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Pseudomonas aeruginosa mucoid strain 8830 binds glycans containing the sialyl-Lewis x epitope

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Abstract *Pseudomonas aeruginosa* infection of patients with cystic fibrosis (CF) is a leading cause of their morbidity and mortality. Pathogenesis is initiated in part by molecular interactions of *P. aeruginosa* with carbohydrate residues in airway mucins that accumulate in the lungs of patients with this disease. To explore the nature of the glycans recognized by a stable, mucoid, alginate-producing strain *P. aeruginosa* 8830 we generated a genetically modified Pa8830 expressing green fluorescent protein (Pa3380-GFP). We tested its binding to a panel of glycolipids and neoglycolipids in which selected glycans were covalently attached to dipalmitoyl phosphatidylethanolamine and analyzed on silica gel surfaces. Among all glycans tested, Pa8830-GFP bound best to sialyl-Le^x-containing glycan NeuAc(α2-3)Gal(β1-4)[Fuc(α1-3)]GlcNAc-R and bound weakly to H-type blood group Fucα1-2Galβ1-4GlcNAc-R, sialyl-lactose, and Le^x, and exhibited little binding toward non-fucosylated derivatives. Interestingly, while Pa8830-GFP bound to the glycosphingolipid asialoGM1, it did not appear to bind to a wide variety of other glycosphingolipids including GM1, GM2, asialoGM2, and sulfatide. These results indicate that *P. aeruginosa* 8830 has preferential binding to sialyl-Le^x-containing glycans and has weak recognition of related fucose- and sialic acid-containing glycans. The finding that Pa8830 binds sialyl-Le^x-containing glycans, which occur at increased levels in mucins from CF patients, is consistent with studies of other strains of *P. aeruginosa* and further suggests that such glycans on CF mucins contribute to disease pathogenesis.

Keywords *Pseudomonas aeruginosa* · Neoglycolipids · Sialyl-Lewis x · Fucose · Conjugate

Abbreviations

CF	Cystic Fibrosis
Sialyl-Le^x	Sialyl-Lewis x antigen
Le^x	Lewis x antigen
GM1 (II^{3}NeuAc-GgOse$_4$Cer)	ganglioside GM1
GM2	ganglioside GM2
LNnT or nLcOse$_4$	Lacto-N-neotetraose
DPPE	dipalmitoyl phosphatidylethanolamine
GFP	green fluorescent protein
CFTR	cystic fibrosis transmembrane conductance regulator
Pa8830-GFP	*P. aeruginosa* strain 8830 stably expressing GFP

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Introduction

Cystic fibrosis (CF), a common genetic disease primarily in Caucasians, is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) [1]. The disease is characterized by excessive accumulation of mucin in the lung leading to colonization by opportunistic pathogens, such *Pseudomonas aeruginosa* [2], is found within a biofilm of alginate slime in the infected lung [3–5]. A high percentage of patients with CF die as a result of pneumonia associated with *P. aeruginosa*. The major mucins in the lung to which *P. aeruginosa* attaches are very high molecular weight glycoproteins comprised primarily of MUC5AC and MUC5B with thousands of sialic acid-containing *O*-glycans in Ser/Thr-linkage per polypeptide chain [6]. These glycans are recognized by *P. aeruginosa* through a number of carbohydrate-binding proteins (lectins or adhesins) produced by the bacteria [7]. While several of these potential carbohydrate-binding proteins from *P. aeruginosa* have been identified, their roles in pathogenesis and the their precise glycan recognition are still largely uncertain [7]. For example, the galactose-binding PA-I lectin (PA-IL) from *P. aeruginosa* is primarily intracellular [8], and recent studies, in fact, show that PA-I lectin binds well to a non-carbohydrate ligand *N*-acyl homoserine lactones [9].

Several elegant studies have shown direct binding and association of *P. aeruginosa* to mucins which is largely dependent on the presence of sialic acid residues [10–14]. *P. aeruginosa* contain lectins in their single polar flagellum, as well as in pili and surface membrane (nonpilus) proteins [7]. However, pili are probably not important in attachment to mucins since *P. aeruginosa* lacking pili bind to mucins [15]. For some strains of *P. aeruginosa* flagellar proteins, such as FliD, along with flagellin are required for binding to Le^x and 3-sulfo-Le^x, sialyl-Le^x, and 6-sulfo-sialyl-Le^x NeuAc(α-3)Gal(β1-4)[*S*-6][Fuc(α1-3)]GlcNAc-R [16–18]. *P. aeruginosa* also possess a flagellar-dependent ability to recognize the specific non-sialylated carbohydrate structure GalNAc(β1-4)Gal-R not found in mucins but found in asialo-GM1 (GgOse4-Cer) [19], a cell-surface glycosphingolipid expressed in airway epithelial cells [20]. The PAO and PAK strains of *P. aeruginosa* bind to both asialo-GM1 and asialo-GM2 (GgOse3-Cer) [21]. However, it was recently shown that asialo-GM1 is not a major target for binding of clinical isolates of *P. aeruginosa* to eukaryotic cells [22], thus causing more attention to now be focused on the mucin-associated glycans bound by *P. aeruginosa*.

