# Sialylation of Outer Membrane Porin Protein D: A Mechanistic Basis of Antibiotic Uptake in *Pseudomonas aeruginosa**

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***Pseudomonas aeruginosa*** **(PA) is an environmentally ubiquitous, extracellular, opportunistic pathogen, associated with severe infections of immune-compromised host. We demonstrated earlier the presence of both $\alpha$2,3- and $\alpha$2,6-linked sialic acids (Sias) on PA (PA$^{+Sias}$) and normal human serum is their source of Sias. PA$^{+Sias}$ showed decreased complement deposition and exhibited enhanced association with immune-cells through sialic acid binding immunoglobulin like lectins (Siglecs). Such Sias-siglec-9 interaction between PA$^{+Sias}$ and neutrophils helped to subvert host immunity. Additionally, PA$^{+Sias}$ showed more resistant to $\beta$-lactam antibiotics as reflected in their minimum inhibitory concentration required to inhibit the growth of 50% than PA$^{-Sias}$. Accordingly, we have affinity purified sialoglycoproteins of PA$^{+Sias}$. They were electrophoresed and identified by matrix-assisted laser desorption-ionization time-of-flight/time-of-flight mass spectrometry analysis. Sequence study indicated the presence of a few $\alpha$2,6-linked, $\alpha$2,3-linked, and both $\alpha$2,3- and $\alpha$2,6-linked sialylated proteins in PA. The outer membrane porin protein D (OprD), a specialized channel-forming protein, responsible for uptake of $\beta$-lactam antibiotics, is one such identified sialoglycoprotein. Accordingly, sialylated (OprD$^{+Sias}$) and non-sialylated (OprD$^{-Sias}$) porin proteins were separately purified by using anion exchange chromatography. Sialylation of purified OprD$^{+Sias}$ was confirmed by several analytical and biochemical procedures. Profiling of glycan structures revealed three sialylated *N*-glycans and two sialylated *O*-glycans in OprD$^{+Sias}$. In contrast, OprD$^{-Sias}$ exhibit only one sialylated *N*-glycans. OprD$^{-Sias}$ interacts with $\beta$-lactam antibiotics more than OprD$^{+Sias}$ as demonstrated by surface plasmon resonance study. Lyposome-swelling assay further exhibited that antibiotics have more capability to penetrate through OprD$^{-Sias}$ purified from four clinical isolates of PA. Taken together, it may be envisaged that sialic acids on OprD protein play important role toward the uptake of commonly used antibiotics in PA$^{+Sias}$. This might be one of the new mechanisms of PA for $\beta$-lactam antibiotic uptake.** ***Molecular & Cellular Proteomics 13: 10.1074/mcp.M113.030999, 1412–1428, 2014.***

Sialic acids (Sias)[1] are nine carbon atom containing acidic residues characteristically found in the terminal position of glycoproteins and glycolipids (1–4). Structural diversity of sialic acids is because of the modification of one or more hydroxyl groups in various positions of the core structure by different groups like acetyl-, methyl-, sulfate-, lactyl-, or phosphate (1, 5–7). More than fifty derivatives of Sias has been reported both in vertebrate and invertebrate systems. It functions as ligand for various cellular communications and also act as masking element for glycoconjugates (8–12).

Sialic acid binding immunoglobulins (Ig)-like lectins (siglecs) selectively expressed on the hematopoetic cells and interact with an array of linkage-specific Sias on a glycan structure express on the same cells or other cells (13). Siglecs can also recognize terminal sialylated glycoconjugates on several pathogens (14–16). After recognizing, they carry out various functions like internalization, attenuation of inflammation, restraining cellular activation along with inhibition of natural killer cell activation (17).

*Pseudomonas aeruginosa* (PA) is a Gram-negative, rod-shaped bacterium. This human pathogen has remarkable capacity to cause diseases in immune compromised hosts. This colonizing microbial pathogen is responsible for infection in

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[1] The abbreviations used are: BLAST, Basic local alignment search tool; IEF, Isoelectric focusing; Gal, Galactose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; HI-NHS, Heat inactivated normal human serum; MAA, *Maackia amurensis agglutinin*; Neu5Ac, *N*-Acetyl neuraminic acid; PA, *Pseudomonas aeruginosa*; Sias, Sialic acid; SNA, *Sambucus nigra agglutinin*; SPR, Surface plasmon resonance; TSB, Trypticase soy broth.

chronic cystic fibrosis, nosocomial infections; severe burn, transplantation, cancer, and AIDS and other immuno-supressed patients (18).

We have reported earlier the presence of linkage-specific Sias on PA. Normal human serum (NHS) is possibly one of the sources of these Sias (19). PA utilizes these Sias to interact through siglecs present on the surface of different immune cells. $PA^{+Sias}$ showed enhanced association with neutrophils through $\alpha$2,3-linked Sias-siglec-9 interaction which facilitated their survival by subverting innate immune function of host (20).

The treatment of PA-infected patient depends upon the extent of the disease and the concerned organs. Conventional $\beta$-lactam, cephalosporins, and aminoglycosides group of antibiotics are most common for such treatment (21). $\beta$-lactam antibiotics inhibit cell wall synthesis by disrupting the synthesis of the peptidoglycan layer of bacterial cell walls (22). When PA showed resistant to $\beta$-lactam antibiotics, new generation of $\beta$-lactam with increased doses or other broad spectrum antibiotics like tetracyclines or fluoroquinolones are prescribed (23). PA isolates from intensive care unit (ICU) patients in general showed higher rates of $\beta$-lactam resistance among other hospitalized patients (24). The increasing frequency of resistance to ceftazidime, piperacillin, imipenem, fluoroquinolone, and aminoglycoside were 36.6%, 22.3%, 22.8%, 23.8%, and 17.8% respectively in PA (25).

The outer membrane of Gram-negative bacteria is, in general, semipermeable through which hydrophilic molecules including antibiotics of below exclusion limit size (0.6 kDa) can pass through the channel-forming proteins generally called porins *e.g.* OprD, OprF, OprG etc. (26, 27). PA shows lower outer membrane permeability with respect to many other Gram-negative bacteria like *Acinetobacter baumannii*, *Stenotrophomonas maltophilia*, *Burkholderia cepacia*, hence the diffusion rate of $\beta$-lactam antibiotics is decreased (27).

Additionally, PA uses MexA-MexB-OprM, MexC-MexD-OprJ, MexE-MexF-OprN, and MexX-MexY-OprM as efflux pumps along with important regulatory factors MexR/NalB, NfxB, NfxC/MexT, and MexZ respectively on their membrane to pump out undesirable chemicals, detergent and antibiotics (28–32). Other Gram-negative bacteria also uses similar types of efflux pumps for such purposes. Moreover, PA produces antibiotic-resistance genes by some mutation (33). Furthermore, $\beta$-lactamase and aminoglycoside-modifying enzymes produced by PA are capable of breaking down the antibiotics (34). Alternatively, these enzymes can directly modify the drug. Hence these antibiotics become functionally ineffective (27).

The presence of lipopolysaccharides (LPS) containing *O*-specific polysaccharides with tri-saccharide repeats of 2-acetamido-2,6-dideoxy-D-glucose, 2-acetamido-2,6-dideoxy-D-galactose, and 5-acetamido-3,5,7,9-tetyradeoxy-7-[(R)-3-hydroxybutyramidol]-3-L-glycerol-L-manno-nonulosonic acid are known for PA serogroup O11 (35). The genes for key enzymes required for complex protein glycosylation are found in the genome of PA14 (36). Moreover, glycosylation in PA1244 has been reported in the form of an *O*-linked glycan in pilin (37). A cluster of seven genes known as the *pel* genes, encode proteins with similarity to components involved in polysaccharide biogenesis. Among these genes, PelF is a putative glycosyltransferase (GT) of the type IV glycosyltransferase (GT4) family (36). PA secreted sialidase in culture medium (38). Genome search reveals that PA14 has the sialidase gene, which may be responsible for cleaving sialic acids (39). PA1 also has sialic acid transporter gene, which possibly transport sialic acids inside the cells (Gene ID: 17688338, Source: http://www.ncbi.nlm.nih.gov/gene/17688338). Additionally, CMP-sialic acid transferase, which is responsible for converting sialic acids to CMP-sialic acid, was purified from PAO12 (40). This enzyme shows close similarity with the enzyme found in *E. coli*.

However, PA being such a notorious organism, it might have many other different mechanisms to fight against antibiotics for their survival. Therefore, it is worthwhile to explore newer mechanism to understand how antibiotics penetrate inside this bacterium. Here we addressed the following questions. Does sialylation of glycoproteins demonstrated on PA play any role in the entry of antibiotics that might facilitate their survival within host?

Accordingly, we have affinity purified a few sialoglycoproteins from PA. Sequence analysis identified twenty six $\alpha$2,3- and $\alpha$2,6-linked sialoglycoproteins. One such identified sialoglycoprotein is OprD porin protein. The presence of Sias on OprD was conclusively confirmed. We have demonstrated that Sias on OprD protein isolated four different clinical isolates hampered its interaction with $\beta$-lactam antibiotics. This might be one of the new mechanisms for $\beta$-lactam antibiotic resistance of PA and thereby facilitates their survival in host.

