa* toxins and competitive factors including β-lactamase, alkaline phosphatase, hemolytic phospholipase C, and CFTR inhibitory factor (Cif) are also packaged into outer membrane vesicles (OMVs) (87, 88). Such vesicles are the result of blebs of the outer membrane and are ubiquitous among Gram-negative bacteria (89). In *P. aeruginosa,*
OMVs are produced in a QS-dependent manner as our group has shown that the quinolone signal PQS is trafficked in and promotes formation of OMVs (90).

The degradative enzymes secreted by *P. aeruginosa* are capable of both attacking host cells and degrading sputum macromolecules. In many cases, the breakdown products of these macromolecules serve as both nutrients and mediators of *P. aeruginosa* pathogenicity. *P. aeruginosa* secretes many extracellular proteases, such as LasA, LasB, and Alkaline protease, whose production are regulated in a QS-dependent manner. These factors work alongside copious host-secreted proteases (91, 92) to profoundly alter the nature of CF sputum and contribute to the inflammatory lung disease phenotype common to nearly all individuals with CF (93, 94). Additionally, a proteomic study by Scott and colleagues examining the transmissible *P. aeruginosa* strain AES-1R found enhanced production of secreted proteases, particularly in an artificial sputum medium described in a later section (95). In addition to altering sputum by cleaving substrates, secreted proteases may also worsen the disease state as Butterworth and colleagues have shown that the alkaline protease of *P. aeruginosa* can activate ENaC channels in the epithelium (96). *P. aeruginosa* also produces several lipases that attack the host (97, 98), and degradation of lipids may be critical for growth and heightened virulence in CF sputum (84, 99, 100). *P. aeruginosa* secretes another toxin that has been shown to inhibit CFTR (CFTR inhibiting factor, Cif), which may play a role in colonization of CF lungs (101). Finally, proteomic studies by Folders and colleagues have identified a staphyloytic/chitin-binding protein and chitinase as QS-induced degradative molecules that act as virulence factors and also likely release free GlcNAc, which potentiates *P. aeruginosa* virulence (49, 94, 102–104). While chitin may not be a common nutrient in CF sputum, the activities of these enzymes may still impact virulence in the CF lung by affecting *P. aeruginosa* interactions with co-infecting yeast and host cells (102, 105). It is clear that *P. aeruginosa* contains a myriad of degradative enzymes that both attack the host and provide nutrients for growth and pathogenicity. More information on how these nutrients affect *P. aeruginosa* virulence can be found in the section “Nutritional cues in CF sputum that affect *P. aeruginosa* metabolism and pathogenicity.”

**Phenazines and Other Redox Active Toxic Factors**

Cultures of *P. aeruginosa* appear a characteristically striking blue/green color, and this is largely due to the liberal secretion of a redox-active phenazine compound called pyocyanin. Pyocyanin production is QS-regulated via the PQS system (106), and it is capable of destroying host cells by altering critical cellular processes (107), generating reactive oxygen species (108), altering cell signaling (109), and inducing neutrophil apoptosis (110). Recently a study by Hunter and colleagues measured levels of pyocyanin and its biosynthetic precursor, phenazine-1-carboxylic acid in expectorated CF sputum samples and found pyocyanin levels negatively correlated to lung function (111). Pyocyanin also appears to act as a signaling molecule, as a study by Dietrich and colleagues demonstrated that the phenazine up-regulates genes for transport and redox control and suppresses genes involved in iron acquisition (112).

Other redox-active factors produced by *P. aeruginosa* include a battery of 4-quinolone molecules with a range of activities (113). Lepine and colleagues used electrospray/mass spectrometry to survey the 4-quinolones produced by *P. aeruginosa* and found 56 unique molecules, which differed by the presence of 3-position substituents, n-oxide groups in place of the quinolone nitrogen, and the alkyl chains at the 2 position (114). The *Pseudomonas* quinolone signal (PQS) is one of the most well studied of these quinolones because of its ability to regulate virulence factor production in a cell-density dependent manner (66–68). In terms of its role in redox balance, PQS is both helpful and harmful to *P. aeruginosa* as Haussler and Becker demonstrated it can induce an anti-oxidative stress response and it also sensitzes cells to oxidative and other stresses (115). Another redox-active toxin produced by *P. aeruginosa* is the cytotoxic poison, hydrogen cyanide, which has been demonstrated to be critical for pathogenicity in a *Caenorhabditis elegans* infection model (116, 117). Regulation of its production is complex and partially dependent on QS (118, 119).

