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PERSISTENT INFECTIONS AND IMMUNITY IN CYSTIC FIBROSIS

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1. ABSTRACT

Cystic fibrosis (CF) is the most common autosomal recessive lethal disease in the Caucasian population. Chronic respiratory infections with *Pseudomonas aeruginosa*, neutrophil-dominated airway inflammation and progressive lung damage are the major causes of morbidity and mortality in CF. Two persistent infection phenotypes expressed by this bacterium are *biofilm* and *mucoidy*. Biofilm, also called the microcolony mode of growth is the surface-associated adherent bacterial community, while mucoidy refers to a phenotype conducive to copious amounts of mucoid exopolysaccharide (MEP)/alginate that provides a matrix for mature biofilms conferring resistance to host defenses and antibiotics. Recent completion of the whole genomic sequence of the standard reference strain *P. aeruginosa* PAO1 has led to discoveries that many clinical isolates of this species possess unique genomic sequences (genomic islands) due to horizontal gene transfer. We propose this type of genetic exchange may play an important role in causing intrinsic genomic diversity of this organism. Therefore, the diversity, as revealed through profiles of restriction fragment length polymorphism (RFLP), may be linked to an array of novel and unexplored pathogenic mechanisms in *P. aeruginosa*. CF mouse models, while displaying many clinical similarities to human CF, have yet to demonstrate a chronic pulmonary disease phenotype. This review is intended to provide an overview of *P. aeruginosa* persistent infection phenotypes (biofilm and mucoidy) and an aerosol infection mouse model for CF. Genomic diversity of *P. aeruginosa* and its implications in the pathogenesis in CF will also be discussed.

2. CYSTIC FIBROSIS

Approximately 30,000 children and adults in the United States have CF. One in 31 Americans, more than 10 million people are asymptomatic carriers of the mutant CF gene. Despite many promising advances in CF medicine, the median life expectancy for CF patients is 30 years (1). Like other genetic diseases, the predisposing factor is a mutation of an otherwise functional gene. The CF gene has been identified, and the gene product is named the CF transmembrane conductance regulator (CFTR) (2). Since the initial discovery, close to 1,000 different types of disease-causing mutations within this gene have been documented (http://www.genet.sickkids.on.ca/). By far the most common, which accounts for almost 70% of all known mutations is a three base pair deletion resulting in the removal of a phenylalanine residue at position 508 of CFTR (deltaF508).

CFTR is a multifunctional protein located on the apical plasma membrane. It is an anion channel and/or a channel regulator that controls the flow of Cl^-, Na^+ and other ions across the membrane. Defects in CFTR result in disruption of normal Na^+ and Cl^- ion transport (3). Recently, CFTR has been linked with the control of Cl^--coupled bicarbonate concentration across the lumen of the

lung epithelium for maintaining the proper acidity (4). The cellular defects associated with the *CFTR* mutations cause many clinical problems, but the majority are confined to those organs rich in exocrine glands such as respiratory, gastrointestinal and genitourinary tracts (5). Approximately 85% of CF patients are characterized with pancreatic insufficiency (PI) while 10–15% of them have a clinical condition called meconium ileus (MI), an intestinal blockage due to malabsorption (6). Patients homozygous for the deltaF508 mutation tend to have more severe pancreatic insufficiency than those carrying other types of mutations (6). Conditions such as vitamin deficiency (A, D, E and K) and chronic malnutrition are common in CF (7). The clinical manifestation of CF is mainly demonstrated through involvement of the respiratory tracts. The electrolytic imbalance combined with acidic pH due to defective bicarbonate transport results in changes in the composition of airway surface liquid (ASL), a thin layer of fluid that covers the upper and lower respiratory tract, and retention of an abnormal dehydrated viscous mucous within the lung (4). These conditions leave the CF lungs prone to becoming infected with microbial organisms (8).

Pulmonary infections in CF are associated with chronic colonization by several bacterial pathogens and debilitating exacerbations as a result of bacterial and viral infections superimposed upon progressive lung damage (9). Children with CF are often infected by *Staphylococcus aureus* and *Haemophilus influenzae*, both within the patient's first 3 years (10). Since the development of aggressive antibiotic therapy, these organisms can be treated before any long-term damage is inflicted. While both *S. aureus* and *H. influenzae* will re-emerge in the lung throughout the remainder of the disease, *P. aeruginosa* ultimately becomes the major inhabitant for the remainder of the CF patient's life (9). In fact, nearly all CF patients will show evidence of *P. aeruginosa* colonizations by age 3 (11). Chronic lung infections with *P. aeruginosa* are responsible for the majority of early deaths in CF (12).

Though CF biogenesis is clear, the relationship between CFTR mutations and pulmonary disease is elusive. Particularly, how the genetic defect leads to the establishment of chronic bacterial colonization in the lungs is not fully understood. Several concurrent and sometimes conflicting proposals have been offered (13-18) to address this relationship. For example, reduced sialylation of glycoconjugates on the surface of epithelial cells has been suggested to promote *P. aeruginosa* adhesion in CF (18). However, a recent paper has questioned the role of this glycolipid as the receptor for fresh clinical isolates of *P. aeruginosa* since the antibodies raised against asialo-GM1 for confirming its interaction with *P. aeruginosa* also bind to multiple *P. aeruginosa* surface antigens (19).

The CF epithelial secretions have been reported to display reduced bactericidal properties due to the altered salt content (14, 17). CFTR has also been proposed to be involved in *P. aeruginosa* uptake by respiratory epithelial cells (16). These models rely mainly on the known functions of *CFTR* as an ion channel and/or channel regulator (6) and on its presumed pleiotropic effects (20). Other studies, focusing on cytokine profiles in broncho-alveolar lavage fluids of CF patients, have suggested that the propensity for excessive inflammation in CF may be attributed to endogenously increased levels of proinflammatory cytokines such as tumor necrosis factor (TNF)-alpha, interleukin (IL)-8, and IL-1 (13), and concurrent decreased level of anti-inflammatory cytokine IL-10 (105). According to some reports, the levels of these cytokines may be altered in CF even before a bacterial infection begins (15, 21). Despite these promising leads, our understanding of host-pathogen interactions in CF is minimal. Even though the most obvious physiological defect in CF is linked with ion transport, pathological consequences due to a *CFTR* mutation appear to be more complex.