## EXPERIMENTAL PROCEDURES

*Bacteria—Pesudomonas aeruginosa* (PA 14) is a wild type, virulent burn-wound isolate, gifted by Prof. Richard D. Cummings, Emory University School of Medicine (Atlanta, GA, USA). Three more stains of PA were isolated from urine ($PA_{Urine}$), pus ($PA_{Pus}$), and sputum ($PA_{Sputum}$) of the patients hospitalized at All Indian Institute of Medical Science, New Delhi, India. The Institutional Human Ethical Committee had approved the study and samples were taken with the consent of the patients. They were grown Trypticase soy broth (TSB, DIBCO) in a microaerobic atmosphere and harvested after overnight growth. Additionally, PA was also grown either in sialic acid free medium [Heme- L-histidine (4.0 ml), RPMI 1640 (191 ml), Minimum essential medium (2 ml, 100 mM), $\beta$-nicotinamide adenine dinucleotide (2 ml, 1.0 mg/ml $H_2O$), uracil (10 ml, 2.0 mg/0.1 N ml NaOH), and inosine (20 ml, 20 mg/ml $H_2O$), pH = 7.5] ($PA^{-Sias}$) or in presence of 10% heat inactivated normal human serum (HI-NHS) used as a source of sialic acids ($PA^{+Sias}$) (20). Bacterial suspensions were counted by using a spectrophotometric method and confirmed by pour plate colony counts. Bacterial suspensions were extensively washed with phosphate buffered saline (0.02 M sodium phosphate, 0.15 M saline, pH 7.2; PBS).

*Survival of PA*—The anti-microbial activities of viable $PA^{+Sias}$/$PA^{-Sias}$ of four clinical isolates including PA14 were measured by using two known $\beta$-lactam antibiotics such as piperacillin and ceftazidime (Sigma). Different doses of ceftazidime (0, 2, 4, 8, 10, 15, and 20

μg/ml) or piperacillin (0, 4, 8, 15, 30, and 50 μg/ml) were added to culture $PA^{+Sias}$/$PA^{-Sias}$ [optical density at 600 nm ($OD_{600\ nm}$) = 0.3] separately and allowed to grow at 37 °C with shaking. Bacterial suspensions were counted by measuring $OD_{600\ nm}$. The survival of PA was further confirmed by pour plate colony counts and the percent survival was calculated by considering bacteria grown in absence of antibiotics as 100%.

*Preparation of Membrane Fraction of Bacteria*—Bacterial Membrane fraction was prepared as describe elsewhere (41). Typically, PA14, $PA_{Urine}$, $PA_{Pus}$, and $PA_{Sputum}$ ($6 \times 10^{14}$ cells) were washed in ice cold Tris-HCl (50 mM), magnesium chloride ($MgCl_2$, 10 mM) dithiothreitol (1 mM), pH 7.2 containing glycerol (0.5%) and subsequently lysed by sonicator (Misonix, Microson) with 4 pulses of 20 s each in ice bath. The bacterial lysate was centrifuged at 10,000 × *g* for 30 min at 4 °C and the supernatant was further centrifuged at 50,000 × *g* at 4 °C for 1 h. The supernatant was discarded and subsequently, pellet was dissolved in solubilizing buffer containing Tris-HCl (0.05 M), 1% (w/v) detergent concentration (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate: β-Octyl-D-glucopyranoside = 1:1), $MgCl_2$ (1.0 mM), calcium chloride (1.0 mM), dithiothreitol (0.2 mM), protease inhibitor mixture, pH 7.2, sonicated (three pulses, 10 s each) in ice-mixture and incubated at 4 °C for 1 h. After centrifugation at 50,000 × *g* at 4 °C for 30 min, the supernatant was collected and dialyzed against Tris-HCl (0.05 M, pH 7.2), saline (0.15 M) containing 0.01% sodium azide (TBS), and the protein content was quantified by Bradford method. This membrane fraction was used for surface plasmon resonance study and also for purification of linkage specific sialoglycoproteins.

*Surface Plasmon Resonance (SPR)*—Two sialic acid binding plant lectins *Sambucus nigra agglutinin* (SNA) and *Maackia amurensis agglutinin* (MAA), that recognize α2,6- and α2,3- sialogalactosyl residues, (Vector labs, Burlingame, CA) respectively were used for this study. The pattern of binding of SNA and MAA with the membrane fraction of PA was determined using SPR (Biacore 2000; Biacore, Uppsala, Sweden). Carboxymethyl-dextran sensor chips (CM5 sensor chips) were equilibrated with running buffer (10 mM HEPES, pH 7.4, 0.15 M KCl and 0.001% Tween 20) for a period of 10 min at 25 °C at a flow rate of 5 μl/min. Four flow cells of a CM5 sensor chip were activated using of a mixture (70 μl, 1:1) of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (200 mM) and *N*-hydroxy-sulfosuccinimide (50 mM) at a flow rate of 5 μl/min in a 14 min pulse. SNA and MAA (200 μg/ml in 10 mM sodium acetate buffer, pH 4.3) were immobilized separately by injecting over the flow cells 2, 3, and 4 respectively to reach 6500 resonance units (RU). Un-reacted groups were blocked with ethanolamine (1 M, 50 μl, pH 8.5) for 7 min. Flow cell 1 was considered as the reference. Dialyzed membrane fractions (10–100 μg/30 μl) against coupling buffer (5 mM $Na_2HPO_4$, pH 7.4 and 150 mM NaCl) at 4 °C were injected for 300 s at a constant flow rate of 5 μl/min. The sample was first injected over the reference surface (flow cell 1) and subsequently on flow cell 2, 3, and 4 with immobilized lectins. Bound ligands were removed with acetate buffer (10 mM, pH 4.3, 30 μl) during a regeneration step.

Similarly, interaction of sialylated and non-sialylated OprD proteins ($OprD^{+Sias}$ or $OprD^{-Sias}$) with β-lactam antibiotics was examined by SPR. $OprD^{+Sias}$ and $OprD^{-Sias}$ (200 μg/ml in acetate buffer) were immobilized separately over the flow cells. Non-covalently bound OprD proteins were removed by two fluxes of HCl (20 mM) for 2 min. β-lactam antibiotics (10–100 μM) such as piperacillin and ceftazidime (Sigma) were injected separately onto $OprD^{+Sias}$ or $OprD^{-Sias}$ coated CM5-sensor chip at a flow rate of 10 μl/min (42, 43). Bio-Evaluation 3.0 software (Biacore) was used to analyze sensorgrams. The dissociation constant ($K_D$) for antibiotic-OprD protein association was calculated as the ratio of the backward ($k_d$) and forward rates ($k_a$).

*Purification of Sialoglycoproteins by Lectin Affinity Chromatography*—Linkage-specific sialoglycoproteins of PA were purified using SNA-Sepharose-4B and MAA-Sepharose-4B column matrix (2.0 ml) separately. SNA and MAA were coupled with cyanogen bromide (CNBr)-activated Sepharose 4B (44).

MAA lectin (2.0 mg) was dissolved in coupling buffer (2.0 ml) containing sodium bicarbonate (0.1 M), sodium chloride (0.5 M), pH 8.5. CNBr-activated Sepharose 4B (0.6 g) was socked with HCl (1 mM) for 15 min at 25 °C and washed with coupling buffer. MAA solution was mixed with the activated Sepharose 4B beads and kept for overnight on gentle shaker at 4 °C. MAA concentration was measured in the supernatant by Bradford method. The active site of Sepharose 4B was blocked with glycine (0.2 M, pH 7.2, 50 μl/5 ml bead) for 2 h at 25 °C. MAA coupled beads were washed with 40 ml of coupling buffer and 40 ml of acetate buffer (0.1 M sodium acetate, 0.5 M NaCl, pH 4) alternatively, followed by 40 ml of TBS. MAA-Sepharose 4B was stored in TBS at 4 °C with sodium azide (0.01%). Similarly SNA was also coupled with CNBr-activated Sepharose 4B. The percentages of coupled MAA and SNA with Sepharose 4B were 83.33% and 87.87% respectively.

The PA membrane protein (2.0 mg) was passed through the SNA-Sepharose and MAA-Sepharose affinity column separately for several times and incubated for overnight at 4 °C. The columns were washed with TBS to remove unbound proteins. Bound sialoglycoproteins were eluted with ethylenediamine (20 mM) at 4 °C, neutralized immediately with dilute HCl (5:3 ratio) and exchanged with TBS by using Viva spine 6 (GE healthcare, 5 kDa cut off). Purified sialoglycoproteins was stored in presence of protease inhibitors mixture at −70 °C for further use (20, 44).