Recently we showed that mucins from CF patients have increased numbers of O-glycans with increased content of sialic acid, fucose, and sulfate residues compared to mucins from non-diseased donors [23], consistent with the increased amounts of sialyl-Le^x in CF mucins compared to mucins from non-diseased donors [24]. These changes in mucin glycosylation may be associated with the inflammatory responses in CF patients. There is strong evidence that the inflammatory response and neutrophil involvement may underlie much of the pathology in CF patients [25, 26]. CF patients have large numbers of neutrophils and high activities of the neutrophil enzymes, such as elastase and cathepsin G, in their sputum [27–30]. In addition, infection by *P. aeruginosa* may lead to altered glycosylation of mucins [23, 31], thereby creating more favorable adhesion receptors for the bacteria.

The adhesion of *P. aeruginosa* to host macromolecules is complex since there are physiological changes in the bacteria themselves during infection that may alter expression of adhesins. Many environmental strains of *P. aeruginosa* are flagellated and motile whereas many isolates from CF patients are non-motile [32]. *P. aeruginosa* isolates from chronically-infected CF patients have a mucoid, alginate-overproducing phenotype [33].

A noted mucoid, alginate-producing strain of *P. aeruginosa* that is able to form biofilms in culture representative of virulence is the 8830 strain, which is derived from an original mucoid isolate in sputum of a CF patient [34]. It was shown earlier that the 8830 strain can bind to asialo-GM1 and that mucins inhibited the binding [35], but there are no direct studies on the specific binding of the 8830 strain to individual mucin-type O-glycans. Here we have genetically engineered the 8830 strain to express the green fluorescent protein and examined its binding to a series of glycolipids and neoglycolipids containing a wide variety of carbohydrate structures including mucin-type O-glycans. Our results show that PA8830-GFP binds well to asialoGM1 but shows strong binding to the sialyl-Le^x antigen and moderate binding to other related glycans. Thus, this mucoid alginate-producing 8830 strain expresses adhesins which recognize sialyl-Le^x antigen of the type found on airway mucins in CF patients.

Materials and methods

The preparation of *P. aeruginosa* strain 8830 expressing green fluorescence protein (GFP)

P. aeruginosa Strain 8830 (Pa8830) was generously provided by Prof. Ananda Chakrabarty (University of Illinois College of Medicine, Chicago, IL). This is a stable alginate-producing organism that was derived from a mucoid isolate obtained from the sputum of a CF patient [34]. The plasmid DNA with GFP ($_P$SMC21) [36] was kindly provided by Dr. George A. O'Toole (Dartmouth Medical School, Hanover, NH). Pa8830 was grown on trypticase soy broth–agar plates. To prepare the GFP-

expressing clone, one clone was grown in 10 mL of trypicase soy broth in 37°C for 18 h with shaking until the density reached an optical density at 600 nm of 0.8 to 0.9. Bacteria were collected by centrifugation at 5,000 rpm/min for 15 min at 4°C. The cell pellet was washed once with cold PBS and the cells suspended in 1 ml of ice-cold Milli-Q water. The cells were washed 3x with 1 ml ice-cold Milli-Q water and then resuspended in a small volume (50–150 μl) of ice-cold 10% glycerol. Approximately 0.5 to 1 μg of $_P$SMC21 DNA was mixed with 50 μl of these cells, and then transferred to a 2-mm-gap electroporation cuvette (Bio-Rad Laboratories, Hercule, CA), and electroporated at 1.8 kV with a Transporator™ plus (BTX, San Diego, CA). The electroporated cells were diluted into 1 ml of LB medium and grown for 1 h before plating on LB agar supplemented with 500 μg/ml ampicillin. The fluorescent GFP-expressing bacterial colonies were observed under a fluorescence microscope. The pa8830-GFP expressing bacteria are highly stable, and in multiple generations no loss of GFP fluorescence was observed.

