Insights on *Pseudomonas aeruginosa* Carbohydrate Binding from Profiles of Cystic Fibrosis Isolates using Multivalent Fluorescent Glycopolymers Bearing Pendant Monosaccharides

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Supplemental File S6

Additional Details and Discussions on the Characteristics of CF Airway and *P. aeruginosa* which may Affect *P. aeruginosa* Survival and Success of Adjunctive Anti-Adhesive Therapeutics

Contents

S6.1. Complex and Variable Airway Milieu in Cystic Fibrosis (CF) Patients

Toward development of effective, novel CF *P. aeruginosa* anti-infectives, it is important to realize that *Pseudomonas* colonization is set in the complex airway milieu that results in part from host-specific genetics [4,5,10,15,s1,s2] and in part from the presence and activities of normal respiratory flora and an array of opportunistic pathogens [1,8,11,19,26,48,s3,s4,s5]. Mutations in the DNA encoding the cystic fibrosis transmembrane conductance regulator (CFTR) in the host exocrine systems lead to insufficient quantity and/or function of this protein at the cell surface [2,4-6,s1]. The severity of disease symptoms differs widely in the CF population, often reflecting the specific mutations present and the corresponding level of functional CFTR [4-6,s1]. Respiratory tract consequences of CFTR-deficits include mucous secretions with: inappropriate levels of pH, salt, and water; altered amounts and structures of high molecular weight mucous glycoproteins; and significant amounts of host immune cell-derived DNA, cell debris, lipids, and cations [2,6,10,43,49,s2]. Impaired mucociliary clearance, increased inflammation,

chronic obstruction and chronic infection are hallmarks of CF pulmonary disease [1,5,15,45,48]. Recently, CF-specific therapeutics that address the basic genetic defect at the cellular level, called CFTR modulators, have become available to individuals with mild to moderate CF disease [s1,4,5]. Many of these patients are experiencing markedly improved airway function and quality of life [s1,4-6]. Despite this therapeutic progress, lung infection with *Pseudomonas* and other pathogens remains a critical issue to address for patients with long-term symptomatic disease and/or severe lung damage, and for individuals for whom these drugs are not effective, not tolerated, or not available [2,4,6,27,48].

S6.2. Character of the CF Airway May Impact Bacterial Colonization

A caveat and context to keep in mind for anti-biofilm and anti-adhesion therapeutics development, is how the character of the CF airway may impact bacterial colonization. Typically, bacterial colonization of a site begins with attachment to a surface then proceeds to the development of a biofilm community [7,12,26,27,42,64,s6,s7]. In the CF respiratory tract insufficient mucociliary clearance may itself begin the aggregation and colonization process as bacteria are trapped in the viscous, dehydrated, often stationary, epithelial mucus gel [6,9,45,49,65,66,67,s8]. *P. aeruginosa* in this highly charged matrix may be temporarily protected from immune components and therapeutics [s8], and be permitted time to adapt to the new environment to further protect themselves, and/or to migrate to the underlying host cell-bound glycoconjugates (mucins, glycosphingolipids, proteoglycans) and invade the epithelium [21,24,36,48,68]. Further complicating potential anti-biofilm therapeutics is the nearly diagnostic presence in chronic CF infections of mucoid *P. aeruginosa* phenotype with abundant elaboration of the polysaccharide alginic acid (an anionic polymer composed of repeating units of acetylated β-1,4-linked D-mannuronic and L-guluronic acids) [7,19,27,s6,s7]. Alginic acid may impact bacterial adherence to mucin oligosaccharides [1,26,s9], increase the viscosity of airway secretions, affect drug diffusion, impair the already-compromised mucociliary clearance, and protect encased organisms (whether producers or not) from dehydration, antibiotics, phagocytosis and killing by host immune cells [1,26,48,s7,s10,s11,s12,s13].

S6.3. *Pseudomonas* Biofilm Exopolysacharide and Adhesin Interactions may Provide Targets for Adjunctive Therapeutics