*Analysis of Purified Sialoglycoproteins and Sialylated OprD Proteins by Gel Electrophoresis*—

*Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)*—Purified sialoglycoproteins (10.0 μg) were separated on gradient SDS-PAGE (7.5–15%) gel in a minigel apparatus (Bio-Rad, Hercules, CA). Gel was stained with Coomassie brilient blue R 250 (Bio-Rad) and distained with methanol, acetic acid, and water mixture (30:15:55). Additionally, purified $OprD^{+Sias}$ and $OprD^{-Sias}$ (4 μg each) were analyzed by SDS-PAGE (10 and 7.5%) and processed similarly. The samples were desialylated overnight with *Arthrobacter ureafaciens* neuraminidase (0.2 mU/mg) at 37 °C and processed similarly (45). Additionally different amounts of purified $OprD^{+Sias}$ protein were analyzed on SDS-PAGE to check their purity.

*Isoelectric Focusing (IEF)*—Purified $OprD^{+Sias}$ and $OprD^{-Sias}$ (2 μg/100 ml) were incubated for overnight on IPG gel strip (7 cm) with a gradient range of pH 4–7. IEF was carried out for 20 min at 250 V at linear mode; 2 h at linear mode from 250 to 4000 V; at 4000 V up to 10000 V-h in rapid mode. Separated proteins according to their iso-electric point (pI) were fixed with methanol-acetic acid-water (30:15:55) and stained by Coomassie. The proteins were desialylated and processed similarly. The pI was determined from the pI of known proteins used as standards (12, 45).

*Two-dimensional Gel Electrophoresis (2DE)*—Purified sialoglycoproteins (120 μg) were initially processed with 2D-clean up kit (Bio-Rad) and subsequently processed for IEF. The focused strips were incubated for 30 min at 25 °C in a first equilibration buffer containing urea (6 M), SDS (2% w/v), Tris-HCl (50 mM, pH 6.8), glycerol (30%), dithiothreitol (2% w/v), and for 30 min in second equilibration buffer containing iodoacetamide (2.5% w/v). The strips were hold in place with low-melting temperature agarose (0.4%) and loaded onto SDS-PAGE (7.5–15%) gel. The 2-D gels were stained with Bio-safe Coomassie (45) and distained with distilled water.

*MALDI-TOF/TOF MS/MSMS*—Coomassie stained spots of affinity-purified sialoglycoprotein were excised from the 2D-Gel. Spots were processed using in-gel tryptic digestion kit (Pierce, Thermo scientific). In brief, they were reduced with Tris (2-carboxyethyl) phosphine in ammonium bicarbonate buffer (25 mM), incubated with iodo-aceta-

mide in dark 1 h and subsequently digested overnight with trypsin (100 ng/per sample) in ammonium bicarbonate buffer.

Gel pieces were washed with acetonitrile, dried and further rehydrated with trypsin containing digestion buffer. Peptides were extracted from gel, dried, and dissolved in acetonitrile (50%) in trifluoroacetic acid (0.1%). Subsequently, they were spotted on a target MALDI plate using $\alpha$-cyano hydroxy cinnamic acid (CHCA) as a matrix (45, 46) and analyzed using MALDI-TOF mass spectrometer (Applied Biosystem, Foster City, CA). Spectra were calibrated using the matrix and tryptic auto-digestion ion peaks of Calmix, a standard mixture of six peptides.

Spectral data were analyzed from PMF in combination with MS/MS spectra by searching against the database using the MASCOT (Matrix Science Ltd., London, UK) version 2.2 and basic local alignment search tool (BLAST) of ABI GPS Explorer software, version 3.6 (Applied Biosystems). For database searching the following parameters were used. Peak list-generating software: 4000 series explorer software version 3.5; taxonomy: all entries; database: MSDB version 2.1.0 dated 27.02.2005; No of entries: Database- MSDB20050227 (1942918 sequences; 629040812 residues); cleavage enzyme: trypsin; variable modifications: oxidation on methionine; fixed modification: carbamidomethylation; missed cleavages permitted: one missed cleavages; minimum signal to noise ratio (S/N): 10; peptide charge: +1; precursor mass tolerance: ± 100 ppm; mass tolerance for the MS/MS search: ± 0.2 Da. Significance of data was selected according to their *p* value ($p < 0.05$) where p is the probability that the observed match in a random event. Therefore Mascot search engine is setting the threshold ions score [-10*Log(p)] on its own based on the type of analysis, number of spectra to be analyzed etc.

To examine the quality and accuracy of data, false discovery rate (FDR) values were determined using Mascot software (Matrix Science). Briefly, raw combined MS and MS/MS data were converted into Mascot generic format (MGF) file using following parameters (MS peak filter: Peak density filter: 65/200 Da; S/N: 10; Area: 50; No of peaks: 200; MS/MS peak filter: Peak density filter: 65/200 Da; S/N: 5; Area: 20; No of peaks: 200). The FDR values of respective proteins were calculated by submitting MGF files in online Mascot website.

*Purification of OprD Proteins by Anion Exchange Chromatography*—For outer membrane OprD porin proteins purification (47), PA14, $PA_{Urine}$, $PA_{Pus}$, and $PA_{Sputum}$ membrane pellets were separately mixed with Tris-HCl buffer (10 mM, pH 8, 5 ml) containing $\beta$-octyl glucoside (68 mM), EDTA (5 mM) and protease inhibitor mixture and sonicated (5 pulses, 10 s each). The supernatant obtained after ultracentrifugation (100,000 × *g*) for 30 min at 4 °C contained the membrane proteins. Diethylaminoethyl (DEAE)-anion exchange column (5.0 ml) was equilibrated with buffer containing Tris-HCl (10 mM, pH 8), octa-ethleneglycol dodecyl ether (5 mM) and 1 mM EDTA (Buffer A, 47). Membrane protein (2 mg) was loaded onto the column and incubated overnight at cold. Unbound or loosely adhered proteins were removed by washing with Buffer A. The bound protein was eluted at 4 °C using a linear gradient of NaCl (0 - 0.50 M) in Buffer A. Eluted fractions (1 ml each/microfuge) were checked by measuring OD at 280 nm. Each twenty fractions were checked on 10% SDS-PAGE through Coomassie brilliant blue staining. OprD protein-enriched fraction was found in #6–10 microfuges (5.0 ml).

OprD protein-enriched fraction was further re-chromatographed using DEAE-anion exchange column. Accordingly, OprD protein-enriched fraction was exchanged with Tris-HCl (10 mM, pH 8), $\beta$-octyl glucoside (34 mM), and 1 mM EDTA (Buffer B). Unbound protein was removed by washing with buffer B, bound protein was eluted (1 ml/microfuge) using a linear gradient of NaCl (0 - 0.3 M) in buffer B. Eluted OprD protein (#6–9 tubes) was combined, concentrated, and concentration was estimated by Bradford protein assay (48). The purity of OprD was checked on SDS-PAGE by Coomassie brilliant blue staining using different amounts of protein.

For further confirmation, gel band was excised, digested with trypsin and identified by MALDI-TOF/TOF-MS. PMF and MS/MS combined spectra were compared with MSDB database sequences.

*Detection of Sialic Acids on $OprD^{+Sias}$*—

*Thin Layer Chromatography (TLC)*—$OprD^{+Sias}$ or $OprD^{-Sias}$ (100 $\mu$g each) protein was subjected to acid hydrolysis with an equal volume of propionic acid (4 M) separately for 4 h at 80 °C to release Sias, cooled on ice for 10 min and purified (11, 49). Liberated Sias was subsequently passed onto cation (Dowex 50WX8, 100–200 mesh) and anion (Dowex 2X8, 200–400 mesh) exchange columns. The eluted free Sias were collected, lyophilized and resuspended in double distilled water ($ddH_2O$, 40 $\mu$l), spotted (5 $\mu$g/10 $\mu$l) onto TLC plates (Merck KGaA, Germany) using 1-propanol/$H_2O$ (7:3 v/v). Sias from bovine submandibular mucin (BSM) along with neuraminic acid (Neu5Ac, Sigma) were used as internal standards. Plates were developed by spraying orcinol/HCl/$FeCl_3$ reagents and heated at 180 °C for 20 min (50).

*Fluorimetric-high Performance Liquid Chromatography (Fluorimetric HPLC)*—An aliquot of the purified Sias from $OprD^{+Sias}$ and $OprD^{-Sias}$ were derivatized with 1,2-diamino-4,5-methylenedioxybenzene (DMB) (11, 19, 45). DMB-Sias were analyzed by fluorimetric reverse-phase HPLC analysis on a RP-18 column (LichroCART, Merck, Germany) using isocratic elution (19, 45) and compared with Sias purified from BSM. Fluorescence was detected using an excitation and emission wavelength of 373 nm and 448 nm respectively. Different forms of DMB-derivatized sialic acids were collected after fluorimetric HPLC, lyophilized for further analysis (50).

*MALDI-TOF MS Analysis*—HPLC fraction of lyophilized DMB-sialic acids purified from OprD protein was dissolved in water (2 $\mu$l) and processed by dried-droplet procedure. An equal volume of 5-dihydroxybenzoic acid (DHB, 10 $\mu$g/$\mu$l) in ethanol (60%) as matrix was mixed with DMB-sialic acids. The mixture (0.50 $\mu$l) was placed on the target and processed as described above (50).