Preparation of glycolipids and neoglycolipids

Oligosaccharides and other reagents used in this study (see Fig. 1b) were obtained from the following sources: lactose and Primulin (Fisher Scientific, Pittsburgh, PA); Lacto-N-neotetraose (LNnT or nLcOse$_4$) (V-Labs, Covington, LA); sialyl-lactose and 2′-fucosyllactose and dipalmitoyl phosphatidylethanolamine (DPPE) (Sigma-Aldrich, St. Louis, MO); human brain asialo-GM1 (GgOse$_4$-Cer), bovine brain GM1 (II^{3}NeuAc-GgOse$_4$Cer), bovine brain bovine brain asialo-GM2 (GgOse$_3$Cer), GM2 (II^{3}NeuAc-GgOse$_3$Cer), and bovine sulfatide (Chembiochem-EMD Biosciences, La Jolla, CA); high-performance TLC plates (HPTLC) (Merck, Darmstadt, Germany). All other chemicals were from Sigma-Aldrich. Each of the free oligosaccharides (100 nmol) was mixed with 110 μl of DPPE (5 mg/ml in chloroform:methanol (1/1 v/v), and sonicated for 5 min at 60°C [37]. After incubation for 1 h at 60°C in a heating block, sodium cyanoborohydride (10 mg/ml in methanol) was added to the mixture and incubation continued for 16 h at 60°C. The mixture was developed by chromatography in HPTLC to purify the desired neoglycolipid away from free DPPE. A small side lane was stained with the lipophilic dye Primulin [38] to identify the positions of individual neoglycolipids using epifluorescence which were then extracted from silica gel by chloroform–methanol–water (130/50/9, v/v/v). The concentration of the purified neoglycolipid was determined by the phenol/sulfuric acid assay [39] using galactose as a standard. The neoglycolipids were stored as ~4 mM solutions in chloroform–methanol–water (130/50/9, v/v/v).

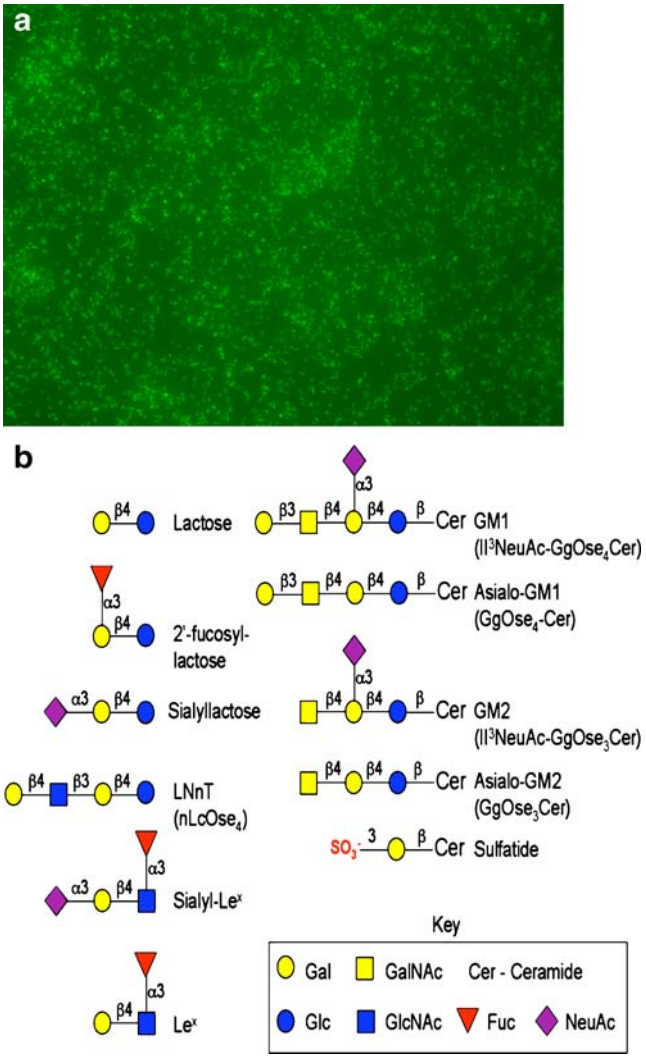

Fig. 1 Preparation of Pa8830-GFP and Structures of Glycolipids Used. (a) P. aeruginosa Strain 8830 was electroporated with plasmid $_P$SMC21, and colonies stably expressing GFP (Pa8830-GFP) were derived. The fluorescence image represents cells prepared for the binding assays, as described in Materials and Methods. (b) Structures of each of the neoglycolipids (*left-hand panel*) and commercial glycosphingolipids (*right-hand panel*) are illustrated using the symbols shown in the key

Overlay assays with Pa8830-GFP

The neoglycolipids or glycolipids were spotted on the HPTLC plates in duplicate or developed by chromatography on HPTLC plates in duplicate. One plate was stained with primulin and photographed under epifluorescence, while the other was used for binding of bacteria. For all glycolipids HPTLC development was conducted in chloroform/methanol/0.2% $CaCl_2$ (60/35/7,v/v/v), except that lactose–DPPE was developed by chloroform/methanol/water (11/9/2,v/v/v). After chromatography or spotting directly, samples were completely air dried for 2 h. The HPTLC plates were immersed in 0.1% polyisobutylmeta-