3. *P. AERUGINOSA*

P. aeruginosa is a ubiquitous Gram-negative environmental bacterium that can grow in a wide variety of moist habitats such as soil, rivers, hospital sinks and oil fields and has an extremely versatile metabolic demand. This organism can colonize humans, invertebrates, insects, and plants. It is considered an opportunistic pathogen since the infections only occur in individuals with underlying compromised host defense systems (22). Besides being associated with fatal pneumonia in CF, *P. aeruginosa* can cause other types of infections ranging from acute and systemic bacterimia to chronic catcher-associated urinary tract infections (8).

The genome of the *P. aeruginosa* strain PAO1 has recently been completely sequenced and released to the public domain (http://www.pseudomonas.com/) (figure 1 and ref. 23). A quick comparison of this genome to a selected group of bacterial genomes is listed in table 1. The genome size of *P. aeruginosa* is the largest among the currently available pathogenic bacteria in GenBank, and has a coding capacity for 5,570 open reading frames (ORF's; ref. 23). Initial analysis of the PAO1 genome suggests the overall gene regulation is more complicated than that of the *E. coli* strain MG1655. For example, the number of the two-component signal transduction systems in PAO1 is 144 (23 per Mb) whereas that of *E. coli* is only 62 (13 per Mb; ref. 23). The inherent drug resistance of this pathogen can be in part attributed to the increased number of efflux pumps encoded within the genome (97). The *P. aeruginosa* strain PAO1 was originally isolated from a wound (24), and is considered a standard reference strain well adapted to the laboratory growth conditions. The O5 serotype to which PAO1 belongs represents a small fraction (2.5%) of the clinical CF isolates surveyed during the late 1980's (personal communication with Dr. Joseph Lam). According to the pulsed-field gel electrophoresis (PFGE) analysis, the genomic profile of PAO1 is not similar to that of the majority of the clinical CF isolates. Therefore, it is unclear how representative this genome is compared to those of clinical isolates since the number of mobile genetic elements (genomic islands, phages and transposons) within each genome of clinical origin remains unknown.

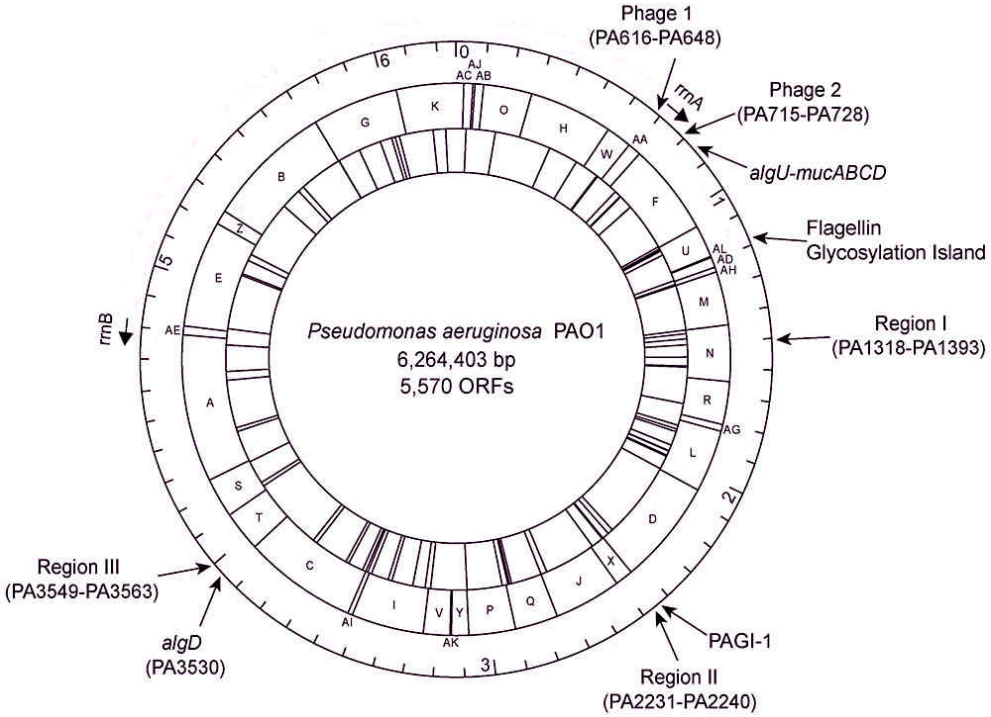

Figure 1. Schematic diagram of the entire genome of *P. aeruginosa* strain PAO1 according to http://www.pseudomonas.com/. The scale on the outmost circle is in Mb. Outer and inner circles consist of *Spe*I and *Xba*I digested fragments assembled in order, respectively. Known *Spe*I fragments (also see table 2) are labeled in the outer circle with respective alphabetic letters. Locations of 2 probable bacterial phages (23), *P. aeruginosa* genomic island-1 (PAGI-1) (78), a flagellin glycosylation island (77), and 3 genetic loci (23) proposed to be involved in the regulation of alginate production along with *algD* and *algU* loci are noted.

4. CHRONIC INFECTION PHENOTYPES: BIOFILM AND MUCOIDY

In response to changing environments, microorganisms have evolved various adaptive mechanisms and as a result they often express different phenotypes. Bacterial biofilms are one of the persistent infection phenotypes (1). *P. aeruginosa* biofilms and CF chronic pulmonary infections are closely related (25, 26). Biofilm is often defined as exopolysaccharide-surrounded bacteria, or microcolonies, growing on biotic or abiotic surfaces (27). Formation of biofilms has been shown to be the preferred mode of bacterial growth in nature as the sessile population exceeds that of planktonic (free floating) biomass by 2-4 $\log_{10}$ units (28). The two modes of growth complement each other; the mobile phase provides a means for spread and colonization, while the biofilms afford protection against protozoans, phages, and antibiotics in environments (28).