With the biofilm central for *Pseudomonas* survival in so many environments [6,7,20,27,53, s6,s7,s10,s14] investigations focused on the interactions within the biofilm matrix have also shed light to suggest potential new targets for CF anti-adhesion and anti-aggregation therapeutics [27,89,130,s14]. For example, LecA and LecB were found to associate with two bacterial-derived exopolysaccharides (EPS) Pel and Psl to help build and maintain the biofilm integrity [22,89]. *P. aeruginosa* Pel was characterized as a cationic polymer composed of *N*-acetylglucosamine (GlcNAc) and *N*-acetylgalactosamine (GalNAc) [24,89,136]. In CF, activity of the bacterial enzyme PelA was shown to alter the degree of Pel acetylation and thereby its positive charge and ability to limit diffusion of charged antibiotics [24,136]. Psl was described as a neutral polymer rich in mannose, glucose, and rhamnose [24,27,89,136]. Using *P. aeruginosa* from various sources, investigations found that for strains with either Pel or Psl as the dominant EPS, the fimbrial adhesin CdrA was also significant in bacterial aggregation and biofilm development [136]. *Pseudomonas* aggregates with Pel and/or Psl were recently demonstrated in CF sputa via immunohistochemical visualizations, showing that their existence *in vivo* is clinically relevant [24], and that both aggregate size and matrix EPS composition varied among CF patients [24]. Additionally, *in vitro* analyses revealed that both partially deacetylated Pel and Psl could associate with extracellular DNA (eDNA) and shield the DNA from therapeutic DNase [24,s15]. These data, and earlier data from others, suggested that aggregated and/or biofilm-growing *Pseudomonas in vivo* may have the protection of a DNA coat or web [s15] as yet another physical means of tolerance to positively charged antibiotics such as tobramycin (as is often seen in CF) [24,27,s6].

S6.4. Overview of Findings and Contributions of *P. aeruginosa* Carbohydrate Binding Profiles Data toward Interventional Agents

In this study we evaluated the glycopolymer binding profile obtained for each isolate or strain relative to its source, phenotypic characteristics, and structural features (i.e. flagella, pili) to assess for informative patterns and predictive trends. α-D-galactose (αGal), β-D-*N*-acetylgalactosamine (βGalNAc), and β-D-galactose-3-sulfate (βGal3S) glycopolymers associated with all *P. aeruginosa* specimens, and this positive binding was accounted for by a small subpopulation within the cultures. Three CF isolates of varied phenotypes showed enhanced associations with αGal, βGalNAc, and βGal3S glycopolymers. Eleven other clinical strains showed this higher binding characteristic to one or two of these glycopolymers. While no phenotype or structural feature guaranteed enhanced binding to any of these glycopolymers, all CF throat isolates and all nonmucoid CF sputum isolates showed high binding to the βGalNAc glycopolymer. These insights, taken in the context of the complex CF airway milieu discussed above and in the main text, are anticipated to inform future anti-infective therapeutic strategies addressing host-pathogen interactions, bacterial aggregation, biofilm matrix development and disruption, and novel agent development for directed-targeting of antibiotics to CF airway *P. aeruginosa*.

S6.5. Significant Additional Factors which may Influence *P. aeruginosa* Carbohydrate Binding *in vivo*

The *P. aeruginosa* variability and fluidity in expression or lack of expression of virulence features and structures further complicates the development of targeted agents for interfering with airway colonization [10,11,15,20,24,26,29,36,58,63,88,90,116,117,131,136,s16]. Apparent microbial microevolution may reflect both reversible phenotypic switches and genetic alterations [13,15,16,19,32,34,135, s17,s18,s19,]. Such fluctuation may occur within a patient and within an original bacterial clone [11,16,32,135]. This flexibility and genetic diversity may in turn affect carbohydrate binding. Virulence structures like flagella, pili, lectins, extracellular polysaccharides (i.e. alginate, Psl, Pel), and other cell-associated and secreted proteins (i.e. CdrA), may separately or coordinately contribute to both *Pseudomonas*-host interactions and to bacterial community assembly [64]. Better understanding of the bacterial carbohydrate interactions that variably partake in colonization and in formation, stabilization, and dispersal of the protected aggregates and complex biofilm architectures will be important to enhancing anti-infective therapeutics.

The *P. aeruginosa* use of redundant and/or complementary mechanisms for attachment, colonization and survival [12,44,131,s6,s11,s20] should be respected in the design of new interventional agents. The presence of mixed phenotype variants of *P. aeruginosa* within a sputum sample is common in CF [9,10,16]. Localized co-existence of non-mucoid and mucoid PA *in vivo* may permit each to benefit from the others' adaptations for surviving the CF host and bacterial competitors [16,19,27,s13,s18, s20,]. Variants, within a biofilm for example, may divide the labors, share the public goods, and jointly evade the host immune effectors [1,20,64,131,142,s13,s21]. Involvement in mixed species biofilms in CF may further benefit *P. aeruginosa* [11,27,s4,s18,s21,s22]. The CF airway microbiome is often patient specific and its diversity is found to fluctuate and adjust over an individual's life [6,8,11,30,s5,48,s4,s21]. Shifts away from the normal microbiome may not only contribute to antibiotic tolerance, but also within polymicrobial communities bring about interspecies interactions that influence *P. aeruginosa* expression of carbohydrate binding features and thereby alter the effectiveness of adhesin-targeted therapies [s3,s21]. These realizations may improve the understanding and treatment of *P. aeruginosa* colonization and persistence in CF.