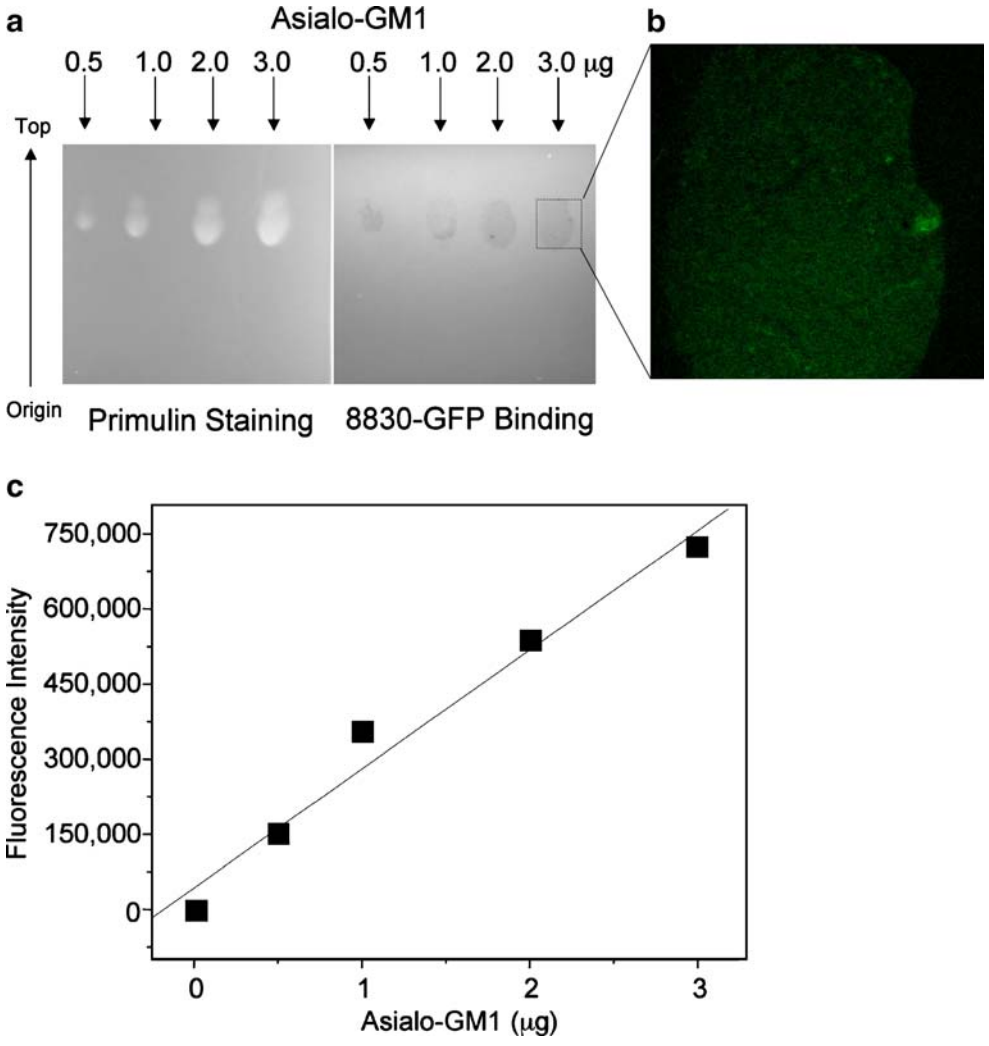

Fig. 2 Binding of Pa8830-GFP to Asialo-GM1. (a) Duplicate HPTLC plates were prepared with asialo-GM1 (0.5 μg to 3.0 μg) were developed and one was stained with primulin and the other overlaid with Pa8830-GFP. Both plates were then imaged on a FluorChem™ FC. (b) A confocal image of one of the spots of bound Pa8830-GFP is shown. (c) The total fluorescence of bound Pa8830-GFP was measured as a function of asialo-GM1 was determined

crylate (Sigma-Aldrich) in acetone for 1.5 min and dried either overnight or for 2 h at room temperature. The plates were blocked with 3% BSA–PBS buffer at room temperature for 2 h. One colony of Pa8830-GFP was placed in 10 ml of trypticase soy broth with 500 μg/ml ampicillin and grown with shaking at 230 rpm at 37°C for 14–16 h. The bacteria were collected by centrifugation, and washed once in 10 ml of cold PBS. Then the Pa8830-GFP were resuspended to ~10^8 CFU/ml in 1% BSA–PBS buffer and overlayed onto the HPTLC plates (~0.3–0.6 ml of solution per cm^2 of plate area), covered with foil, and incubated at room temperature for 2 h. After incubation, the plates were gently rinsed 3 times with cold PBS, and air dried. Plates and spots stained with primulin or incubated with Pa8830-GFP were photographed under a confocal microscope (Leica TCS NT confocal) or the imager by FluorChem™ FC (Alpha Innotech Corporation, San Leandro, CA). Data analysis was done by with Leica TCS software or densitometric scanning using the NIH Image program (rsb.info.nih.gov/nih-image/). In some experiments the relative intensity of the primulin staining by epifluorescence was compared to the fluorescent intensity of the bound Pa8830-GFP, and these relative ratios were determined by the NIH Image program.