Biofilm formation is a dynamic and complex process that can be roughly divided into four phases (1): i) initial attachment to the surface (transition from planktonic cells to transiently attached cells); ii) cell proliferation forming a monolayer; iii) formation of a microcolony; and iv) development of mature biofilms. In terms of biofilm formation within the CF lungs, it is still not fully understood how this process is initiated. Even so, a hypothetical and perhaps oversimplified scheme is presented in figure 2. Based on several studies monitoring biofilm formation on abiotic surfaces, motility is important for the initial attachment to the surface since it provides the forces required to overcome the surface repulsion for bacteria (29). Type IV pili mutant of *P. aeruginosa* forms thin and undifferentiated monolayers, but fails to differentiate into microcolonies (30). Other factors may also be required since a hyperpiliated *P. aeruginosa* strain without twitching ability is still able to form thick biofilms (31). Some surface-associated proteins such as curli and outer membrane proteins (32) also play a role in the initial attachment. Very recently, through screening the Tn5 insertion library for surface adhesion deficiency in the type IV pili mutant background of *P. aeruginosa* PAK, Vallet *et al*. (33) reported the identification of a novel chaperone/usher pathway involved in the assembly of new classes of adhesins that are needed for early biofilm formation, suggesting that multiple adhesion factors are present on the cell surface of *P. aeruginosa*. Furthermore, many global regulators such as the *crc* gene involved in carbon metabolism (34) and polyphosphate kinase (35), and more recently, a two-component regulatory system GacA/GacS (36), are also known to participate in the initial biofilm formation.

After initial attachment, *P. aeruginosa* biofilm formation requires participation of a cell density-dependent response, known as quorum sensing consisting of two distinct but interrelated systems, *las* and *rhl* (37). These

Table 1. Completed genomes from a representative group of pathogenic bacteria[1]

Organism	*Genome size*	*ORF #*	*Accession #*	*Release Date*
H. influenzae Rd	1,830,138	1,709	NC_000907	07/25/95
E. coli MG1655	4,639,221	4,289	NC_000913	10/13/98
Mycobacterium tuberculosis H37Rv	4,411,529	3,918	NC_000962	6/11/98
Vibrio cholera O1 El Tor	4,033,464	2,736 1,092	NC_002505 NC_002506	6/14/00
P. aeruginosa PAO1	6,264,403	5,570[2]	NC_002516	5/16/00
E. coli O157:H7	5,529,376	5,283	NC_002655	1/25/01
M. leprae	3,268,203	2,720	NC_002677	2/20/01

[1] based on http://www.ncbi.nlm.nih.gov/PMGifs/Genomes/bact.html, [2] based on http://www.pseudomonas.com/

systems work coordinately to regulate a number of factors that enable this pathogen to survive in highly diverse environments. Two molecules produced are *N*-(3-oxododecanoyl)-L-homoserine lactone ($3OC_{12}$-HSL) and *N*-butyryl-L-HSL (C_4-HSL), which serve as signals for regulation of the cell-to-cell communication (38, 39). Particularly, $3OC_{12}$-HSL is involved in microcolony differentiation (40). Recently, using a sensitive radiometric assay coupled with a reverse phase high performance liquid chromatography to directly measure the level of acyl-HSLs, Singh *et al.* (26) reported that CF sputum carrying *P. aeruginosa* has more C_4-HSL than $3OC_{12}$-HSL, which is similar to PAO1 grown in the biofilm mode, suggesting that C_4-HSL may serve as a biomarker for mature biofilms (figure 2). Although alginate helps to maintain three dimensional structure of biofilms, alginate per se does not appear to be required for the initial *P. aeruginosa* biofilm development (31, 41). Furthermore, the wild type and *lasI* mutant of PAO1 produce an equal amount of alginate when forming biofilm *in vitro* (40). This appears to support the notion that control of alginate production is independent of at least $3OC_{12}$-HSL mediated quorum sensing pathway. Therefore, the initial biofilm formation and MEP based biofilms may represent two separate phenomena and/or two different stages of biofilm development. To distinguish the initial biofilm formation from those microcolonies embedded with a mucoid coat as commonly seen in CF, we introduce a term called "mature" biofilms to describe those covered with excessive amounts of alginate (figures 2 and 4).

The major component of the mucoid capsule produced by *P. aeruginosa* is alginate that consists of negatively-charged polyuronic acid (9). During chronic respiratory tract colonization, a subset of initial nonmucoid colonizing *P. aeruginosa* strains may acquire mutations causing conversion to mucoidy (42). The transition from the initial non-mucoid strain to the mucoid variant has recently been reproduced *in vitro* with repeated exposure to a sublethal concentration of hydrogen peroxide, a common oxidant in polymorphonuclear neutrophils (PMN's) (43). Overproduction of alginate in *P. aeruginosa* facilitates development of mature biofilms that contribute to immune evasion and antibiotic resistance (figures 2 and 4) (1). This unique phenotype (figure 3) is the main diagnostic indicator for the chronic onset of *Pseudomonas* lung infections in CF (44). The mucoid phenotype is usually unstable, but it is possible to maintain this phenotype *in vitro* using certain laboratory media (figure 3). Mucoidy is of importance to the persistence of *P. aeruginosa* in CF. Protective, opsonizing antibodies against alginate are present only in uncolonized patients (45). The mucoid variant can influence the pulmonary outcome in CF patients (46). Alginate also has other pathogenic roles such as inhibition of phagocytosis, suppression of neutrophil chemotaxis, and scavenging of oxidative radicals (9).

Persistence of *P. aeruginosa* and mucoidy are two inseparable clinical conditions since emergence of mucoid phenotype in CF-affected lungs correlates with onset of pulmonary function deterioration (47). One of the molecular mechanisms related to mucoidy conversion is up-regulation of an alternative sigma factor (AlgU/AlgT) that in turn activates expression of *algD*, the biosynthetic gene for alginate production (48). AlgU, a stress-related sigma factor with extracytoplasmic function (ECF), is negatively regulated by its immediate downstream gene cluster, *mucABCD* where MucA is the cognate antisigma factor for AlgU (48). The AlgU ortholog in *E. coli* is RpoE ($sigma^E$) (66% identity and 91% overall similarity). The *E. coli rpoE* gene can functionally complement the *algU* mutation and restore mucoidy in *P. aeruginosa* (49). Furthermore, up-regulation of *algU* inhibits flagellum synthesis (50). Recently, we have found that inactivation of *algU* and *rpoE* in *P. aeruginosa* PAO1 and *E. coli* K12, respectively, could lead to increased flagellar activity in both organisms, thereby causing increased initial biofilm formation *in vitro* and *in vivo* (51). Conversely, overproduction of $sigma^E$ in *E. coli* K12 causes reduced initial biofilm formation (51). This is interesting since an earlier report indicates that inactivation of *algU* in PAO1 caused increased virulence in a systemic infection mouse model (52). This elevated virulence as a result of *algU* inactivation may be due to increased initial colonization of the mutant. Two recent analyses using proteomics to compare global protein expression coupled with mucoidy indicate that production of several proteins such as outer membrane protein porin F (OprF) and disulfide bond isomerase (DsbA), which may not be involved in alginate production, is affected (53, 54). These candidates may play a role in induction of mucoidy-coupled host-derived human beta-defensin (55). As a result of the bioinformatic analysis of the PAO1 genomic sequence, three additional genetic loci in this genome (figure 1) have been proposed