*Quantitation of Sias by Fluorimetric Acetyl Acetone Method*—The total Sias content of $OprD^{+Sias}$ or $OprD^{-Sias}$ (50 $\mu$g each) of PA14, $PA_{Urine}$, $PA_{Pus}$, and $PA_{Sputum}$ was measured by mild oxidation with sodium metaperiodate separately using pure Neu5Ac as standard (51). The fluorogen formed was detected at 510 nm upon excitation at 410 nm with an F-4010 spectrofluorimeter (Hitachi, Tokyo, Japan).

*Enzymatic Release of N-Linked Glycans*—Sialylated and non-sialylated OprD proteins were purified from $PA^{+Sias}$ and $PA^{-Sias}$ respectively. Proteins were de-salted using spin filter (10 kDa cut off, MilliPore, Billerica, MA) by Glycocore. *N*-glycans were isolated from 200 $\mu$g of protein using peptide *N*-glycosidase F (New England Biolabs, Ipswich, MA). Briefly, the samples were denatured using denaturing buffer (New England Biolabs) at 100 °C for 10 min, followed by blocking of excess SDS using Nonidet P-40 buffer (New England Biolabs) and finally treating the samples with 5 mU of PNGaseF (New England Biolabs) in enzyme reaction buffer at 37 °C for 20 h. *N*-Glycans were purified using sequential passing of the reaction mixture over SepPak C18 (100 mg, 1 ml, Waters, Milford, CA) and Poly-Graphitized-Charcoal cartridge (50 mg, 1 ml, Thermo Scientific, Waltham, MA). *N*-glycans trapped in the PGC was eluted with 15–30% acetonitrile containing 0.1% TFA and dried down on speed vac. *N*-Glycans isolated from known amount of proteins were used for their chemical characterization. For monosaccharide analysis by high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) 20 $\mu$g of protein was hydrolyzed using 2NTFA at 100 °C for 4 h. Acid was removed by dry nitrogen flush and samples dissolved in known volume of water and injected on CarboPac PA-1 column attached to PAD detector (ICS3000,Dionexnow Thermo Scientific, Sunnyvale, CA). Sialic acid was done using an ultra

performance liquid chromatography with fluorescence (UPLC-FL, Aquity UPLC System, Waters, Milford, MA) on 10 μg of protein. Briefly, samples were hydrolyzed using 2 M AcOH at 80 °C for 3 h, followed by removal of the acid in speed vac and 10 kDa spin filtration. The filtrate was dried and reacted with DMB (Sigma Aldrich, Milwaukee, WI) and injected on a BEH-C18 column (2.1 × 50 mm) (Waters). For fluorescent tagging purified *N*-glycans equivalent to 20 μg of protein was used. Fluorophore 2-AB (Sigma Aldrich) was used to reductive couple on the pure *N*-glycans followed by removal of excess reagents by Glycoclean S-cartridge (Prozyme, Hayward, CA). Fluorescent labeled *N*-glycans were also identified by HPAEC coupled with an online fluorescent detector (HPAEC-FL). 2-AB-labeled glycans were dissolved inwater and separated by using a PA-1 column (4 × 250 mm; Dionex, Sunnyvale, CA). For high mannose glycans the elution profile was matched with the retention times of 2-AB-labeled *N*-glycans from RNaseB (Sigma Aldrich) and for sialylated glycans the spectra was compared with *N*-glycans from bovine fetuin (Sigma Aldrich).

Purified *N*-glycans isolated from 150 μg of protein were characterized by MALDI-TOF mass spectrometry (4800 Plus MALDI-TOF/TOF, AB-Sciex). Prior to doing MALDI, *N*-glycans were permethylated following by modified Ciucannu and Kereks method (52). Briefly, dried *N*-glycans were dissolved in anhydrous DMSO (Sigma Aldrich) followed by addition of NaOH slurry in DMSO and Methyl-iodide (Sigma Aldrich). After vigorous stirring for 45 min the reaction was stopped adding ice-cold water and permethylated glycans were extracted using chloroform-water partitioning. The chloroform later containing permethylated glycans were dried using dry nitrogen flush and dissolved in 200 μl of methanol l.50 μl of sample was dried in speed vac and re-dissolved in 5 μl of methanol followed by addition of1 μl of water containing 0.1% TFA. 1.2 μl of this sample solution was mixed with super-DHB matrix (Sigma Aldrich) in 1:1 ratio and spotted on stainless steel MALDI plate. All the spectra were acquired in positive mode using 337 nm solid state lasers (53).

*Chemical Release of O-Linked Glycans*—Reductive beta elimination reaction was performed for *O*-glycan release on 150 μg of protein sample. Briefly samples were treated with 50 mM NaOH in presence of 1 M $NaBH_4$at 45 °C for 16 h on a stirring cum heating block (ReactiTherm, Pierce, Rockford, IL). The samples were cooled over ice-water bath, neutralized with ice-cold 30% acetic acid and desalted using cation-exchange resine (Dowex-50W, BioRad) and lyophilized. Dried sample was co-evaporated three times each with acidified methanol (MeOH: AcOH 9:1 v/v mixture) and MeOH and to remove the borate passed over SepPak C18 cartridge (Waters) and permethylated prior to doing mass spectral analysis using MALDI. Plausible N- and O-glycan structures were searched and annotated by selecting the consortium for functional glycomics (CFG) data base of GlycoWorkbench software version 1.2.4105 (53- 55).

*Liposome-swelling Assay*—Egg phosphatidylcholine (6.2 μmol) and dicetylphosphate (0.2 μmol) were dissolved in acetone and dried at the round bottomed flask by vacuum centrifugation. The phospholipids film was resuspended in double distilled water (0.2 ml) and mixed with purified $OprD^{+Sias}$/$OprD^{-Sias}$ or sialidase-treated-$OprD^{+Sias}$/$OprD^{-Sias}$ proteins of $PA_{Urine}$, $PA_{Pus}$, and $PA_{Sputum}$ along with PA14 or BSA (10 μg) separately. The mixtures were sonicated (5 s pulse, five times) in an ice bath until suspension turn from opaque to translucent. Suspensions were then dried in the same tubes by warming them in a water bath (45 °C) and further using vacuum pump via a copper sulfate ($CuSO_4$) tube. Purified channel-forming porin protein OprD was incorporated into the phospholipid bilayer. The liposomes were stored overnight in an evacuated desiccator containing $CuSO_4$. Finally, the film was suspended in a solution (0.40 ml) typically containing dextran T-20 (Sigma, 17%, W/V), sodium nicotinamide adenine dinucleotide (NAD, 4 mM, pH 6) and imidazole-NAD (1 mM, pH 6.0) buffer. The suspension in the tubes was kept for 2 h at 25 °C without agitation and then gently resuspended by hand shaking. The suspended liposome solutions were then passed through Millipore membrane filter (8 μm) to remove larger particles. The filtered suspension (20 μl for each tube) was diluted (0.6 ml) with antibiotic (piperacillin or ceftazidime, 20 mM) solution separately. Because of presence dextran T-20 (17%) of $OprD^{+Sias}$ protein-containing liposome solution increased the turbidity of the liposome suspension after a rapid dilution into the solutions of antibiotics. Because of the osmotic pressure applied by the dextran in the intravascular space, penetration of piperacillin or ceftazidime through liposomes caused the liposomes to swell and the swelling was detected as a decrease in turbidity. The optical density was measured at 400 nm with a spectrophotometer (Shimadzu, Japan) for 90 s (56).

*Statistical Analysis*—Values show the means ± standard deviations of triplicate results from single representative experiments. For all data shown, three independent experiments were routinely carried out, unless indicated otherwise. Student's *t* test was used to determine the level of statistical significance.

## RESULTS

*$PA^{+Sias}$ Showed Increased Survival Against Antibiotics*—We have reported earlier the presence of linkage-specific Sias on PA. As increased frequency of multi-drug-resistance in PA is very common, here we wanted to address whether the Sias on PA play any role toward β-lactam antibiotic resistance. Accordingly, PA were treated with two different types of β-lactam antibiotics and their survival was quantitated by colony-forming unit (CFU). Near minimum inhibitory concentration required to inhibit the growth of 50% ($MIC_{50}$, 8 μg/ml) of ceftazidime antibiotics, $PA^{+Sias}$ ($5.92 \pm 0.25 \times 10^5$) showed higher CFU compared with $1.43 \pm 0.18 \times 10^5$ shown by $PA^{-Sias}$ (Fig. 1*A*). In parallel, $PA^{+Sias}$ with piperacillin ($6.79 \pm 0.34 \times 10^5$) also showed enhanced CFU as compared with $PA^{-Sias}$ with piperacillin ($3.12 \pm 0.21 \times 10^5$) in the vicinity of $MIC_{50}$ (15 μg/ml) indicating Sias on $PA^{+Sias}$ may play some crucial role for their resistivity toward antibiotics (Fig. 1*B*). However, both $PA^{+Sias}$ and $PA^{-Sias}$ showed low but similar CFU value ($0.26 \pm 0.06 \times 10^5$) in presence of higher doses of either ceftazidime (20 μg/ml) or piperacillin (50 μg/ml). CFU counts indicated that all three clinical isolates namely $PA_{Urine}$ (Fig. 1*C*, 1*D*), $PA_{Pus}$ (Fig. 1*E*, 1*F*), and $PA_{Sputum}$ (Fig. 1*G*, 1*H*) showed similar degree of resistance against those β-lactam antibiotics like PA14.