*P. aeruginosa* is a deadly opportunistic pathogen, and this stems from the extensive array of virulence factors it is capable of producing. We have seen that many of these virulence factors are QS-regulated, which facilitates colonization of host environments like the CF lung. It is also clear that the nutritional environment of the CF lung is radically different from that of healthy individuals, and we will see how the presence of these nutrients impacts *P. aeruginosa* virulence. CF lung infections are unique in that they are chronic, and generally persist throughout the individual’s lifetime. Thus, it is critical to study *P. aeruginosa* in the context of the CF lung over extended periods of time, and the next section will examine methods to recapitulate these chronic CF lung infections in the laboratory setting.
APPROACHES AND CHALLENGES TO DEVELOPING CF SPUTUM PATHOGENESIS MODELS

*P. aeruginosa* causes decades-long, chronic infections in the lungs of individuals who have the genetic disease cystic fibrosis. These infections are the leading cause of morbidity and mortality for individuals with CF (1, 2, 120). The complexity of CF sputum and the lung environment in which these infections occur has already been addressed in this chapter; however, we have yet to describe the physiology of these infections. An obvious place to begin this study is to isolate bacteria from genuine CF infections, and much information has been gained from studying these clinical isolates (121–127). Additionally, studying sequential isolates over time has yielded information on the adaptive responses of *P. aeruginosa* to the CF lung environment (122, 128–130). While these studies have been instrumental in understanding critical aspects of the physiology of *P. aeruginosa* infections, they are entirely retrospective and could potentially miss heterogeneity within the population causing the infection. An ideal alternative would involve use of an animal model system that is capable of mimicking the CF lung as well as the chronic infections observed there. Several model systems have been used to study *P. aeruginosa* infections, and somewhat surprisingly, these studies indicate there is conservation of the virulence factors required for colonization of a broad range of eukaryotic hosts (131–137). As the focus of this chapter is *P. aeruginosa* infections in the lungs of humans with cystic fibrosis, the following discussion of model systems will focus on mammalian CF model organisms. This section will detail different approaches to solving this problem, and particular emphasis will be placed on overcoming the challenges of accurately replicating the environment/physiology of human CF-related lung infections.

**Mouse Models**

Mammalian animal models are a reasonable first approach to recreating the chronic *P. aeruginosa* infections of the CF lung, and the mouse has been a workhorse model organism for a range of CF-related studies reviewed in the following reference (138). In 1992, several groups generated mice with a homozygous disruption of the murine CFTR gene, and phenotypic characterization of these mutants demonstrated similarities to the human CF disease, including aberrant ion transport across epithelial surfaces, meconium ileus, failure to thrive, and histological abnormalities (138–142). Subsequent mouse mutants have been made that more closely replicate the human mutations responsible for CF disease, and the ability to over-express CFTR in different tissues has been employed to mitigate these mutations (143). While these models have been able to replicate CF lung disease and colonization with *P. aeruginosa* to varying degrees of success, they generally require artificial enhancements like repeated aerosolized exposure of *P. aeruginosa* to mice missing a second Cl– ion channel (144, 145), or inoculating with *P. aeruginosa* coated agar/agarose beads to establish infections (146, 147). Importantly, these infections were largely transient and failed to model the chronic infections that are hallmarks of CF lung disease. Coleman and colleagues developed the most successful protocol for chronic infection of ΔF508-CFTR mice (143), though only ~33% of mice retained *P. aeruginosa* in their lungs 24–53 weeks after inoculation (148). Hoffmann and colleagues reported a chronic infection model that utilizes a stable mucoid strain of *P. aeruginosa*, though these experiments were not carried out beyond 2 weeks, and genuine CF infections last years to decades (149). A conditional mouse CFTR mutant has been made in which CFTR null phenotypes can be examined tissue by tissue (150, 151). These mice have been used to show that the loss of expression of CFTR in myeloid-derived cells results in mice that are more susceptible to challenge with *P. aeruginosa* embedded in agarose beads (152). This study lends credence to the hypothesis that the impaired innate immune system of the CF lung contributes to CF lung disease in addition to the physical and nutritional nature of CF sputum.

**Pig and Ferret Models**

The inability of mouse models to accurately replicate important CF phenotypes like chronic *P. aeruginosa* infections has led researchers to pursue alternative animal models that may possess physiologies closer to humans. One of the most intriguing alternative models to the mouse was a CF pig that was first developed in 2008 (153, 154). New insights are beginning to be gained from the CF pig model, as a study by Pezzulo and colleagues demonstrated that newborn CF pigs are less able to kill *Staphylococcus aureus*, a common pathogen present in young individuals with CF (155). The authors attached *S. aureus* to gold grids, surgically placed them into the trachea, and used live/dead staining to evaluate *S. aureus* killing. The authors determined diminished killing was due to the lower pH of CF airway surface fluids, which inhibited antimicrobial factors like lysozyme and lactoferrin (155). Additionally, a homozygous ΔF508-CFTR pig has been developed (156). Despite the
fact that these ΔF508-CFTR pigs retain some CFTR activity in the airway epithelia, some bacteria (10 CFU [colony forming units]/g to 743 CFU/g of lung tissue) were able to be cultured from the lungs of these animals, while no bacteria were able to be cultured form wild-type controls (156).