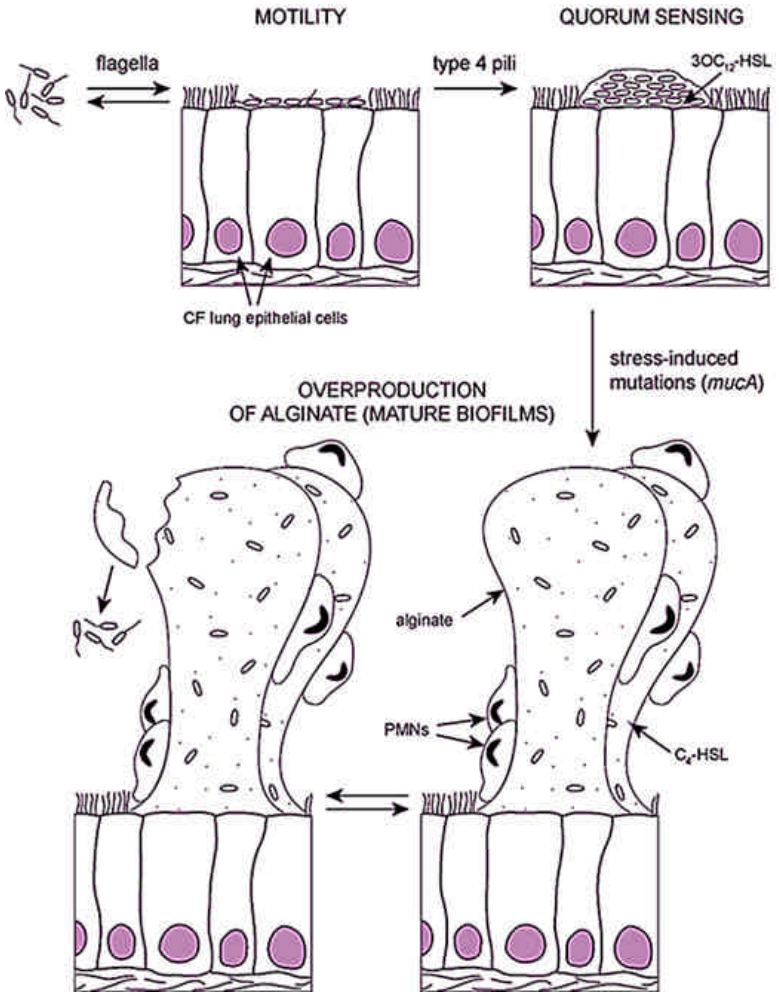

Figure 2. A hypothetic process of *P. aeruginosa* colonization leading to biofilm formation in CF. Flagella-mediated motility is involved in the initial attachment (30, 51). Type 4 pili (30) and a quorum sensing signal ($3OC_{12}$-HSL) (40) participate in microcolony differentiation. Overproduction of alginate, which results in the formation of mature biofilms, can be caused by the *mucA* mutations induced through stresses such as oxidants released by PMN's (43). The level of C_4-HSL is increased in mature biofilms (26). Sessile cells can be sloughed off as planktonic cells due to mechanical factors (ex. coughing). Biofilms formed as a result of mediations through motility, quorum sensing and overproduction of alginate may be different from each other physiologically, and may represent various snapshots of a bacterial developmental process.

to be involved in production of exopolysaccharide in *P. aeruginosa* (23). It will be interesting to investigate if any of these genes may indeed be required for alginate regulation since there is an alternative AlgU-independent pathway that involves RpoN (sigma^{54}) for the control of MEP production (56).

5. GENETIC DIVERSITY AND GENOMIC ISLANDS

The genomes of the clinical CF and environmental isolates (figure 5) of *P. aeruginosa* are highly polymorphic. This phenomenon is also referred to as genomic diversity (57-59). From an evolutionary viewpoint, genomic diversity enhances metabolic versatility of the organism. To investigate the diversity, a DNA scoring method is often used, which depends on macrorestriction digestion of the chromosome coupled with physical separation of DNA fragments in a PFGE system (60). The distinctive banding pattern generated in this system, known as RFLP (figure 5), represents a unique genetic fingerprint and once assembled, reflects the overall genomic structure of each isolate. Besides being used for investigation into the diversity (57-59, 61), the *P. aeruginosa* RFLP profiles coupled with linkage analysis have been applied towards construction of a genomic map (62-65), comparative genomic analysis (66), and investigation of genetic differences among clinical isolates (67). The RFLP profiles have also been utilized for epidemiological analysis to track the sources of clinical isolates (68-70). Another common DNA scoring technique, based on the polymerase chain reaction (PCR), is random amplification of polymorphic DNA (RAPD) which has been applied for a similar purpose (71, 72). While these methods provide a quick means to generate

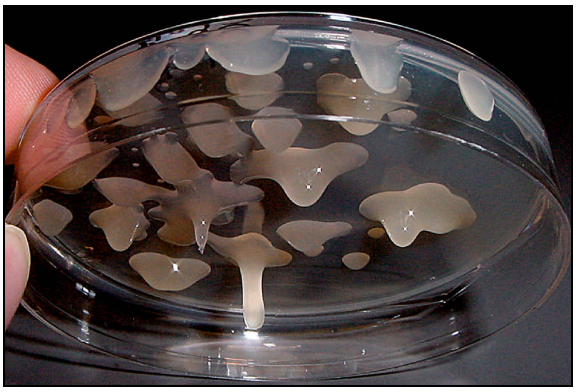

Figure 3. A persistent infection phenotype expressed by *P. aeruginosa* due to the excessive production of alginate. Alginate helps to maintain the three dimensional architecture of mature biofilms known to be resistant to host defenses and antibiotics. Shown is a mucoid colony morphology from a clinical CF isolate of *P. aeruginosa* growing on a laboratory media.