Results

Preparation of Pa8830 strains expressing GFP

To aid in quantifying and imaging the binding of *P. aeruginosa* Strain 8830, we sought to develop stable clones expressing the green fluorescent protein. The 8830 strain was transformed with plasmid DNA with GFP ($_P$SMC21) [36], and stably-expressing colonies of Pa8830-GFP were identified. A fluorescent image of the isolated Pa8830-GFP

used for the subsequent binding experiments reported below is shown in Fig. 1a. All bacteria were highly fluorescent and the level of fluorescence has been maintained indefinitely by the original colonies.

Preparation of glycolipids and neoglycolipids

As a method of identifying direct binding of bacteria to glycoconjugates, a method of choice is the overlay assay, originally developed by Ginsberg's laboratory [19]. In this assay glycolipids are spotted or chromatographed on HPTLC plates and overlayed with labeled bacteria (originally using ^{125}I-labeled bacteria), and binding is visualized following washing of the plate surface. Strongly bound bacteria can then be identified relative to the positions of individual glycolipid, and both the glycolipid and bound bacteria can be quantified.

To facilitate this assay, we prepared a series of neoglycolipids by reductive amination of free glycans with DPPE [37, 40]. Although this approach sometimes gives variable efficiencies of conjugation with different glycans, the degree of conjugation is adequate except for larger sized anionic glycans. In addition, the synthesized neoglycolipids may be purified by HPTLC [37], if necessary, as we have done here. The neoglycolipids we prepared are shown in Fig. 1b. In addition, a number of glycosphingolipids were obtained commercially, and their structures are shown also in Fig. 1b.

Binding of Pa8830-GFP to asialo-GM1

In order to validate the assay technique, we examined the binding of Pa8830-GFP toward asialo-GM1, a previously defined receptor for this mucoid strain [35]. Different amounts of asialo-GM1 were chromatographed on HPTLC and then incubated with Pa8830-GFP. As shown in Fig. 2a, the bacteria bound in a proportional manner to the amount of asialo-GM1 as determined by primulin staining by epifluorescence and a fluorescence image of the bound bacteria from one of the lanes is shown in Fig. 2b. A graphical representation of the binding (Fig. 2c) shows a nearly linear relationship between the amount of asialo-GM1 chromatographed and the binding of Pa8830-GFP, as measured by fluorescence intensity. These results indicate the binding assay is valid, and little if any non-specific binding is observed to the silica matrix.

Specific binding of Pa8830-GFP to asialo-GM1 (GgOse4-Cer) compared to other glycosphingolipids

Using this approach, we then examined the comparative binding of Pa8830-GFP to other glycosphingolipids. As

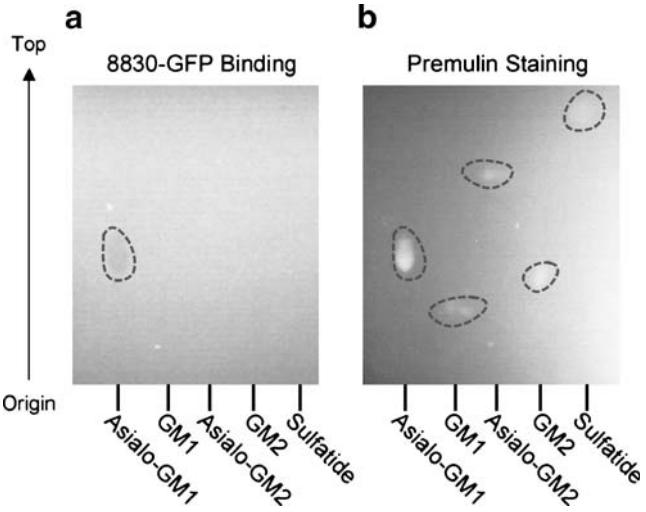

Fig. 3 Binding of Pa8830-GFP to Glycosphingolipids. Each of the indicated glycosphingolipids (1 μg) were developed by in duplicate HPTLC plates and (a) one was overlaid with Pa8830-GFP and (b) the other was stained with primulin and each stained glycolipid is denoted by dotted circles. Detectable binding by Pa8830-GFP was only observed toward asialo-GM1, as indicated by the dotted circle in the (a)

shown in Fig. 3, Pa8830-GFP bound to asialo-GM1, but showed no detectable binding to GM1, GM2, asialo-GM2, or sulfatide (see structures in Fig. 1b). Thus, Pa8830-GFP exhibits specific binding to asialo-GM1 among all glycosphingolipids tested. The results indicate that the binding is highly specific to the terminal sequence of asialo-GM1. This is interesting, since it has been reported that the *P. aeruginosa* stains PAK and PAO bind to both asialo-GM1 and asialo-GM2 [21].