*$PA^{+Sias}$ Membrane Showed Increased Association with Sias-binding Lectins*—Encourage by this observation, we aimed to understand the various sialoglycoproteins present on $PA^{+Sias}$ at molecular level. Accordingly, at the initial phase, we determined the binding capacity of membrane protein isolated from $PA^{+Sias}$ with two Sias-binding lectins (SNA and MAA). For analysis of such interaction, SNA and MAA were separately immobilized on the activated CM5 sensor chip to reach 6500 RU. The best level of activation of CM5 chips was observed following activation for 14 min with freshly prepared carbodiimide and hydroxysuccinimide buffers, used immediately after mixing.

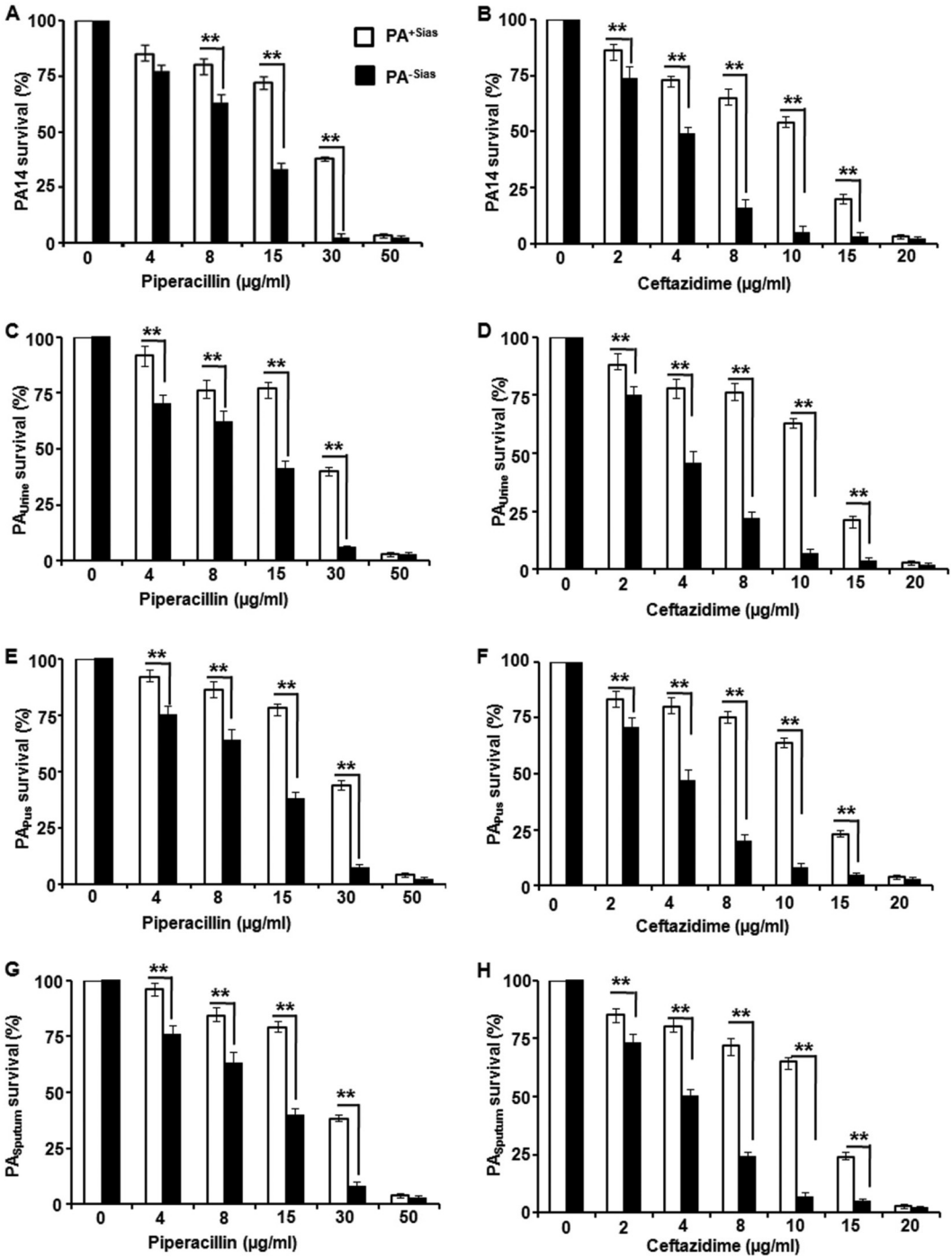



FIG. 1. **$PA^{+Sias}$ showed increased survival against antibiotics.** PA14, $PA_{Urine}$, $PA_{Pus}$, and $PA_{Sputum}$ were grown in heat inactivated human serum containing ($PA^{+Sias}$) and Sias free medium ($PA^{-Sias}$). The anti-microbicidal activity of two known drugs piperacillin (*A, C, E, G*) and ceftazidime (*B, D, F, H*) on $PA^{+Sias}$ or $PA^{-Sias}$ were measured. $PA^{+Sias}$ or $PA^{-Sias}$ were grown in presence of ceftazidime (0, 2, 4, 8, 10, 15 and 20 $\mu$g/ml) or piperacillin (0, 4, 8, 15, 30 and 50 $\mu$g/ml) at 37 °C overnight. PA was counted by turbidity method using $OD_{600\ nm}$ and further confirmed by CFU counts. Data was presented as percent survival of PA. The values represent the means from three separate experiments ± S.D. *p* values (**$<0.005$) are from one-tailed t tests comparing $PA^{+Sias}$ with $PA^{-Sias}$ in respective dose.

The sensorgram was obtained by chip activation and immobilization of lectins followed by deactivation of the remaining sites with ethanolamine. A representative sensorgram was shown for immobilization of SNA (supplemental Fig S1). Bacterial membrane fractions (10–100 $\mu$g/30 $\mu$l) were injected for 6 min over the covalently bound SNA and/or MAA surface separately. Representative sensorgrams showed that both SNA and MAA exhibited a similar pattern and magnitude of

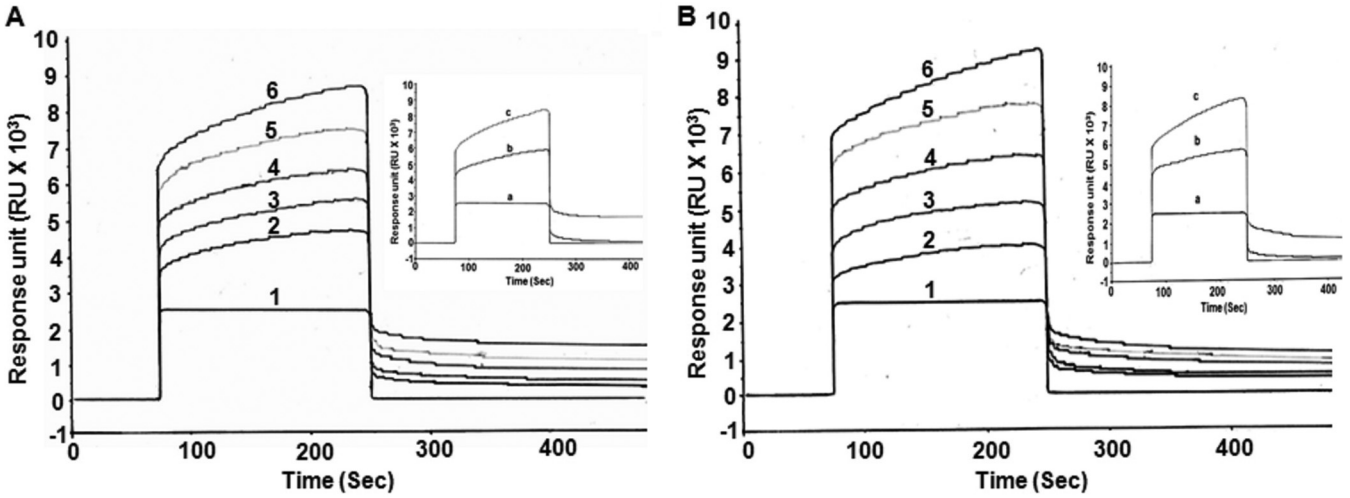



FIG. 2. **Interaction of PA with sialic acid binding lectins by Biacore analysis.** SNA and MAA were covalently coupled over a Biacore CM5 sensor chip. Different analytes such as coupling buffer (30 $\mu$l, 1) and increasing concentrations of PA proteins [10 $\mu$g (2), 20 $\mu$g (3), 30 $\mu$g (4), 50 $\mu$g (5), and 100 $\mu$g (6)] in 30 $\mu$l were injected separately on immobilized SNA (*A*) and MAA (*B*) respectively. The extent of binding was expressed in arbitrary resonance units (RU). The reduction in binding of the bacterial membrane fraction to SNA and MAA in the presence (b) and absence (a) of sialidase is shown in inset of *A* and *B*.

binding (Fig. 2*A*, 2*B*). Dose dependent increased association of PA membrane protein (10, 20, 30, 50, and 100 $\mu$g/30 $\mu$l) with SNA (648, 872, 1107, 1431, and 1675 RU) as well as MAA (755, 847, 966, 1135, and 1342 RU) respectively was observed. The bacterial membrane-lectin interactions with both SNA and MAA reduced significantly after de-sialylation as represented by a lower RU value indicating specificity of the binding (Inset, Fig. 2*A*, 2*B*).