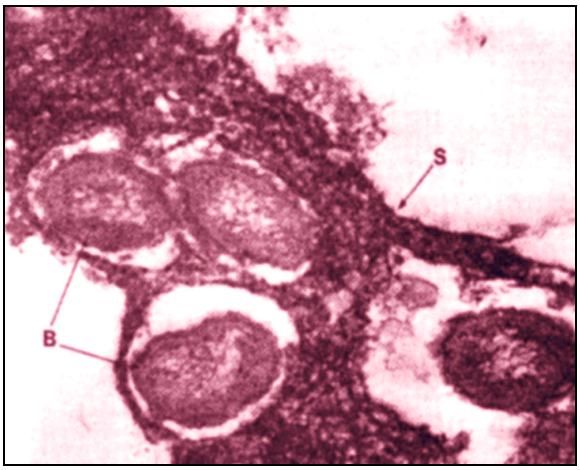

Figure 4. Transmission electron micrograph of a thin-section of a postmortem lung sample from a CF patient. Shown is *P. aeruginosa* in mature biofilms, a persistent infection phenotype closely associated with chronic bacterial lung infections in CF. *P. aeruginosa* is embedded within a mucous matrix produced by bacteria and host [Reproduced with permission from Lam *et al*. (25)].

genomic comparison, they are often limited by lack of information on individual DNA fragments produced within these systems.

The availability of the PAO1 genomic sequence facilitates utilization of PFGE more effectively to study genomic diversity among various clinical isolates. For example, it has been previously reported that complete digestion of the PAO1 genome with restriction endonuclease *Spe*I generated 37 fragments in a PFGE system (64). While the estimation of fragment sizes was not always accurate, one can deduce a precise range of fragments based on the PAO1 genomic sequence. These bands, listed in table 2, may be used as a standard reference for comparison with clinical CF isolates. In our initial PFGE analysis of 101 clinical CF isolates, which were collected over a wide range of geographical regions and compared with the standard banding pattern of PAO1 (table 2), we identified 75 unique *Spe*I-digested genomic profiles with 23 ± 12 bands (n = 75; 99% confidence interval; ref. 73). Of these isolates, 51% have at least one band significantly larger than the largest fragment of PAO1 (Spe-A = 539,862 bp). We observed 74% genomic diversity among the CF isolates in our collection. This number perhaps under-estimates the genomic diversity of *P. aeruginosa* since the remaining 26% non-unique genomic profiles were sequential, sibling or same-source isolates. It appears that a particular isolate remains fairly stable in its genome after it colonizes the CF lung (figure 5), which is consistent with a previous observation that the majority of CF patients are colonized with one strain (74). However it is possible for a single patient to be colonized by 2 different strains at the same time (71, 74). Horizontal transmission between patients is possible since identical genomic profiles have been seen between CF isolates and an isolate from aquatic habitats (59, 66).

The ultimate cause of genomic diversity that exists throughout clinical CF isolates of *P. aeruginosa* is attributed to mutation (75). One of the virulence mechanisms employed by many pathogenic bacteria is the acquisition of auxiliary DNA sequences from within or between species or even genera (76). There is compelling evidence to suggest that the event of horizontal gene transfer occurs in *P. aeruginosa* (77-79). Mobile genetic elements such as bacterial phages are probably present in the PAO1 genome (figure 1 and ref. 23). Some of the *Pseudomonas* phages have the genetic capacity of serotype conversion (80). Tummler *et al*. (66) were among the first to notice that the genome of one aquatic/CF isolate of *P. aeruginosa* was significantly larger than that of PAO1. A subsequent study with a clinical wound isolate of *P. aeruginosa*, UCBPP-PA14 (PA14), has reported the discovery of several unique chromosomal regions found in this strain but not present in the PAO1 genome (81-83).

Recently, Liang *et al*. (78) have reported the identification of the first genomic island (PAGI-1; figure 1), in the genome of a clinical urinary tract infection isolate *P. aeruginosa* X24509. This genetic island is absent in PAO1, but is present in 85% of the clinical isolates including those from CF patients. It is composed of 48,893 bp with a coding capacity for 51 ORF's. Through recombination, this island replaces a region of 6,729 bp sequence derived from the PAO1 genome that is thought to be prone to genetic alterations. Interestingly, this PAGI-1-replaced locus is located within a previously identified region with unusually low G+C content (PA2221-PA2228, G+C: 49.2% vs. 66.6% overall; figure 1) (23). While the functions of PAGI-1 need to be elucidated, there are at least two potential transcriptional regulators located on the island, one of which is the homologue of RpoN-dependent transcriptional activators. Furthermore, two homologs of *E. coli*, which are known to be involved in counteracting oxidative stress, are also encoded on PAGI-1. Since RpoN has been implicated in the regulation of mucoidy (56) and other surface molecules such as flagella and pili, it is

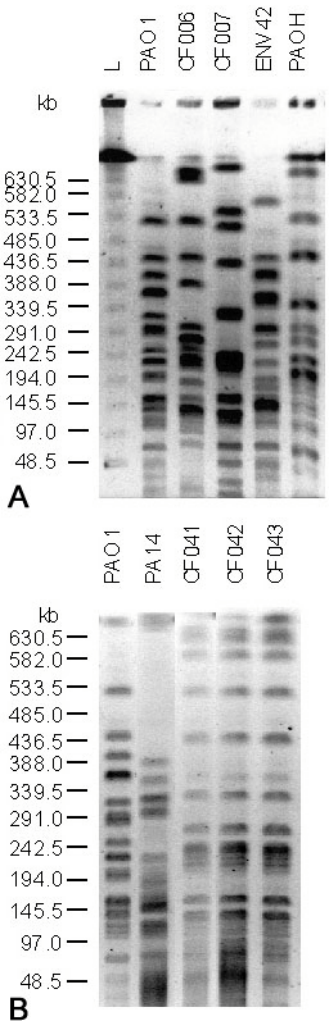

Figure 5. Separation of *Spe*I digested *P. aeruginosa* chromosomal DNA by PFGE. (A). CF006 and CF007, two clinical CF isolates display the genomic diversity that exists throughout the majority of clinical isolates. Environmental strains (ENV42: Japan, and PAOH: Ohio River) of *P. aeruginosa* also exhibit a diverse chromosome. (B). Burn isolates PAO1 and PA14 also possess diversity along the chromosome. Clinical sequential isolation of *P. aeruginosa* shows conservation of genome throughout colonization (CF041: initial isolate in 1990, CF042: isolated in 1994, CF043: isolated in 1998).

conceivable that some genes encoded on PAGI-1 may have specific virulence roles including modulation of chronic infection phenotypes.