Binding of Pa8830-GFP to neoglycolipids containing sialyl-Le^x and Le^x antigens

The remarkable specificity of binding of Pa8830-GFP to asialo-GM1 compared to other glycosphingolipids, prompted us to test whether the bacteria could recognize other glycan structures as neoglycolipids. To this end, we tested a variety of neoglycolipids prepared with DPPE having the structures shown in Fig. 1b. In each assay, asialo-GM1 was used as a positive control. As shown in Fig. 4a,b, Pa8830-GFP bound to asialo-GM1, but bound better to a neoglycolipid containing the sialyl-Le^x antigen (sialyl-Le^x–DPPE), and very weak binding was observed toward related conjugates sialyl-lactose–DPPE and LNnT–DPPE. Because of the possible different amounts of glycolipids loaded into each lane, we normalized the binding by comparing the ratio of primulin staining of the lipid moiety with that of the fluorescence derived from the binding of Pa8830-GFP. This relative binding ratio (Fig. 4c) shows

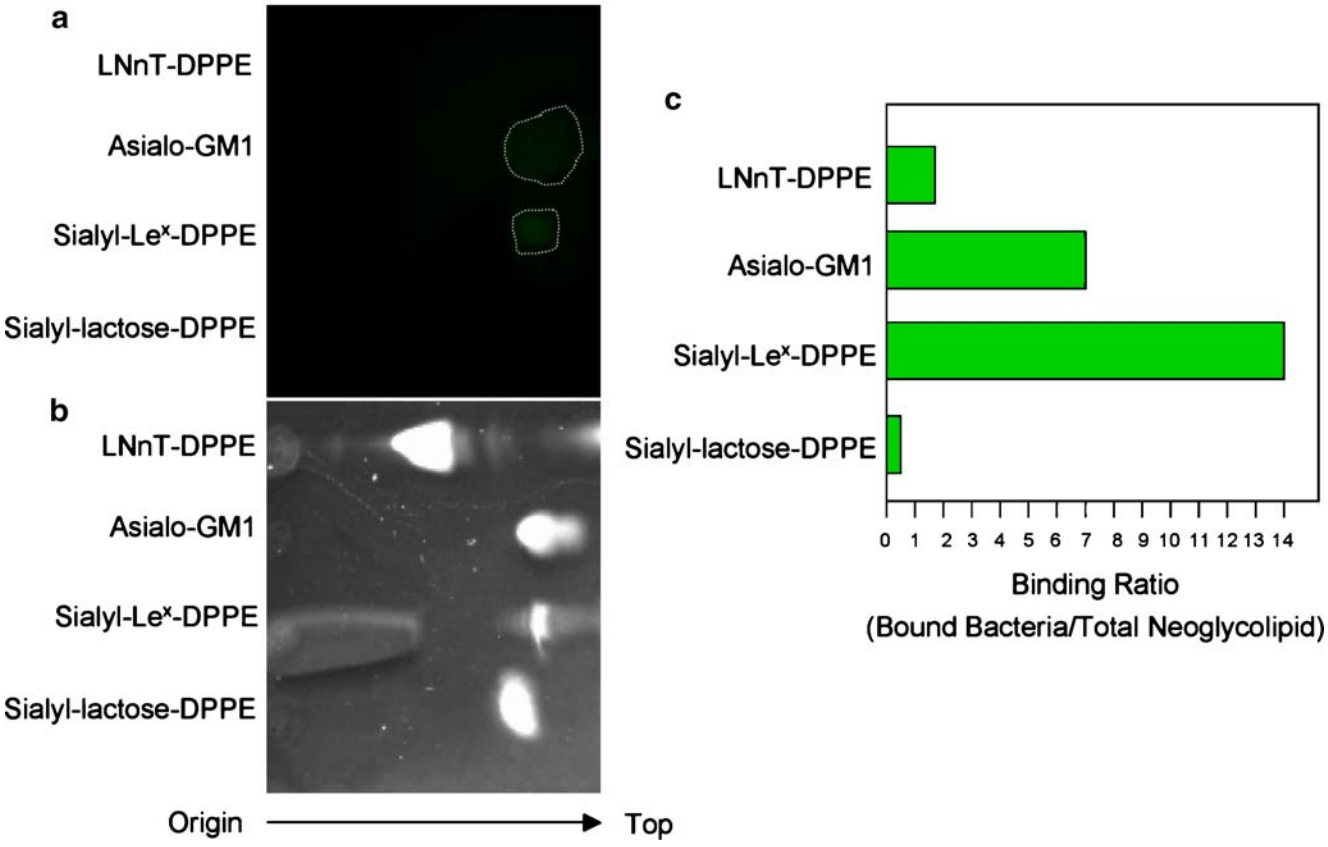

Fig. 4 Binding of Pa8830-GFP to Asialo-GM1 and Neoglycolipids. Approximately 2 μg of each glycolipid was chromatographed on HPTLC and analyzed as in Fig. 3 by (a) overlay with Pa8830-GFP and (b) staining with primulin. (c) The relative fluorescence in primulin versus the GFP was compared and the ratio of total fluorescence is shown

that Sialyl-Le^x–DPPE is well recognized by Pa8830-GFP compared to asialo-GM1.