*$PA^{+Sias}$ Exhibited More Number of $\alpha$2,6-linked Compared with $\alpha$2,3-linked Sialylated Glycoproteins*—Cell surface sialoglycoproteins of pathogens play essential role in disease biology. To check which glycoproteins of PA become sialylated, all sialoglycoproteins having $\alpha$2,6- and $\alpha$2,3-linkages were purified from total membrane protein of $PA^{+Sias}$ by using SNA and MAA as their respective affinity ligands. Approximately 8.9 $\pm$ 1.23% and 7.45 $\pm$ 1.38% of total membrane proteins are $\alpha$2,6-linked and $\alpha$2,3-linked of purified sialoglycoproteins respectively.

Equal amount (10 $\mu$g) of total membrane proteins from $PA^{+Sias}$, purified $\alpha$2,6- and $\alpha$2,3-linked sialoglycoproteins as well as unbound proteins from affinity column were separated by SDS-PAGE (Fig. 3*A*). As expected a few bands in purified fractions were coincided with total membrane proteins whereas a few of them were absent in unbound fraction. Purified SNA/MAA bound sialoglycoproteins (120 $\mu$g) were separately analyzed by 2D-SDS-PAGE and images were taken. There were 22 spots corresponding to $\alpha$2,6-linked sialoglycoproteins (Fig. 3*B*) whereas $\alpha$2,3-linked sialoglycoproteins showed only 14 spots (Fig. 3*C*) indicating PA membrane fractions contained more number of $\alpha$2,6-linked compared with $\alpha$2,3-linked sialylated glycoproteins.

*Linkage-specific Sialoglycoproteins Identified on $PA^{+Sias}$*—Molecular identification of these linkage-specific sialoglycoproteins is necessary to understand their role in $PA^{+Sias}$. Accordingly, Coomassie-stained 2D SDS-PAGE gel spots were excised, destained and treated with trypsin. Tryptic fragments of all the spots were analyzed by MALDI-TOF/TOF-MS. Using the MASCOT software, the resulting combined PMF (supplemental Figs S6–S31) and MS/MS (supplemental Figs S58–S83) spectrum was compared with the MSDB sequence database. A few $\alpha$2,6-linked, $\alpha$2,3-linked (Table I) and both $\alpha$2,3- and $\alpha$2,6-linked sialylated proteins (Table II) were matched with the MSDB database sequences of PA using search criteria "All entries" that will consider mammalian, bacteria, fungi etc. together. Additionally, to examine the quality and accuracy, false discovery rate (FDR) of all data was calculated. FDR values emphasized that 22 out of 26 proteins were identified with zero false positive identifications (Table I and II). Information about the identified peptides for each protein and their individual scores have documented in supplemental Figs S32–S57. Although we believed that these identified proteins possibly are sialylated based on their purification using lectin-affinity chromatography, however, details investigation of each protein is needed to further validate this observation.

*Purification and Characterization of $OprD^{+Sias}$ Protein*—Antibiotic resistance of PA is very common in hospitalized patients especially in underdeveloped country. After careful monitoring of these linkage-specific sialoglycoproteins identified on $PA^{+Sias}$, we have selected OprD porin proteins, having both $\alpha$2,3- and $\alpha$2,6-linked sialic acids, to understand the detailed functional role of such acquired sialic acids with respect to the entry of $\beta$-lactam antibiotics inside the cell.

Accordingly, in search for the possible reasons behind antibiotic resistance of PA in protein level, we have purified OprD proteins form $PA^{+Sias}$ ($OprD^{+Sias}$) and $PA^{-Sias}$ ($OprD^{-Sias}$)

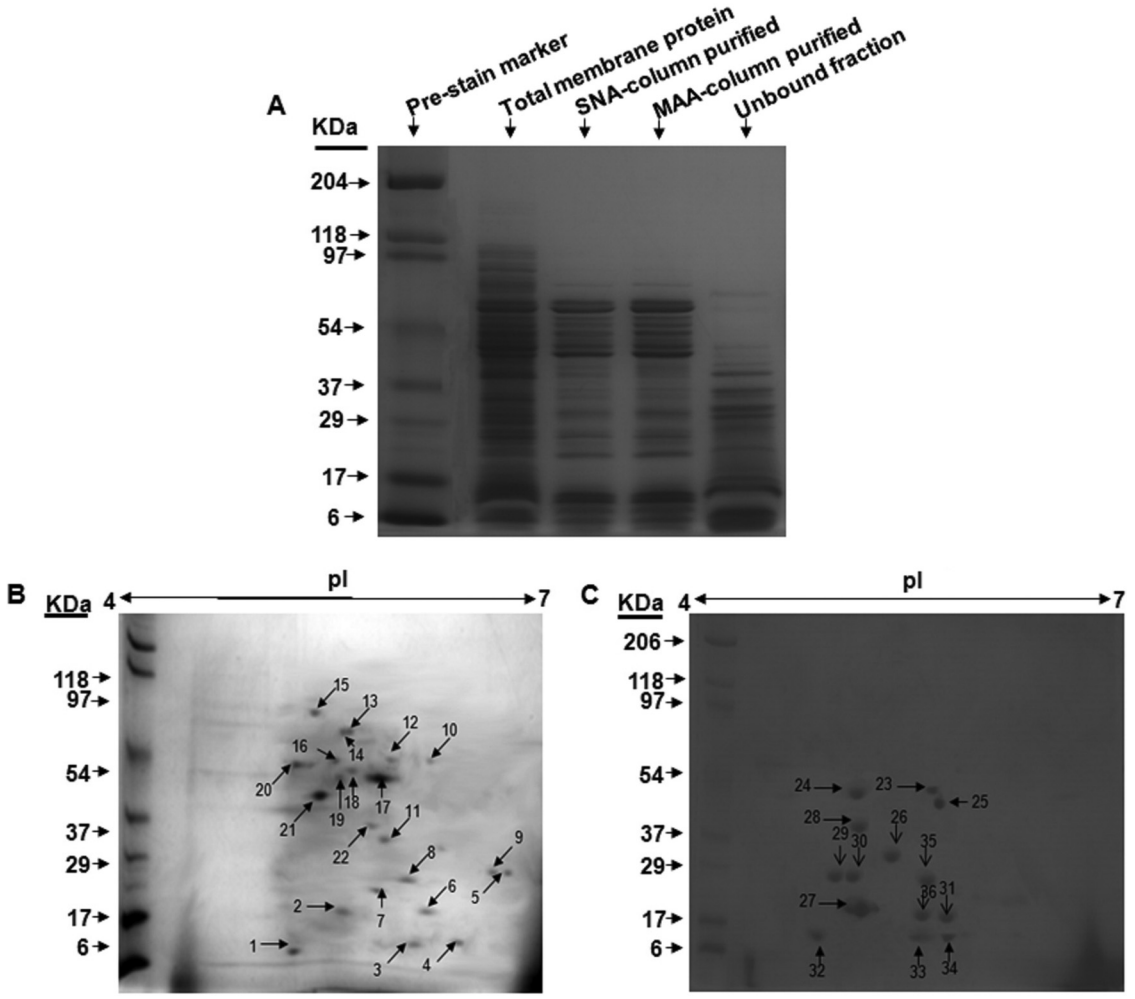



FIG. 3. **Purification of linkage specific sialoglycoproteins by affinity chromatography.** $\alpha$2,6- and $\alpha$2,3-linked sialoglycoproteins were purified by using Sepharose bound SNA and MAA as their respective affinity matrixes. A representative SDS-PAGE (7.5%, *A*) was shown to elucidate the profile of total membrane proteins (15 $\mu$g, lane 2), purified sialoglycoproteins (15 $\mu$g, lane 3–4) of PA and unbound fraction (15 $\mu$g, lane 5). Purified (120 $\mu$g) $\alpha$2,6-linked (*B*) and $\alpha$2,3-linked sialoglycoproteins (*C*) were separated over pI 4–7 strip according to their isoelectric points, ran on 7.5–15% gradient SDS-PAGE gel as second dimension and stained bio-safe Coomassie.

separately by using classical method like DEAE-cellulose as a matrix of anion exchange chromatography. The enriched fraction of OprD protein was further purified by changing buffer composition and salt gradient using another column. The yield of purified $OprD^{+Sias}$ and $OprD^{-Sias}$ was 0.045 $\pm$ 0.08 mg and 0.056 $\pm$ 0.10 mg from $6 \times 10^{14}$ cells, corresponding to 2.25 $\pm$ 0.07% and 2.80 $\pm$ 0.08% respectively. Coomassie brilliant blue stained SDS-PAGE (10%) of purified $OprD^{+Sias}$ and $OprD^{-Sias}$ appeared as a single band (supplemental Fig S2*A*). Different amounts of purified protein also showed single band on SDS-PAGE (data not shown). These proteins exhibited single band in IEF ( supplemental Fig S2*B*).