The conditions of the individual host may further affect the processes of colonization and adaptation of *P. aeruginosa* [1,9,20,31,34,82,83,133,s2,s3, s23]. Host condition is suggested to trigger the divergent evolutionary paths of *Pseudomonas* variants [16,19,34]. In addition to the altered chemistries of CF, the local inflammatory and infectious microenvironments, and the variably impaired mucociliary clearance, host status effectors also include pre-existing and progressing airway pathologies [20,23,34,83,133,s2,s3,s23]. The specific and variable characteristics of the complex carbohydrates present in CF respiratory tracts are mechanistically important to bacterial binding and/or clearance from the

airway [9,60,65,67,78,79,81,82]. Distinctions and differences are expected in mucosal glycoconjugates reflecting the host genetic background, the functional impacts of the specific CFTR mutations, the history of exacerbations, the degree of inflammation, the current infections, and the ongoing therapies [9,34,67,82,83,133,s2,s3,s23,s24]. Active viral or bacterial infections, for example, may also alter what host glycans *P. aeruginosa* adhesins may access. Neuraminidases from influenza viruses and/or bacteria may enhance opportunities for *P. aeruginosa* binding by removing sialic acids from epithelial and mucosal glycoconjugates to increase the density of cell surface asialo-glycosphingolids GM1 and/or GM2 and expose galactose on membrane-bound MUC1 mucin [9,79,85,145,s25]. *P. aeruginosa* sialidase itself is suggested to be important in biofilm formation [s25]. Sub-inhibitory concentrations of antibiotics may also elicit *P. aeruginosa* changes that affect mucin binding [88,137,138] and production of virulence factors, such as the cytotoxic phenazine pyocyanin [139]. Pyocyanin at non-lethal levels may increase airway epithelial cell production of specific types of sialyl-Lewis antigens, such as sialyl-LeX shown important to bacterial binding [67]. As might be seen with lytic virus infection or following other injurious conditions, wounded and regenerating epithelial margins undergoing remodeling are also anticipated to show increased amounts of asialo-glycosphinogolipids and other exposed glycocalyx components for *Pseudomonas* binding [60,68,148]. Increased cytotoxicity observed *in vitro* for the CF therapeutic agent colistimethate [48] when coincident with pyocyanin [140] suggests that inadvertent damage to the CF epithelium during antimicrobial treatment may also set the remodeling stage and provide even more sites for binding, aggregation, and biofilm establishment.

The well-known, well-respected, adhesive and protective biofilm provides much *Pseudomonas* heterogeneity that may affect host interactions and effectiveness of targeted anti-infective agents [6,7,14,21,23,26,27,40,42,45,130,136,s6,s7,s10,s17,s19,s26]. Proteins involved in biofilm formation and interactions, such as CdrA, LecA and LecB, show genetic variations [117,136,147]. The dominant exopolysaccharide (EPS) elaborated in *Pseudomonas* biofilms varies, as does the amount [10,18,130,136, s11]. Mucoid isolates typically produce an abundance of the acidic polysaccharide alginate 14,27,136], while other phenotypes elaborate the neutral matrix polysaccharide Psl, and/or the cationic pellicle EPS Pel [10,14,27,36, s11]. The airway biofilm structures and complexities, as seen upon analysis of sputa, also include variable and potentially large amounts of bacterial and neutrophil-derived extracellular DNA (eDNA) and other host elements [7,21,43,45,s2,s6]. Heterogeneous chemical gradients within bacterial biofilms foster microenvironments such that local adaptations of *Pseudomonas* cells are not equivalent across sister cells in other areas of the matrix [7,15,21,27,42,64,134,s10,s27,s26]. Varied distribution of therapeutic agents within the biofilm, as well as within the airway, may further modulate virulence [21,42,132,139,s26]. Sub-lethal levels of antimicrobials within the biofilm can lead to the generation of non-replicating antibiotic tolerant persister subpopulations and may include a phenotypic shift to the small colony variant morphology [7,18,20,21,s10,s17]. Such regional biological flexibility is expected to contribute to *Pseudomonas* survival.