The discovery of PAGI-1 suggests that an island of this nature may contribute to causing the genomic diversity observed with PFGE. For example, within the PAGI-1 DNA sequence (GenBank accession# AF241171), there are five positions recognizable by endonucleases *Spe*I and *Xba*I (4 sites) that are often used for PFGE analysis of *P. aeruginosa* genomes. Therefore, a genomic island is linked with a cause of genomic diversity of *P. aeruginosa*. A recent publication of the *E. coli* O157:H7 genome sequence indicates that this virulent strain, unlike the laboratory counterpart *E. coli* K12, has an additional 1.34 Mb encoding 1,387 new genes in 177 O157:H7-specific genomic islands interspersed in a K12 genome (84). Based on the overall sequence homology between *P. aeruginosa* PAO1 and *E. coli* K12 (23), it is reasonable to assume that more genomic islands are to be identified throughout the *P. aeruginosa* genomes. It is known that severity of the pulmonary disease in CF is poorly correlated with the genotype (6). This variability can be attributed to many factors of environmental and/or host origin. Perhaps it can also be caused by factors of bacterial origin through the diversity. Genomic diversity resulting from horizontal gene transfer suggests a possibility of acquisition of novel virulence traits in *P. aeruginosa*. For example, flagella of some *P. aeruginosa* strains are often glycosylated (85). Very recently, it has been reported (77) that another genomic island of ca. 16 kb located immediately upstream of the *fliC* gene encoding the structural flagellin protein is responsible for this action. Although the pathogenic significance of this glycosylation is still unknown, this discovery may become the first example demonstrating that horizontal gene transfer introduces a novel virulence mechanism into this organism.

6. AEROSOL INFECTION MOUSE MODEL

An appropriate animal model is essential for simulating the respiratory tract infection in CF. The availability of *CFTR* transgenic mice has enabled us to investigate CF-related bacterial pulmonary infections in a surrogate host. Currently, there are several genetic mouse models for CF (86-93). These CF mice, while displaying several similar characteristics of intestinal disease in human CF, fail to develop respiratory infections or other signs of overt lung disease. This disappointing limitation of the *CFTR* transgenic mice, has been linked to the presence of alternative Cl^- and Na^+ channels in mice that could compensate for the loss of the *CFTR* lesion (94). No apparent differences in the ionic compositions of ASL have been found between CFTR mutant mice and their littermate controls (95). With the recent development of sensitive methods to directly measure the contents of ASL such as the use of a cryoprobe (96) and staining with fluorescent ion indicators (97), there is still no significant difference in ASL salt content between wild type and CF mice. More importantly, spontaneous colonization with *P. aeruginosa* has not been detected in the CF animals (98). While there are numerous mouse infection models for CF, each model has its pros and cons (8, 99). For example, the agar-bead model, in which *P. aeruginosa* is artificially embedded in agar beads, simulates a situation similar to mature biofilm infection. On the other hand, the aerosol infection model described below may be more useful in modeling the innate host response immediately after initial *Pseudomonas* colonization.

A Bacterial Aerosol Induced Lung Infection (BAILI) mouse model has been developed for simulation of bacterial pulmonary infections in CF (100-102). This model utilizes the aerosol technology originally developed for tuberculosis studies (103). The core of this model is an

Table 2. *Spe*I digest fragments based on the published PAO1 genomic sequence[1]

Genome location (bp)		Fragment size (bp)	Fragment name
4,250,766	- 4,790,627	539,862	A
5,266,156	- 5,726,542	460,387	B
3,538,448	- 3,962,321	423,874	C
2,053,672	- 2,444,424	390,753	D
4,819,853	- 5,206,702	386,850	E
725,196	- 1,062,491	337,296	F
5,726,543	- 6,041,639	315,097	G
281,799	- 587,953	306,155	H
3,254,467	- 3,527,969	273,503	I
2,511,495	- 2,761,444	249,950	J
6,041,640	- 21,723	244,487	K
1,827,181	- 2,053,671	226,491	L
1,246,699	- 1,461,937	215,239	M
1,461,938	- 1,644,798	182,861	N
99,954	- 281,798	181,845	O
2,919,511	- 3,089,800	170,290	P
1,644,799	- 1,803,569	158,771	Q
2,761,445	- 2,919,510	158,066	R
4,101,947	- 4,250,765	148,819	S
3,962,322	- 4,101,946	139,625	T
1,062,492	- 1,187,488	124,997	U
3,157,585	- 3,254,466	96,882	V
587,954	- 681,774	93,821	W
2,444,425	- 2,511,494	67,070	X
3,089,801	- 3,151,305	61,505	Y
5,206,703	- 5,266,155	59,453	Z
681,775	- 725,195	43,421	AA
63,068	- 99,953	36,886	AB
21,724	- 56,415	34,692	AC
1,192,398	- 1,226,237	33,840	AD
4,790,628	- 4,819,852	29,225	AE
1,803,570	- 1,827,180	23,611	AG
1,226,238	- 1,246,698	20,461	AH
3,527,970	- 3,538,447	10,478	AI
56,416	- 63,067	6,652	AJ
3,151,306	- 3,157,584	6,279	AK
1,187,489	- 1,192,397	4,909	AL

[1]Analysis of PAO1 genomic sequence (97) was performed using the software OMIGA; *Xba*I digestion generated 99 fragments ranging from 237,113 to 25 bp.

inhalation exposure system where artificially generated *P. aeruginosa* aerosols are evenly introduced to murine lungs within the system (figure 6A and 6B). This apparatus, which has also been described elsewhere (8), has a nebulizer-Venturi unit into which a bacterial suspension is introduced (figure 6B). *Pseudomonas* suspension is atomized and mixed with room air, and this cloud of bacterial aerosol is introduced into the chamber. The whole system is negatively pressurized with a microprocessor to control cycles of preheating, nebulizing, exposure, and bacterial cloudy decay. The exhaust air is passed through a HEPA filter and an incinerator in the rear of the unit (figure 6B). Two built-in bactericidal UV lamps within the system are used at the cycle end for chamber decontamination. Using this model, the following mice have been tested for respiratory tract infections, based on a regimen of single (innate) and repeated (chronic) exposures to *P. aeruginosa* aerosols, and investigated for bacterial clearance and inflammatory responses. These mice include C57BL/6J, BALB/cJ, DBA, IL-10 and TNF-alpha knockout mice (100-102).