For further confirmation, gel band (lane 4, Fig. 4*A*) was excised, digested with trypsin and identified by MALDI-TOF/TOF-MS. Combined PMF (Fig. 4*B*) and MS/MS (supplemental Fig S3) spectra were compared with MSDB database sequences using search parameter "All entries" which matched with OprD of *P aeruginosa*. Database search identified fifteen proteins from these spectra according to the protein score (supplemental Fig S4). Among these 15 proteins, protein score of OprD precursor is 669 as compare with other protein score being around 50–60. Sequence coverage is 24% (Fig. 4*C*, shown in red color) and protein identity is S23771. Matched peptide sequences are also shown in Fig. 4*D*.

*$OprD^{+Sias}$ is Highly Sialylated*—Before and after neuraminidase treatment, $OprD^{+Sias}$ showed variation in mobility when analyzed on 10% SDS-PAGE (supplemental Fig S2*A*). Difference in molecular mass (1.25 kDa) were more prominent in 7.5% gel (Fig. 5*A*). On contrary, $OprD^{-Sias}$ showed negligible difference in molecular mass upon neuraminidase treatment indicating presence of less Sias. Such dissimilarity in mobility between $OprD^{+Sias}$ and $OprD^{-Sias}$ further suggested that they may differ also in charge and/or structure.

TABLE I
*Identified α2,6- and α2,3-linked sialoglycoproteins by MALDI-TOF MS/MSMS*

| Spot no. | Linkage specificity | NCBI accession number | **Protein name | Mol wt (Da) | pI | Mascot Score | Sequence Coverage (%) | FDR values (%) |
|---|---|---|---|---|---|---|---|---|
| 1 | α2,6- | F83062 | Hypothetical protein | 18,382 | 5.87 | 413 | 71 | 0.00 |
| 2 | | E83059 | Ketol-acid reductoisomerase | 36,743 | 5.57 | 278 | 41 | 0.00 |
| 3 | | F83029 | 50S ribosomal protein | 15,522 | 5.47 | 373 | 63 | 0.00 |
| 4 | | 1BEXA | Azurin chain A | 14,108 | 5.72 | 65 | 74 | 0.00 |
| 5, 9 | | F83448 | Succinate dehydrogenase | 22,765 | 6.59 | 339 | 44 | 0.00 |
| 7 | | C83316 | NADH-dehydrogenase β-chain | 25,694 | 5.31 | 74 | 18 | – |
| 8 | | T44454 | Arginine binding protein | 28,106 | 6.43 | 202 | 49 | 0.00 |
| 10 | | gi 15600749 | ATP-synthase subunit A | 55,530 | 5.33 | 84 | 6 | 0.00 |
| 11 | | Q9AGI3_PSEAE | Hypothetical protein | 28,624 | 5.28 | 55 | 27 | – |
| 13, 14 | | G83005 | Glutamine synthase | 52,140 | 5.14 | 266 | 54 | 0.00 |
| 15 | | F83250 | 30S ribosomal protein | 61,946 | 4.83 | 825 | 57 | 0.00 |
| 17 | | E82952 | ATP-synthase α chain | 55,530 | 5.33 | 209 | 26 | 0.00 |
| 19 | | G83299 | Probable outer membrane protein | 46,879 | 5.54 | 298 | 38 | 0.00 |
| 20 | | C82952 | ATP synthase β chain | 49,526 | 4.98 | 111 | 47 | 0.00 |
| 22 | | Q83WT8_PSEAE | Flic-*pseudomonas aeruginosa* | 39,408 | 4.78 | 96 | 12 | 50.00 |
| 25 | α2,3- | 024779_PSEAE | OprE3 | 46,865 | 5.54 | 277 | 33 | 0.00 |
| 26 | | B83189 | Elongation Factor Tu | 30,691 | 5.22 | 224 | 43 | 0.00 |
| 27, 35 | | G83204 | Probable peroxidase | 21,922 | 5.37 | 514 | 61 | 0.00 |
| 29, 30 | | C83139 | Outer membrane protein OprG precursor | 25,178 | 4.85 | 60 | 25 | – |
| 32 | | F83110 | 50S ribosomal protein | 12,472 | 4.71 | 108 | 59 | 0.00 |
| 33 | | A83113 | Bacterio ferritin | 17,986 | 5.01 | 249 | 70 | 0.00 |
| 34 | | A83000 | Conserved hypothetical protein | 15,451 | 5.45 | 216 | 57 | 0.00 |

TABLE II
*Identified sialoglycoproteins having both α2,6- and α2,3-linkages*

| Spot no. | NCBI accession number | [a]Protein name | Mol wt (Da) | pI | Mascot score | Sequence coverage (%) | FDR values (%) |
|---|---|---|---|---|---|---|---|
| 6, 31, 36 | H83231 | Conserved hypothetical protein | 16,543 | 5.50 | 666 | 70 | 0.00 |
| 21, 24 | S23771 | Outer membrane porin protein OprD precursor | 48,331 | 4.96 | 170 | 26 | 0.00 |
| 12, 16, 23 | E83507 | Flagellin type B | 49,231 | 5.4 | 336 | 48 | 0.00 |
| 18, 28 | S39156 | Outer membrane protein F (Opr F) precursor | 37,844 | 4.98 | 283 | 36 | 0.00 |

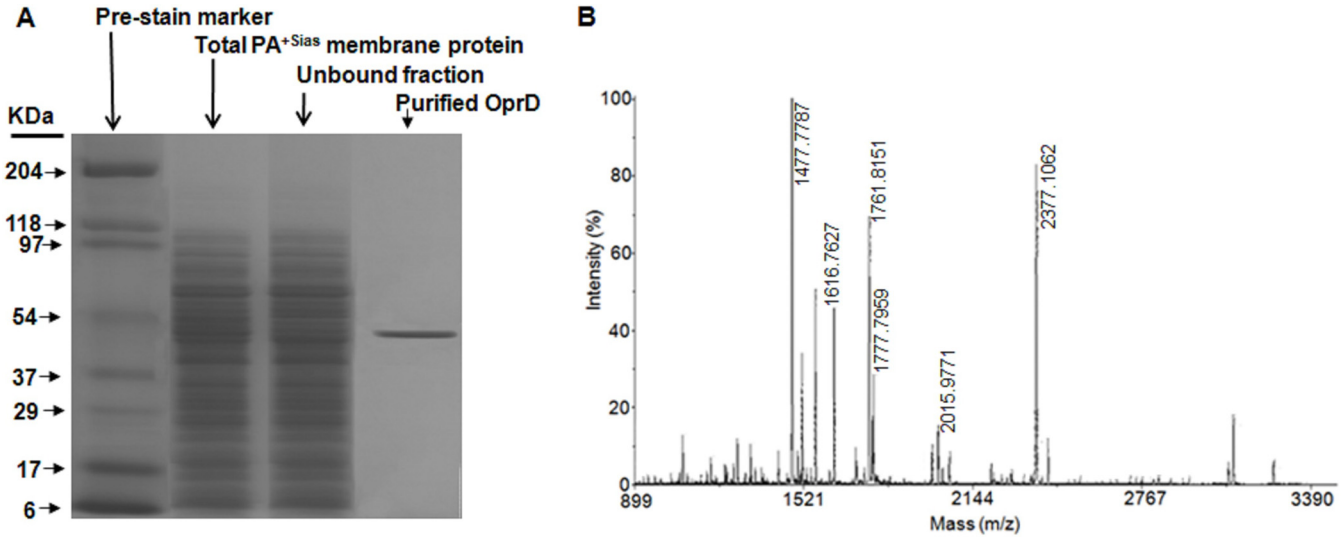
[a] Identified peptides with their score were given in supplemental Figs. S32 to S57.

Charge heterogeneity of $OprD^{+Sias}$ and $OprD^{-Sias}$ was subsequently investigated by IEF. In general, both the proteins showed a single band confirming their purity and the presence of a single molecular entity (supplemental Fig S2*B*). Interestingly, neuraminidase-treated $OprD^{+Sias}$ showed increase in pI from 4.95 (lane 2) to 6.05 (lane 3) demonstrating the presence of more Sias. On the other hand, pI of $OprD^{-Sias}$ is only 6.10, which did not change appreciably after neuraminidase treatment suggesting minimal presence of Sias (supplemental Fig. S2*B*, lane 5).