With all these environmental influences and *P. aeruginosa*'s adept adaptive abilities the rational design of agents to universally limit this opportunistic pathogen's negative effects on its host seems quite the daunting challenge. However, results of this carbohydrate binding profile study of a heterogeneous collection of *P. aeruginosa* may shed light on common ground from which to build broadly effective adjuvants to current therapies.

S6.6. Bacterial Subpopulations may be Particularly Important for Persistence of CF Chronic Infections

The concept of subpopulations providing the desired function for the whole population is a familiar theme for microorganisms [1,64,132,142,143,s19,s26]. Examples in CF include portions of infecting populations persisting as metabolically inactive cells tolerant to antibiotic challenges (such as small colony variants), nonmucoid organisms living among mucoid variants protected from dehydration, antibiotics and host recognition, and metabolically deficient cells surviving as social cheaters benefitting from the activities of the larger community [1,6,8,14,21,64,132,142,s10,s18,s19,s25,s26, s27]. For opportunistic pathogens like *P. aeruginosa*, whether division of labor is intentional by species design, is driven by adaptation to oxygen level, sub-inhibitory antibiotic(s) or other chemical gradient(s) or

signal(s), or is generated stochastically (randomly), the heterogeneity of character among cells undoubtedly contributes to species survival and chronic colonization of the vulnerable host [1,8,11,21,42,64,132,142,s10,s18,s19,s25,s26,s28].

S6.7. Specifics of Some of the Many Opportunities for *P. aeruginosa* Carbohydrate Interactions in the CF Airway

In the CF airway, *P. aeruginosa* likely participate in numerous complex carbohydrate interactions at epithelial surfaces [148], within respiratory mucus, and in colonizing bacterial aggregates and biofilm community matrices.

For the host, the terminal and penultimate carbohydrate residues and their linkages impose important definitive characteristics which may influence bacterial association, including blood group specificity [9,49,74,s18,s24]. Structures common to respiratory mucin oligosaccharides are the terminal, and often repeating internal, disaccharide units of galactose linked to *N*-acetyl-glucosamine as βGal(1-3)βGlcNAc or βGal(1-4)βGlcNAc, with or without additional sialic acid, fucose or sulfate entities [9,49,72,74,83,84,149,s24]. Terminal structures common to host cell gangliosides include galactose and *N*-acetyl-galactosamine in linkages of βGal(1-3)βGalNAc (as in asialo-GM1) and GalNAcß1-4(NeuAcα2-3)Galß1-4Glcß-Cer (as in GM2), with the series of glycosphingolipids carrying varying numbers of sialic acids [77,s9,s29]. As noted above, host conditions may affect what complex carbohydrates are elaborated and how they are modified [9,34,67,82,83,133,s3,s23,s24]. Where disease, damage, infection, and/or inflammation occur, the relative amounts and locations of sialic acids, fucose, and sulfate esters are anticipated to vary [9,49,74,100,s24].

The pendant monosaccharide structures positive in this study for whole cell binding of *P. aeruginosa* may be found in nature as follows: the *α*-Gal structure can be found at the termini of carbohydrate residues of blood group B active oligosaccharides and globoseries glycosphingolipid Gb3 on plasma membranes; the ß-GalNAc at termini of gangliosides like GM2; and the ß-Gal3S on CF tracheobronchial mucins oligosaccharides [47,49,147,149,s29,s30]. Of note, in branched CF respiratory mucin oligosaccharides, it is also possible to have the blood group B antigen at the terminus of one branch and a ß-Gal3S residue at the terminus of another branch [49], positioning two binding epitopes in potentially close proximity.

For anti-adhesion therapeutics, the first thought may be to address this host attachment. In CF however, the concentration of organisms per location in the human host may be more related to stagnant mucus trapping and protective bacterial cell-bacterial cell interactions with aggregation and biofilm community structure development than to the numbers of available host cell receptors for the bacteria. Each type of interaction may provide sites for anti-adhesion related anti-infective interventions. As noted above, it should also be respected in therapeutics design that, as discussed above, the varied permutations of the condition of the host, the adaptations of the colonizing microbes, and the treatments being received are likely to keep the infectious landscape ever-changing [8,20,43,133,s24,s31].

S6.8. Addressing Specific Types of Carbohydrate Related Intra-Biofilm Interactions for Therapeutic Interventions

It is increasingly appreciated that establishment of chronic *P. aeruginosa* colonization in the CF host likely includes persistence in a protective biofilm. Prevention and destabilization of *Pseudomonas* biofilms *in vivo* via interventions addressing simple sugars interactions with cell surface and biofilm bound adhesins is an enticing avenue of investigation.