To provide better quantitative analysis of bacterial binding to different neoglycolipids, we purified each neoglycolipid by HPTLC and extraction, and then spotted increasing and equivalent amounts of each one on HPTLC plates, and directly measured the binding of Pa8830-GFP. As shown in Fig. 5a and b, strong binding of Pa8830-GFP was observed toward sialyl-Le^x–DPPE, but we observed relatively weaker binding toward sialyl-lactose–DPPE and the H-antigen-containing 2-fucosyllactose–DPPE. A quantitative relationship between binding of Pa8830-GFP versus primulin staining in Fig. 5c indicates that sialyl-Le^x–DPPE is preferentially recognized. We further tested the binding of Pa8830-GFP to three other structurally related neoglycolipids to further define the specific sugar determinants and the possible recognition of Fuc(α1-3)GlcNAc moiety that are occurring in the sialyl-Le^x–DPPE. We observed significant binding to Le^x–DPPE and relatively weaker binding to LNnT–DPPE and lactose–DPPE (Fig. 5a,b). These results indicate that the interaction of Pa8830-GFP is strongest toward sialyl-Le^x–DPPE, but the bacteria also weakly bound to Le^x–DPPE. Only low level binding was observed toward the other neoglycolipids.

Discussion

The molecular nature of the recognition of glycans by *P. aeruginosa* is highly important to understanding the role of the bacteria in causing lung disease in patients with CF [41]. Previous studies have documented that some strains of *P. aeruginosa* bind to airway mucins and that the binding is dependent on the sialic acid residues in mucins [7]. It has been shown that in autopsy specimens of CF lungs, the identifiable *P. aeruginosa* are usually associated with stagnant luminal mucin and exudate, but not associated with mucosal surfaces [42].

Here we examined the specific interaction of stable, mucoid, alginate-producing strain *P. aeruginosa* 8830 with a defined set of glycoconjugates and demonstrated that the bacteria strongly recognize sialyl-Le^x–DPPE and less strongly fucose- and sialic acid-containing glycans. Interestingly, the 8830 strain bound well to asialo-GM1, consistent with an earlier study [35], but bacteria did not bind several other glycosphingolipids, including GM1, GM2, asialo-GM2, and sulfatide. The ability of *P. aeruginosa* to recognize the glycosphingolipid asialo-GM1, a likely component of lipid rafts, may be important in the bacterial binding and subsequent entry into epithelial cells