In 2010, Sun and colleagues were able to generate a ferret CFTR knockout mutant that displayed several phenotypes similar to those of humans with CF, though these animals also did not suffer severe lung infections beyond their first week of life (157). A study by Fisher and colleagues compared processing of CFTR in humans and ferrets and found that the ferret CFTR protein was processed more efficiently and displayed a longer half-life in the epithelium than human CFTR, and the authors argued this could yield approaches to stabilize human CFTR (158). The same study examined processing of a ferret ΔF508-CFTR, and while maturation of the ferret mutant protein was enhanced compared to human ΔF508-CFTR, expression of neither protein resulted in cAMP-dependent chloride ion conduction in airway epithelial cells (158). Like the pig, the ferret serves as another useful model for studying initiation of CF lung infections, (157) however, neither animal model has been spontaneously or chronically colonized by *P. aeruginosa*, which bodes poorly for use of these organisms to study chronic infections (151).

**CF Sputum Media**

Due to the modest success of generating animal models that accurately recapitulate chronic CF lung disease, several groups have mimicked the CF lung environment by generating sputum-based growth media. An early example involved the use of sputum as the growth substrate for mucoid strains of *P. aeruginosa*, and the observed phenotypes of growth in this medium were consistent with the hypothesis that mucoidy is an adaptive aspect of chronic infection (159). Our own group used a medium featuring sputum as the sole source of carbon and energy for *P. aeruginosa* transcriptome profiling, and the results indicated that the production of several key virulence factors is enhanced in sputum (71). The same medium was also used to demonstrate that a *P. aeruginosa* membrane-bound nitrate reductase is critical for anaerobic growth in CF sputum (160). While oxygen is not a nutrient for the purposes of this work, several studies have suggested that cells grow in microaerophilic pockets within sputum, and anaerobic *P. aeruginosa* biofilms are more resistant to antibiotics, making them more difficult to eradicate from the lung (160–162).

The complexity of sputum and the difficulty in obtaining it from lavage, expectoration, or extralunged lungs led several groups to investigate synthetic sputum media that nutritionally (and rheologically) mimic the contents of genuine CF sputum (15, 163, 164). Ghani and Soothill used such a medium to determine whether nutrients present in CF sputum would affect the ability of combinations of antibiotics to kill biofilm-grown *P. aeruginosa*, and the authors were able to identify a cocktail of ceftazidime, gentamicin, and rifampicin as a successful combination (163). Building off this work, Sriramulu and colleagues developed a similar artificial sputum medium (ASM) that spontaneously promoted *P. aeruginosa* growth as microcolonies or the sputum component-attached biofilms believed to in part explain the difficulty of eradicating *P. aeruginosa* from the CF lung (164). Fung and colleagues generated an enhanced version of ASM that included albumin and altered the concentrations of mucin and DNA to reflect more recent definition of genuine CF sputum (165). This medium was used for *P. aeruginosa* transcriptome profiling and identified several metabolic and virulence genes that were up-regulated in the presence of CF sputum nutrients (165). Another recent study by Haley and colleagues used this medium to determine optimal concentrations of several nutrients for biofilm formation as well as to dissect the role of QS in the same process (166).

Our group has measured the concentration of several nutrients in sputum samples expectorated from individuals with CF and used these concentrations to develop an alternative synthetic CF sputum medium (SCFM) (15). The particular utility of this medium stems from the fact that its entire nutrient composition is defined, thus nutrient utilization can be quantified. Using SCFM as such a tool, we were able to delineate a hierarchy of nutrient preference for *P. aeruginosa* in sputum where proline, alanine, arginine, glutamate, aspartate, and lactate were among a set of preferentially consumed carbon sources (15). Another utility of SCFM is the ability to add and subtract nutrients from this medium in order to understand their roles in *P. aeruginosa* pathogenicity, which will be discussed in the following section.

*P. aeruginosa* infections in the CF lung are unique in that they are chronic and generally last for years. While this makes for an excellent system to study bacterial adaptation to infection sites, our ability to adequately replicate these infections in model systems has been a significant challenge to the field. That said, progress has been made using various CF animal models and syn-
thethic media for in vivo and in vitro experiments, as these tools have been invaluable in expanding our knowledge of the roles played by specific nutrients in CF sputum in cuing P. aeruginosa pathogenicity. The next section will summarize our current knowledge of the roles certain nutrients play in cuing P. aeruginosa pathogenicity in CF sputum.

**NUTRITIONAL CUES IN CF SPUTUM THAT AFFECT P. AERUGINOSA METABOLISM AND PATHOGENICITY**

The introduction to this chapter outlined the significant differences between healthy and diseased lungs, and it was made clear that there is a substantial increase in the availability of nutrients for microbial growth in diseased lungs like those of individuals with cystic fibrosis. This is likely due to the inability of these individuals to clear the dehydrated airway secretions as well as the release of charged, polymeric molecules like mucin, DNA, and proteins from lysed host and microbial cells. We have also seen that many P. aeruginosa virulence factors are capable of degrading these macromolecules to generate an even more complex extracellular environment at these infection sites. The following subsections will detail what is known about how these nutrients specifically cue P. aeruginosa behaviors that are essential for colonization and maintenance of infections in the CF lung (for summary see Table 1).