While this study did not address biofilms directly, the binding results appear translatable to this arena. As highlighted above: extracellular matrices of *P. aeruginosa* vary between patients in the type and amounts of EPS [24]; LecB may bind mannose side chains in Psl [89]; CdrA may associate with *N*-acetyl-galactosamine in Pel biofilms [24,136]; and glycomimetic inhibitors may interfere with bacterial outer membrane bound [90,92] or biofilm located lectins interactions to disperse intact *Pseudomonas* biofilms

[104,106]. Combining these findings and many others with the insights of the current whole cell glycopolymer binding study suggests that combinations of inhibitors designed to affect (a) LecA binding to galactose and a lesser degree *N*-acetylgalactosamine [47,96], (b) LecB affinity for fucose and mannose, and/or (c) fibrillar protein CdrA association with *N*-acetylgalactosamine, may jointly affect biofilm integrity *in vivo* for a variety of individuals colonized with *P. aeruginosa*.

S6.9. Drug Delivery Challenges and Opportunities in the CF airway Mucus Environment

The highly charged and dense natures of both the CF airway mucus environment and the *P. aeruginosa* biofilm emphasize the challenges of limited diffusion and reduced penetration of charged therapeutics en route to their bacterial targets [6-8,21,26,27,40,42,43,45,48,52,130]. Designing new adjunctive agents and their delivery solutions, especially for inhaled preparations, to remain as neutral and as small as possible will likely aid in their successful distribution and deposition [40]. The smaller sized carbohydrate-based therapeutics would also be less likely to elicit a host immune response to the agent. Further investigation is required to assess whether small relatively neutral molecules possessing galactose and/or *N*-acetyl-galactosamine in the appropriate buffer conditions may penetrate and diffuse through the airway mucus and the variable and potentially highly ordered complex biofilm matrices and channels, to compete with carbohydrate interactions at the bacterial cell membranes, at the host cell surfaces, and within the biofilms themselves.

Co-administration of aerosols of carbohydrate-based agents to disrupt bacterial interactions with proven antibiotics continues to represent a promising anti-*Pseudomonal* therapeutic strategy [37,46,52]. In an *in vitro* model of combination inhalation therapy, a nebulized ciprofloxacin-mannitol formulation demonstrated improved antibiotic effectiveness against a *Pseudomonas* biofilm [37]. This synergy and that suggested by early clinical reports of benefit of inhaled simple sugars to antibiotic treatments [100,101] imply that simultaneous application of antibiotic with a carbohydrate-based biofilm destabilizing agent is an actionable goal for adjunctive therapeutics [37,113]. This cooperative benefit may be realized regardless of the mechanism of action of biofilm destabilization, i.e. via interfering with lectin binding, by water influx as expected with therapeutic osmotic agents such as dry powder mannitol, via enzymatic disruption of the exopolysaccharide matrix or the eDNA within the biofilm, and etc [7,42,46,51,s12].

S6.10. To Conclude these Additional Details and Discussions on the Characteristics of CF Airway and *P. aeruginosa* which may Affect *P. aeruginosa* Survival and Success of Adjunctive Anti-Adhesive Therapeutics

These insights and data, taken in the context of the complex CF airway milieu, are anticipated to inform future anti-infective therapeutic strategies addressing host-pathogen interactions, bacterial aggregation, biofilm matrix development and disruption, and novel agent development for directed-targeting of antibiotics to CF airway *P. aeruginosa*.

Technical Note:

This bacterial carbohydrate binding series of experiments relied heavily on synthetic water soluble polyacrylamide-based fluorescent glycopolymers representing the pendant monosaccharides anticipated at termini of mucosal oligosaccharides either decorating airway mucins or epithelial cells.

Historically, synthetic glycopolymers/particles as experimental tools have taken many forms and been produced in many ways [105,108,s21,s23]. Functionalized glycopolymers have had widespread use in detecting pathogens and cancers, and more recently in research on targeting therapeutics [22,54,105,s28,s32,s33]. Fluorescence-tagged glycopolymers specifically have been proven versatile aides in investigations of host-pathogen interactions and bacterial lifestyles [22,65,75,81,s33].

Previous studies in our laboratories have shown the well-defined predictably uniform glycopolymers produced by controlled polymerization reactions can provide anomeric specificity important by understanding bacterial lectin binding [119].

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