The wild type C57BL/6J mice were first tested for single and multiple exposures in this BAILI model

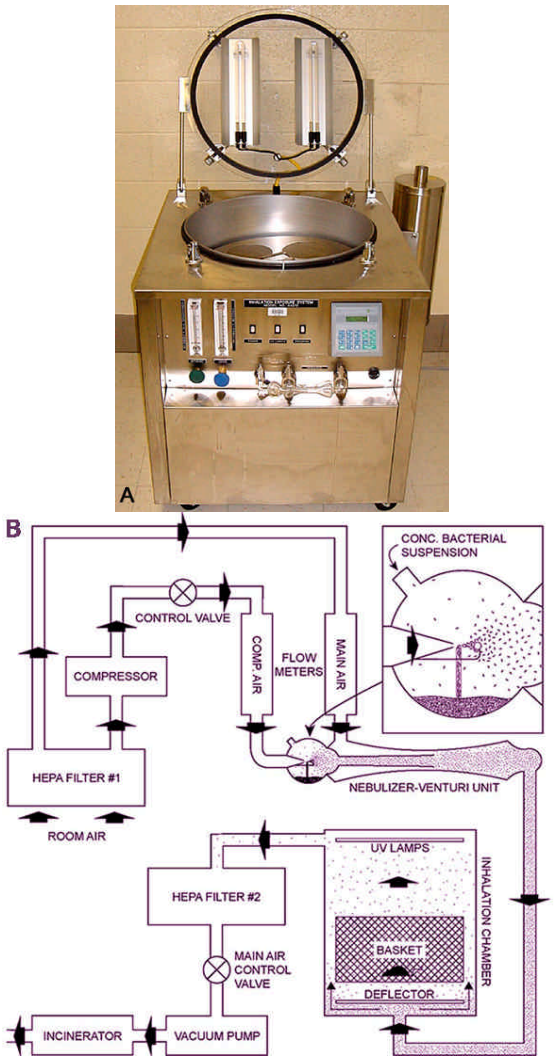

Figure 6. A. The core of the bacterial aerosol infection mouse model (BAILI): An inhalation exposure system. The BAILI mouse model (6, 115, 116) is a whole-body aerosol-based infection model where artificially generated *P. aeruginosa* aerosols can be evenly introduced to the murine lungs within the chamber, thereby causing a lung-specific infection and inflammation in mice. The chamber holds up to 100 mice within 5 separate compartments and can be used as an efficient high-throughput screening device for monitoring pulmonary clearance and inflammatory responses from the host.B. Schematic diagram of the aerosol inhalation machine system. Utilizing a negative pressured system powered by a vacuum pump at the end of the arrangement, room air is passed through a HEPA filter (#1) before entering the system. A compressor produces the air pressure needed to aerosolize the bacterial suspension, as shown in the inset. A valve controls the flow of the compressed air, regulating the level of aerosolization. The main air is responsible for carrying the aerosol from the nebulizer-Venturi unit to the inhalation chamber. Exhaust air with bacteria is filtered (HEPA filter #2) and incinerated. UV lamps destroy residual bacteria within the chamber as well as on the animal coats.

(101). While a single exposure to *P. aeruginosa* aerosol resulted in only mild histopathological changes, repeated exposures caused significant lung pathology in C57BL/6J mice. Mucoid cells (*mucA22*) were cleared several-fold less efficiently than isogenic nonmucoid (*mucA*$^{+}$) cells during the initial stages of aerosol exposure. However, microscopic pathology findings and proinflammatory cytokine levels were similar in mice exposed to nonmucoid and mucoid *P. aeruginosa* throughout the infection. Lung histopathology and proinflammatory cytokines were tested in IL-10 deficient transgenic mice (IL-10T). Significant mortality was seen in the IL-10T mice on initial challenge with *P. aeruginosa*. Increased pathology was detected upon repeated challenge with *P. aeruginosa* in the IL-10T mice relative to the C57BL/6J mice. In an chronic infection agar-bead model with IL-10 knockout mice, a similar finding was noted (104). These observations suggest that anti-inflammatory cytokines may play a role in suppressing *P. aeruginosa*-induced tissue damage during chronic infection, and that reduced IL-10 levels in the lungs of CF patients (105) may be of significance for the respiratory sequelae in this disease.

The CF mice have also been tested in this model for their ability to clear *P. aeruginosa* (102). This result is apparently in keeping with other observations using the agar-bead chronic infection model (106, 107) or another aerosol infection model (108). These mice presented two extremes: either clearing or not clearing *P. aeruginosa*. This finding seems to be associated with variations in mouse body weight and nutritional status. When *CFTR*$^{mIUNC-/-}$ mice had their intestinal defect corrected by a functional human *CFTR* gene expressed from a rat intestinal fatty acid-binding protein gene promoter *CFTR*$^{mIUNC-/-}$ (FABP-h*CFTR* mouse; ref. 92), *P. aeruginosa* was efficiently cleared from the lung and variability was no longer observed. While these observations may suggest that repairing the *CFTR* defect in the intestinal tract improves lung defense against *P. aeruginosa*, they also show that CF transgenic mice may be incapable of clearing *Pseudomonas* lung infections under certain conditions.