**Iron**

Iron is typically sequestered within a host, and many pathogens have evolved extensive mechanisms to acquire iron while causing infections (167, 168). These mechanisms include production of energetically expensive iron scavenging molecules called siderophores, and a study by Martin and colleagues indicated that the P. aeruginosa siderophore pyoverdine is an important, though nonexclusive means of iron acquisition in CF sputum (169). Additionally our group has proposed that co-culture with S. aureus results in P. aeruginosa killing S. aureus to acquire iron (170). This may be particularly relevant in the CF lung, which is frequently co-colonized by P. aeruginosa and S. aureus. Iron levels also tend to be higher in CF airways compared to healthy airways (17, 18), and there is evidence that this helps P. aeruginosa infections beyond simply being a more available essential nutrient, as iron is a critical component of the extracellular matrix of biofilms (171). In a study by Moreau-Marquis and colleagues, P. aeruginosa biofilms grown on airway epithelial cells were 25-fold more resistant to tobramycin than those grown on an abiotic surface (14). Additionally, growth on ΔF508-CFTR mutant epithelial cells enhanced biofilm production compared to wild-type epithelial cells. The authors attributed this to greater iron levels that were observed outside these cells, because removal of excess iron abolished the biofilm phenotype (14). The picture of iron in the CF lung has been complicated with studies by

**TABLE 1** Summary of nutrients present in CF sputum and their roles in P. aeruginosa pathogenicity

<table>
<thead>
<tr>
<th>Nutrient (concentration*)</th>
<th>Carbon source?</th>
<th>Pathogenic cue(s) and other notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron (0.9–63 μM)</td>
<td>–</td>
<td>Important component for microcolony formation; iron acquisition key to infection, may explain transmissibility of Australian epidemic strain</td>
</tr>
<tr>
<td>Mucins (~7–14 mg/mL)</td>
<td>+</td>
<td>May play a role in attachment/colonization; decreases motility and induces microcolony formation</td>
</tr>
<tr>
<td>Phospholipids (281–53 μg/mL)</td>
<td>+</td>
<td>Carbon source during high cell density growth; phospholipase mutants less virulent in animal models; chemotaxis toward PE; degradation products (phosphorylcholine, choline) also cue virulence</td>
</tr>
<tr>
<td>DNA (400–700 μg/mL)</td>
<td>+</td>
<td>Crosslinks sputum, treated with rhDNase; aids microcolony formation; may sequester neutrophil proteases</td>
</tr>
<tr>
<td>Small acids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactate (3–15 mM)</td>
<td>+</td>
<td>Preferred carbon source, levels correlated with inflammation, lactate consumption may be adaptive strategy</td>
</tr>
<tr>
<td>Sugars</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GlcNAc (~300 μM)</td>
<td>+</td>
<td>Induces expression of pyocyanin and other virulence factors; hyaluronic acid is antagonistic</td>
</tr>
<tr>
<td>Glucose (2.9 mM)</td>
<td>+</td>
<td>Induces heterogeneous, mushroom-shaped microcolonies; simple sugars may also inhibit biofilm formation</td>
</tr>
<tr>
<td>Amino acids (~20 mM total)</td>
<td>+</td>
<td>Several preferred carbon sources; aromatic amino acids involved in QS-mediated virulence; alanine catabolism aids in rat infection model; promote tight microcolony formation; promote swarming motility</td>
</tr>
</tbody>
</table>

*Concentrations reflect consensus of reported values. See “nutrients present in the lung: healthy versus diseased state” for references.
Koley and Hunter and colleagues demonstrating ferrous iron is generated near P. aeruginosa biofilms (172) and that a proportion of ferrous relative to ferric iron increases with the progress of infection (173). The presence of ferrous iron has implications for redox active virulence factors like phenazines and biofilm formation (173). Finally, a proteomic study by Hare and colleagues comparing the Australian epidemic strain 1 (AES-1R) to a burn wound/septic strain PA14 and lab strain PAO1 grown in a synthetic CF sputum medium found an increase in enzymes for synthesis of pyocyanin and siderophores (174). The authors argued that iron acquisition could explain why AES-1R is associated with person-to-person transmission of P. aeruginosa, which is more commonly thought to be acquired from the environment (174).

**Mucins**

Mucins are substantial components of sputum, and they can serve as a source of carbon and energy for P. aeruginosa, as Sriramulu and colleagues observed that growth in ASM was substantially diminished in the absence of mucin (164). Another study by Henke and colleagues demonstrated that host and microbe-derived serine proteases are responsible for degrading mucins in CF sputum, and their degradation may contribute to airway inflammation by removing a barrier to the epithelium (175). Finally, a study by Aristoteli and Wilcox characterized mucin degradation by ocular infection strains, demonstrating that the ability to metabolize mucin provided a long-term growth advantage (176). Thus, mucin may be a critical nutrient for sustaining infections.