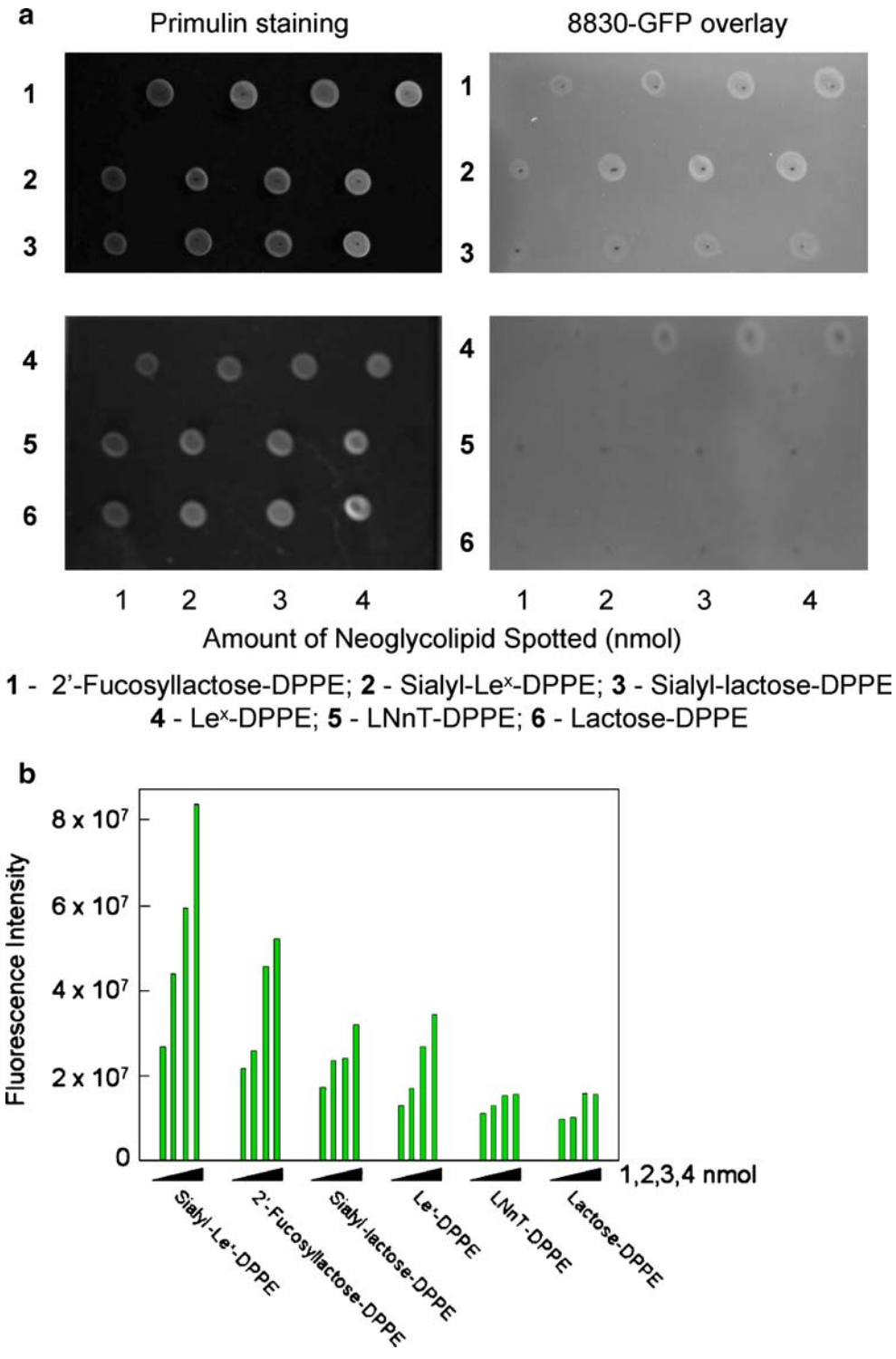

Fig. 5 Binding of Pa8830-GFP to Sialyl-Le^x–DPPE versus Different Neoglycolipids. (a) Different amounts indicated of each neoglycolipid (1,2,3, and 4 nmol) were spotted directly on HPTLC plates and analyzed by primulin staining and overlay with Pa8830-GFP. (b) The relative fluorescence of bound Pa8830-GFP was directly determined for each spot from (a) by confocal microscopy and the fluorescence intensities are shown

[43]. Thus, adhesion by Pa8830-GFP is highly specific and, in regard to sialyl-Le^x, the binding is toward specific glycan determinants known to occur in airway mucins. Sialyl-Le^x is a common determinant found in airway mucins from patients with CF [24].

Previous studies showed that non-mucoid and non-piliated strain 1244-NP of *P. aeruginosa* and several mucoid producing strains isolated from patients bound to polyacrylamide derivatives containing sialyl-Le^x and sulfated forms of sialyl-Le^x [17]. *P. aeruginosa* contains a number of carbohydrate-binding proteins or adhesins [7] but their specific binding to glycan determinants are not well understood. In fact, it is interesting that each strain may have differences in their recognition of different

glycans. One elegant study [17] showed that the flagellar cap protein FliD of the non-mucoid strain PAO1 may recognize glycoconjugates bearing Le^x or sialyl-Le^x determinants, while flagellin binding may be restricted to the glycoconjugate-bearing Le^x epitope. By contrast, another study [44] found that flagellin from the non-mucoid *P. aeruginosa* strain PAK is an adhesion receptor for MUC1 expressed in epithelial cells and a recombinant form expressed in Chinese hamster ovary cells (CHO), which do not express Le^x-related antigens.

Our recent study on airway mucins from human CF patients showed that the mucins contained a higher number of O-glycans per polypeptide (higher density) compared to airway mucins from normal, non-diseased individuals [23]. In addition, there was a substantial increase in sulfated, sialylated, and fucosylated O-glycan structures, and over 260 compositionally different O-glycans were identified in the mucins by matrix-assisted laser/desorption ionization mass spectrometry (MALDI) [23] which is consistent with previous studies showing that airway mucins from CF patients have a high content of a variety of O-glycans containing sialylated, sulfated, and fucosylated glycans in addition to the sialyl-Le^x epitope and its sulfated derivatives [45–57]. The high density presentation of sialyl-Le^x epitope on CF mucins may be especially favorable for *P. aeruginosa* attachment and colonization within the lung space. Efforts are underway in our laboratory now to identify the specific adhesins or glycan-binding proteins in the mucoid *P. aeruginosa* strain 8830 that recognize the sialyl-Le^x epitope.

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