Recently, we have tested the role of malnutrition in host pulmonary defenses against *P. aeruginosa* colonizations in a group of C57BL/6J mice which was first induced with protein energy malnutrition (PEM) and then exposed to *P. aeruginosa* aerosol (102). PEM resulted in a 11-fold increase of *P. aeruginosa* survival in the lungs of the C57BL/6J mice. PEM also contributed to excessive inflammation upon chronic infections with *P. aeruginosa*. The repeatedly infected malnourished mice did not produce IL-10 in their lungs**.** In addition to increased levels of proinflammatory cytokines and neutrophil infiltration, another CF hallmark is a low level of the major anti-inflammatory cytokine IL-10 in the bronchio-alveolar fluid (105). Using a model of a repeated exposure with *P. aeruginosa* (101), we observed significant amounts of IL-10 production in the well-nourished mice 22 days following the initiation of the regimen of repeated exposures. In contrast, the malnourished animals had no detectable IL-10. These results demonstrate that malnutrition compromises pulmonary defenses against *P. aeruginosa* colonization and

is conducive to excessive inflammation in response to *P. aeruginosa* infection, resembling the situation in CF. Furthermore, neutrophil infiltration in the lungs of malnourished animals did not result in increased bacterial lung clearance and instead was a correlate of an unproductive inflammatory response. In two separate reports using a sepsis model induced by *P. aeruginosa* (109) and a chronic infection model with *Pseudomonas*-laden agar beads (110), respectively, increased neutrophil infiltration and increased bacterial load in the lungs were detected at the same time. These results suggest that neutrophils under these conditions (e.g., PEM, sepsis and chronic infection) may not be fully activated or functional in the process of eliminating *Pseudomonas* from the lungs. Therefore, neutrophil-mediated bactericidal mechanisms need to be further investigated since pulmonary alveolar macrophages have recently been shown to play a marginal role in defense against *P. aeruginosa* infections in mice (111).

7. PERSISTENT INFECTION PHENOTYPES AND IMMUNITY

The major characteristic of chronic lung infections in CF is the persistence of bacterial pathogens despite excessive immune response from the host (112). The normal innate pulmonary defense systems involve mucociliary clearance and cough, phagocytes and antimicrobial factors such as defensins (113). In normal individuals, an effective inflammatory response involving proinflammatory cytokines is needed immediately after exposure to *P. aeruginosa*. However, the successful initial colonization of *P. aeruginosa* in the CF lungs suggests that the innate immunity that is required for *Pseudomonas* clearance is defective in CF. When this fails, biofilm formation, accompanied by excessive inflammation, occurs resulting in chronic infection. Chronic pulmonary inflammation, caused by excessive neutrophil infiltration and an unproductive immune response, is one of the major clinical manifestations in CF. The CF patients apparently have the ability to elicit humoral and cell-mediated immune responses. The phagocytic cells of CF patients appear to lack any major functional abnormalities (44). Furthermore, CF patients are able to mount an antibody response since the proteinaceous antigens of *P. aeruginosa* are processed by antigen presenting cells and presented to T-cells as evidenced by the presence of anti-toxin (112) and more recently, anti-type-III-protein antibodies (114) in the serum of CF patients. The problem encountered in combating *P. aeruginosa* persistent infections is due to the deficiency of an effective removal system of MEP enclosed microcolonies of *P. aeruginosa* and therefore causing increased and prolonged host inflammatory responses (115). The antibody-bacterial complex aggregates, which are too large to be phagocytized, could act as foci for "frustrated phagocytosis" (116). Fc-receptor mediated degranulation and oxidative burst due to the presence of continuous infiltration of neutrophils occur, resulting in excessive inflammation and tissue destruction.

Biofilm formation, particularly conversion to the mucoid phenotype leading to development of mature biofilms, impairs the normal immune response process in CF patients. Alginate is immunogenic, and can act as an efficient polyclonal B-cell activator (117) leading to a clinical condition mainly present in older CF patients: hyperimmunoglobulinemia (44). Another peculiar property of alginate is that excessive amounts in the experimental animals induces production of non-protective and low opsonizing antibodies (45). Using serum from a patient undergoing an exacerbation, it was demonstrated by cross immunoelectrophoresis that the serum recognized more than 30 distinct *P. aeruginosa* antigens (118). In contrast, serum from a patient in remission, who had been infected for 16 years, only recognized five antigens (119). This provided prima facie evidence of masking *P. aeruginosa* antigens by biofilm-forming bacteria.

The seminal work performed to establish the basic concept for *P. aeruginosa* biofilms was done in the early 1980s. Using direct electron microscopic analysis of post-mortem lung tissues from CF patients, Lam *et al*. (118) demonstrated that *P. aeruginosa* in CF lungs form aggregates or microcolonies (figure 4). Excessive amounts of alginate surrounding microcolonies during the course of chronic infections in CF directly impair antigen presentation. Antibody response to iron-regulated outer membrane proteins (IROMPs) in CF patients does not appear early in the infection and is apparently associated with the advanced stage of the disease (120). The rough LPS mutants of *P. aeruginosa* emerge during the course of chronic infections in CF patients who have a high titer of anti-LPS antibodies, but the immune systems of CF patients fail to eradicate the serum-sensitive mucoid variant *P. aeruginosa* infections (44). Efforts for vaccine development have been mainly focused on the following candidates: O-polysaccharide, toxins, flagella, outer membrane proteins and alginate (121). Though some candidates are promising, an effective anti-*Pseudomonas* vaccine is still not available. Recently, it has been reported that a DNA vaccine carrying *oprF* allows the immunized mice to produce a significantly higher level of opsonic IgG1 antibody, confering protection in these mice (122). Genetic vaccines combined with molecular adjuvants may offer a new approach for future development of vaccines against *P. aeruginosa* infections in CF.

8. FUTURE CHALLENGES

Recent completion of the entire PAO1 genomic sequence accompanied by discoveries of genomic islands from clinical isolates spurs further investigation of the genetic diversity in *P. aeruginosa*. Some of the relevant questions to now address include i) the number of islands present per genome; ii) the relationship between the islands and diversity; iii) identification of any CF- and/or other disease-specific islands; and iv) elucidation of specific virulence roles for the islands including their possible involvement in biofilm formation. Some of the challenging topics related to biofilm research include i) identification and characterization of new adhesins, and ii) control of biofilm formation by quorum sensing and other global regulators. To establish a relationship between *in vitro* and *in vivo* biofilm formation, it is necessary to evaluate biofilm

formation defective mutants in a proper animal model. A good CF infection mouse model will allow us to reproduce the chronic infection process, i.e., from initial nonmucoid cell colonization to *in vivo* mucoidy conversion leading to development of mature biofilms coupled with excessive neutrophilic inflammation and inflammatory cytokine profiles similar to what is seen in human CF. Development of a true CF disease animal model and elucidation of the molecular mechanisms underlying the transition to the persistent infection phenotypes in this organism could lead to novel therapeutic strategies against chronic *P. aeruginosa* lung infections in CF.

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