Several studies have demonstrated that P. aeruginosa binds mucin, and this may be the critical first step in colonizing CF airways (177–179). Interestingly, a study by Sajjan and colleagues indicated that P. aeruginosa interactions with mucin were not necessarily specific to mucin itself, arguing that colonization of the CF lung is not based on tropism for mucin (180). In support of the theory that pathogen-mucin interactions are nonspecific, several groups have reported that other CF-associated opportunistic pathogens bind mucin (181–183). Investigations comparing CF and non-CF mucin indicated that CF mucins are bound better by P. aeruginosa, though this is likely due to the fact that CF-mucins are degraded in the presence of P. aeruginosa into a form to which the pathogen more readily adheres (186). Mucin may also help facilitate the maintenance of P. aeruginosa infections, as a study by Landry and colleagues demonstrated that the presence of mucin on a surface diminished the organism’s motility, resulting in large cellular aggregates with increased resistance to the antibiotic tobramycin (187). By contrast, more recent reports have indicated that mucin promotes motility across surfaces (188), and mucin biopolymers can enhance motility of planktonic cells, preventing their attachment to underlying surfaces (189). These studies collectively implicate mucin as a critical nutrient for both initiating and sustaining P. aeruginosa infections.

**Lipids**

Multiple studies have indicated that lipids may be a significant carbon source for P. aeruginosa in CF sputum, and this may be particularly true in high cell density (HCD) infections like those with >10⁸ CFU/mL observed during exacerbations in the CF lung (1, 190, 191). It is believed that the many secreted lipases of this organism cleave off fatty acid chains in sputum, which can then be internalized and oxidized as carbon sources (190, 192, 193). Another study by Wargo and colleagues demonstrated that a mutant in hemolytic phospholipase C, PlcHR, caused less severe infections in a mouse model, and inhibition of this enzyme with the drug miltefosine also decreased the effect of PlcHR on mouse pulmonary surfactant (193). Son and colleagues were able to perform transcriptome profiling of P. aeruginosa in vivo undergoing HCD exacerbations, and found genes for lipid metabolism to be up-regulated (190). In a subsequent study, the same group identified several fatty acid synthetases (Fads) that are involved in metabolism of fatty acids in sputum. The ability to catabolize fatty acids seems to be important for virulence as mutants in these Fads were diminished in their ability to cause infections in a mouse model (192).

A study by Krieg and colleagues found P. aeruginosa increased capsule formation and adhesion during growth on phosphorylcholine, a degradation product generated by phospholipase C acting on phosphatidylcholine (194). Subsequent work by the same group demonstrated that growth on phosphorylcholine promotes conversion to mucoid variants of P. aeruginosa, which is believed to be an adaptation consistent with long-term colonization (195). P. aeruginosa possesses an enzyme capable of degrading phosphorylcholine to choline (196), and choline also appears to play a role in P. aeruginosa virulence, as it has been shown to induce phospholipase C activity (197, 198). Additionally, a study by Wargo determined that mutants defective in catabolism of choline to glycine betaine displayed a diminished capacity to infect mouse lungs (199). Phosphatidylethanolamine (PE) is another sputum lipid that likely promotes P. aeruginosa
virulence. A study by Kearns and colleagues demonstrated that *P. aeruginosa* is capable of twitching motility-mediated chemotaxis up a PE gradient and that the rate of twitching motility was increased in the presence of this nutrient (200). The role this movement may play in *P. aeruginosa* virulence is not entirely clear, but the fact that the organism displayed chemotaxis toward both host- and microbe-derived PE suggests it may be both a signal for movement toward host cells, and/or an auto-aggregative signal (200). Subsequent work by Barker and colleagues characterized a novel PE phospholipase that is required for this chemotaxis (100).

A final example of sputum lipids involved in *P. aeruginosa* pathogenicity is the sphingomyelin degradation product ceramide. A study by Grassme and colleagues demonstrated that the presence of *P. aeruginosa* stimulates the release of ceramide, which promotes formation of sphingomyelin rafts that are critical for internalization of *P. aeruginosa*, inducing apoptosis, and regulating the release of cytokines (201). A subsequent study by the same group found that the ceramide accumulates with age in the airways of CF mice, and this accumulation of ceramide was correlated with increased susceptibility to *P. aeruginosa* infection (202). Enhanced susceptibility of these mice to infection is likely due to ceramide-induced cell death, which releases DNA on the epithelium and facilitates *P. aeruginosa* colonization (202). The complex roles of sphingolipids and ceramides in the CF lung was reviewed recently by Becker and colleagues (203).

**DNA**

DNA was known to be present in the polymeric extracellular matrix (ECM) that coats *P. aeruginosa* biofilms, but a significant study by Whitchurch and colleagues demonstrated that DNA is actually required for effective biofilm formation (204, 205). Much of the DNA in sputum has been attributed to lysed host cells (40), though *P. aeruginosa* is also known to release DNA for biofilm formation (206). A study by Walker and colleagues demonstrated that the presence of lysed neutrophil DNA and F-actin enhances *P. aeruginosa* biofilm formation (207), and these DNA-containing biofilms appear to be more resistant to antimicrobial peptides and aminoglycosides (208, 209). DNA secreted from neutrophils as neutrophil extracellular traps (NETs) may also sequester neutrophil derived proteases, and it has been argued that DNase treatment releases these proteases (210), thus like the ECM of biofilms, the presence of DNA protects *P. aeruginosa* in the CF lung. These authors also suggest DNase treatment could be accompanied by protease and/or elastase inhibitors to more effectively combat *P. aeruginosa* CF lung infections (210).

**Organic Acids and Carbohydrates**

Work from our group indicated that lactate is among six preferred carbon sources that are readily consumed by *P. aeruginosa* in synthetic sputum medium, and the pathogen is known to readily utilize organic acids as sources of carbon and energy (15). This may be particularly relevant in sputum as a study by Wolak and colleagues found that an increase in lactic acid in sputum correlated with increased inflammation; though, it is not clear if this lactic acid increase is due to the inflammation, the generation of hypoxic areas in the lung, or some combination of the two (48). Another study by Bensel and colleagues found that lactate levels decreased with antibiotic treatment of exacerbations (47). Organic acids have been also implicated in *P. aeruginosa* pathogenicity as a study by Petrova and colleagues indicated that the presence of pyruvate and its fermentation induces microcolony formation (211). The authors argued that pyruvate fermentation is an adaptive strategy used by *P. aeruginosa* in the CF lung (211), and this is consistent with work by Eschbach and colleagues, which demonstrated that pyruvate fermentation is important for long-term survival under anaerobic conditions, such as the hypoxic zones with in the CF lung (212).

Simple and modified sugars also appear to affect *P. aeruginosa* virulence and persistence as growth of *P. aeruginosa* on glucose is known to affect biofilm formation. Klausen and colleagues noted that several studies have reported that glucose promotes formation of heterogeneous, mushroom-shaped microcolonies, while growth on citrate or amino acids results in dense, flat biofilms (213–215). Other studies have shown that simple sugars like fructose, galactose, mannose, and fucose, mitigate *P. aeruginosa* infections by inhibiting attachment and diminishing lung damage (216, 217). Aminoglycosides are a common class of antibiotics used to treat *P. aeruginosa* infections in the CF lung, and several studies have indicated that subinhibitory concentrations of these drugs (and other antibiotics) may enhance *P. aeruginosa* biofilm formation and virulence (218–220).

Studies from our own group have indicated the importance of another amino-modified sugar, N-acetylglucosamine (GlcNAc), for *P. aeruginosa* pathogenicity. These studies began with the observation that genes for GlcNAc catabolism were up-regulated when *P. aeruginosa* was grown in CF sputum medium, suggesting
both that GlcNAc is present in sputum and that it is a relevant carbon source for P. aeruginosa in the CF lung (71). Subsequent work by our group determined that GlcNAc is indeed present in CF sputum at hundred micromolar levels, and that it cues enhanced production of pyocyanin (49). There are many potential sources of GlcNAc in sputum and additional work by Korgaonkar and colleagues used colonization of P. aeruginosa in the fly gut to demonstrate that shedding of the GlcNAc-containing polymer peptidoglycan by Gram-positive bacteria is responsible for enhanced virulence factor production (104). The authors also identified a gene (PA0601) required for GlcNAc sensing, and found that mutants unable to sense GlcNAc were less competitive when co-infected with S. aureus in a mouse chronic wound model (104). These studies emphasize the importance of studying pathogens in the context of co-infection with other microbes since most infections in nature are polymicrobial, including the CF lung. Finally, while it appears that GlcNAc activates virulence and makes P. aeruginosa more competitive in the CF lung, other studies have indicated that the GlcNAc-containing polymer hyaluronic acid is antagonistic to the pathogen (221–223).

Amino Acids

The many proteases released into sputum by host and bacterial cells result in high levels (~20 mM) of free amino acids in the infection site. Work from our own group has demonstrated that aromatic amino acids are both a significant source of carbon and energy in sputum, but also cue enhanced virulence factor production in a QS-dependent manner (15, 71). While growing P. aeruginosa in genuine CF sputum medium, our group noticed enhanced production of the QS-signal PQS, which regulates a range of virulence factors (71). This combined with the observation by Farrow and colleagues that tryptophan enhances PQS production by serving as a source of anthranilate for PQS biosynthesis, led us to question whether aromatic amino acids could serve as metabolic cues for PQS production (71, 224). We tested this hypothesis by measuring the concentration of many nutrients in sputum samples, finding essentially no free tryptophan was present, though phenylalanine and tyrosine were present at hundred micromolar levels (15). Enhanced production of PQS in SCFM was abolished when these aromatic amino acids were replaced with equimolar amounts of serine (15). Subsequent work in our group has determined that enhanced PQS production in the presence of aromatic amino acids is not due to co-regulation of aromatic amino acid biosynthesis and PQS production (225). Thus, we favor a hypothesis in which the presence of aromatic amino acids results in altered central metabolite flux toward PQS biosynthesis (15, 225). When aromatic amino acids are present, their biosynthesis is feedback inhibited, resulting in greater flux of the central metabolite chorismate toward PQS. When aromatic amino acids are absent, more chorismate must be channeled toward synthesis of these critical amino acids, resulting in less PQS biosynthesis. Given that PQS signaling has been demonstrated to be critical for virulence in several animal models (116, 226, 227), we believe that enhanced PQS production in sputum is a powerful contributor to enhanced P. aeruginosa virulence in the CF lung.

In addition to aromatic amino acids, several other amino acids have been identified as cues for P. aeruginosa pathogenicity in sputum. Sriramulu and colleagues used artificial sputum medium (ASM) supplemented with amino acids (ASM+) resulted in “tight” microcolonies, while the standard ASM- medium resulted in “loose” microcolonies (164). A later study by Bernier and colleagues may explain this phenomenon as they used physiologically relevant concentrations of amino acids to demonstrate that arginine, ornithine, isoleucine, leucine, valine, phenylalanine and tyrosine promote P. aeruginosa cyclic di-GMP-mediated biofilm formation (228). Among these amino acids, arginine also inhibited P. aeruginosa swarming motility, suggesting that it is a potent nutritional cue for promoting the antibiotic-resistant, sessile lifestyle believed to be common in the CF lung (228).

After demonstrating that alanine was a preferred source of carbon and energy in sputum (15), our group has gone on to characterize alanine catabolism in P. aeruginosa, and we demonstrated that a mutant unable to catabolize DL-alanine was diminished in its capacity to cause infection in a rat lung infection model (229). A study by Rau and colleagues demonstrated that leucine catabolism genes were up-regulated in longitudinal clinical isolates of P. aeruginosa from individuals with CF (129). While leucine was not a preferred carbon source in SCFM (15), it was present at high levels (low millimolar), and the authors suggest optimization for growth on leucine may play a role in maintaining P. aeruginosa infections in the CF lung (129). Finally, Kohler and colleagues noted that growth with glutamate, aspartate, histidine, or proline as the sole source of nitrogen promoted P. aeruginosa swarming motility (230), which may also impact colonization and maintenance.
FUTURE AREAS OF RESEARCH

P. aeruginosa infections in the CF lung are the leading cause of morbidity and mortality for individuals with this genetic disease (1, 2), and we have seen that a great deal of research has been conducted to better understand the basic physiology of these infections. The importance of nutritional cues in sputum for P. aeruginosa is rapidly becoming more appreciated. As a consequence, the field is working to develop better in vitro and in vivo models that more accurately represent the nutritional environment of CF sputum. With a renewed emphasis on studying the nature of these infections in situ and newly available technologies for studying these infections, the future of these studies appears bright. This section will highlight some new areas of research, including experiments involving next-generation sequencing technologies, examining multispecies interactions in the CF lung, and the possibility of metabolism-based therapeutics for eradicating these infections.

Next-generation Sequencing Technology

Next-generation sequencing technologies are revolutionizing many groups’ approaches to understanding P. aeruginosa infections. The ability to rapidly and inexpensively sequence bacterial genomes has resulted in a blossoming of available draft genome sequences (231–233). Another recently available technology, RNA-seq, involves sequencing of cDNA generated from total RNA preparations and mapping those sequence reads back to the genome (234–236). This type of transcriptome profiling has advantages over other methods like DNA microarrays in that it accounts for transcripts not present on a gene chip, can easily identify small RNAs, antisense transcripts, transcriptional start sites, and importantly, can be used on more than one species at a time (i.e. to study the host and pathogen or multispecies infections) (234–236). Some examples of RNA-seq transcriptome profiling experiments include investigation of planktonic versus biofilm growth (237), global identification of small RNAs (238), investigation of QS-regulated gene expression from different strains (239), and determining the effect of temperature on virulence (240).

Transposon insertion site sequencing (Tn–seq) is another example of a transformative technique that takes advantage of next-generation sequencing technology. Tn-seq can be used to identify fitness determinants by generating a saturated library of insertion mutants and counting the relative numbers of these mutants present after applying a selective pressure (e.g., mutant pool is grown in a mouse infection model) (241, 242). Insertions that are over-represented in the output pool represent genes that are not required for fitness, while insertions that are under-represented or absent in the output pool are important for fitness. Using this kind of approach, Skurnik and colleagues determined the contribution of every nonessential gene in the P. aeruginosa genome toward fitness in a murine model infection (242). Additional Tn-seq work by Skurnik and colleagues identified P. aeruginosa mutants in oprD that in addition to being carbapenem-resistant, also displayed an enhanced survival in a murine model infection (241). As we will see in the next section, several groups are also taking advantage of next-generation sequencing technologies to survey the microbiome of the CF lung, and these studies are generating a greater appreciation for the role that multispecies interactions play in CF lung infections.

Polymicrobial Infections and Multispecies Interactions

Several studies have used next-generation sequencing technology to examine diversity of the CF airway microbiome (243–250). In general, these studies have reported considerable numbers of organisms, which is changing the perception that CF lung disease is dominated by handful of common opportunistic pathogens, like P. aeruginosa, S. aureus, Haemophilus influenzae, and Burkholderia cepacia. On the other hand, a recent report by Goddard and colleagues that compared 16S rRNA pyrosequencing data from sputum samples taken from transplanted lungs to those taken from throat found that much of the diversity observed in other microbiome surveys could be explained by oropharyngeal contamination (247). Regardless of how diverse the CF microbiome may be, it is clear that these infections are not monocultures, and several groups have started investigating multispecies interactions in the CF lung. A study by Hubert and colleagues found that individuals co-colonized with P. aeruginosa and S. aureus displayed greater declines in respiratory function than those colonized with S. aureus alone (251). Another example of these types of interactions comes from a study by Twomey and colleagues, which demonstrated that diffusible signaling factors from Burkholderia cenocepacia and/or Stenotrophomonas maltophilia were present in CF sputum samples, promoted P. aeruginosa persistence in a mouse CF model, and enhanced antibiotic resistance of P. aeruginosa biofilms grown on airway epithelial cells (252). Studies from our own group have shown that the presence of other pathogens can enhance
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*P. aeruginosa* infections (104, 170). As more of these cross-species interactions are delineated, we will have a better understanding of the entirety of the infection that causes CF lung disease, and these discoveries will likely lead to novel approaches for mitigating these infections.

**Metabolism-based Therapeutics in the CF Lung**

As we continue to learn more about the role of metabolism in *P. aeruginosa* infections in the CF lung, several targets for metabolism-based therapeutics have been identified. We have already seen that inhibition of phospholipase activity by miltefosine protects pulmonary surfactant from *P. aeruginosa* degradation in mouse model infections, and depriving *P. aeruginosa* of lipids as potentially significant carbon sources may contribute to the drug’s positive effect in the CF lung (193). While it is obvious that the activity of degradative enzymes in sputum could be culpable for enhanced *P. aeruginosa* virulence, the use of recombinant human DNase (rhDNase) as an inhaled therapeutic capable of breaking up sputum cross-linked by DNA improves outcomes in individuals with CF (41, 44, 45). The successful use of this therapeutic suggests that other enzymes capable of degrading key nutrients in sputum may also help mitigate CF lung disease. rhDNase treatment combined with an alginase lyase that degrades the extracellular matrix of mucoid biofilms, enhanced aminoglycoside activity in CF sputum (253). Our own studies of the role phenylalanine and tyrosine play in cueing virulence factor production in the CF lung suggest that degradation of these nutrients could mitigate the capacity of *P. aeruginosa* to damage lung tissue (14). The phenylalanine- and tyrosine-degrading enzyme phenylalanine ammonia lyse from *Anabaena variabilis* is currently being prepared for use as a therapeutic to treat the buildup of phenylalanine associated with the genetic disease phenylketonuria (254–256). A therapeutic like this may also help to treat *P. aeruginosa* infections in the CF lung. The advantage of these metabolically-based therapeutics is that, unlike traditional antimicrobials which kill or inhibit the growth of a pathogen, these new therapeutics alter the environment around bacteria to diminish virulence. This removes the immediate incentive to evolve/acquire resistance. As the rise of antibiotic-resistant strains continues to threaten the global health community, it will require alternative approaches like metabolism-based therapeutics to develop the antibiotics of the future, and this makes a strong case for studying the metabolism and physiology of all infections.

**SUMMARY AND CONCLUSIONS**

This chapter has reviewed the current state of knowledge regarding how nutritional cues in CF sputum affect *P. aeruginosa* colonization and persistence. These devastating, life-long infections are incredibly difficult to treat, and the nutrients available in CF lung secretions have a profound influence on the infection. The nutritional contents of CF and healthy sputum were compared, and the struggles to generate both *in vitro* and *in vivo* systems for recapitulating chronic *P. aeruginosa* infections were described. Specific nutritional cues in sputum, such as mucin, lipids, DNA, and amino acids, promote *P. aeruginosa* virulence. The future of research in this field was discussed with particular emphasis on the ways in which next-generation sequencing technologies are driving our capacity to study *P. aeruginosa* infections systematically, and importantly, examine the polymicrobial nature of these infections. The potential for new antimicrobial therapeutics based on knowledge gained from these metabolic studies is also of interest, given the rise of strains resistant to traditional antibiotics. While much is already known about *P. aeruginosa* infections of the CF lung, these infections continue to be incredibly difficult to defeat. However, continued study of *P. aeruginosa* metabolism at the site of infection has significantly enhanced understanding of the physiology of these infections and substantially increased the likelihood that this organism will cease to be a problem in the future.

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