*Presence of Enhanced Sias on $OprD^{+Sias}$ was Confirmed by Different Analytical Methods*—To check the status of glycosidically bound Sias, $OprD^{+Sias}$ were subjected to acid hydrolysis and purified through Dowex cation and anion exchange columns. The eluted free sialic acids were DMB derivatized and separated by fluorimetric-HPLC (Fig. 5*B*). The chromatogram of DMB-Sias of $OprD^{+Sias}$ exhibited well-resolved intense peak of *N*-acetyl neuraminic acid (Neu5Ac), co-migrating with Neu5Ac, *N*-glycolyl neuraminic acid (Neu5Gc), 5-*N*-acetyl-9-*O*-acetyl neuraminic acid ($Neu5,9Ac_2$) derived from BSM and used as internal standards. In parallel $OprD^{-Sias}$ was similarly processed. As expected, DMB-Sias from $OprD^{-Sias}$ exhibited a small peak of Neu5Ac corroborating presence of negligible Sias.

Additionally, fluorimetric-HPLC fraction corresponding to Neu5Ac was analyzed by MALDI-TOF MS (Fig. 5*C*) and yielded the expected signal for the sodium cationized molecular ion having *m/z* at 448.7 convincingly demonstrated their occurrence on $OprD^{+Sias}$.

In parallel, Sias on $OprD^{+Sias}$ were shown using orcinol-stained TLC plates, further demonstrating the presence of Neu5Ac (supplemental Fig. S2*C*). The $R_F$ values corresponded to standard Neu5Ac and free Sias purified from BSM. No such spot was observed with $OprD^{-Sias}$.




C

OprD protein, S23771

1 MKVMKWSAIA LAVSAGSTQF AVADAFVSDQ AEAKGFIEDS SLDLLLRNYY
51 FNRDGKSGSG DRVDWTQGFL TTYESGFTQG TVGFGVDAFG YLGLKLDGTS
101 DKTGTGNLPV MNDGKPRDDY SRAGGAVKVR ISKTMLKWGE MQPTAPVFAA
151 GGSRLFPQTA TGFQLQSSEF EGLDLEAGHF TEGKEPTTVK SRGELYATYA
201 GETAKSADFI GGRYAITDNL SASLYGAELE DIYRQYYLNS NYTIPLASDQ
251 SLGFDFNIYR TNDEGKAKAG DISNTTWSLA AAYTLDAHTF TLAYQKVHGD
301 QPFDYIGFGR NGSGAGGDSI FLANSVQYSD FNGPGEKSWQ ARYDLNLASY
351 GVPGLTFMVR YINGKDIDGT KMSDNNVGYK NYGYGEDGKH HETNLEAKYV
401 VQSGPAKDLS FRIRQAWHRA NADQGEGDQN EFRLIVDYPL SIL

XXXX - Sequenced tryptic fragments
XXX – Identified fragments

D

| Miss match | Sequence |
|---|---|
| 0 | K.GFIEDSSLDLLLR.N (Ions score 129) |
| 0 | K.GFIEDSSLDLLLR.N (No match) |
| 0 | K.WGEMQPTAPVFAAGGSR.L (Ions score 128) |
| 0 | K.WGEMQPTAPVFAAGGSR.L (No match) |
| 0 | K.WGEMQPTAPVFAAGGSR.L Oxidation (M) (Ions score 51) |
| 0 | K.WGEMQPTAPVFAAGGSR.L Oxidation (M) (No match) |
| 1 | K.SRGELYATYAGETAK.S (No match) |
| 0 | R.GELYATYAGETAK.S (No match) |
| 0 | R.YAITDNLSASLYGAELEDIYR.Q (Ions score 230) |
| 0 | R.YAITDNLSASLYGAELEDIYR.Q (No match) |
| 0 | R.QYYLNSNYTIPLASDQSLGFDFNIYR.T (Ions score 119) |
| 0 | R.QYYLNSNYTIPLASDQSLGFDFNIYR.T (No match) |
| 0 | R.YDLNLASYGVPGLTFMVR.Y (Ions score 26) |
| 0 | R.YDLNLASYGVPGLTFMVR.Y (No match) |
| 0 | R.YDLNLASYGVPGLTFMVR.Y Oxidation (M) (No match) |

FIG. 4. **Purification of OprD$^{+Sias}$ by anion exchange chromatography.** *A,* OprD$^{+Sias}$ were purified by using DEAE-Sepharose as matrix. Total membrane proteins (15 $\mu$g, lane 2), unbound fraction (15 $\mu$g, lane 3) and purified OprD$^{+Sias}$ (4 $\mu$g, lane 4) of PA were separated on SDS-PAGE (10%). *B,* PMF spectra of tryptic fragments of OprD$^{+Sias}$ was identified by MALDI-TOF MS. Each fragment was denoted by their respective m/z values. *C*, OprD$^{+Sias}$ were identified from the database using the MASCOT algorithm, BLAST of ABI GPS Explorer software, version 3.6. Red colored amino acids were identified tryptic fragments. *D.* Matched and mis-matched peptides were shown.

Presence of Sias on OprD$^{+Sias}$ or OprD$^{-Sias}$ was quantitated by fluorimetric acetyl acetone method (Fig. 5*D*). OprD$^{+Sias}$ showed significantly ($p < 0.0001$) higher amount of Sias (1.98 $\pm$ 0.15 $\mu$g) compared with OprD$^{-Sias}$ (0.15 $\pm$ 0.02 $\mu$g). Additonally, we have also purified sialylated and non-sialylated OprD proteins from three more clinical isolates of

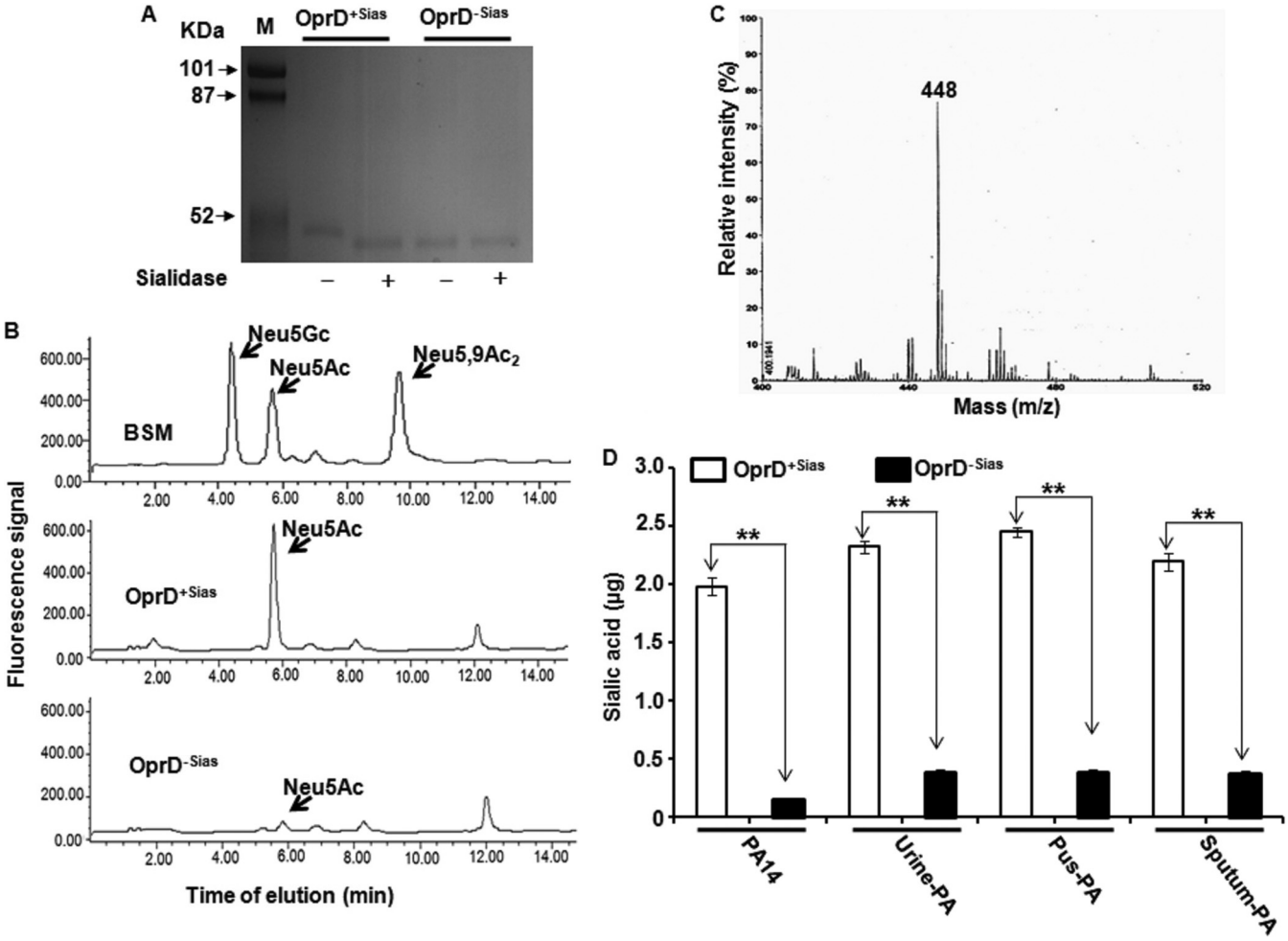



FIG. 5. **Identification of Sias on OprD$^{+Sias}$.** *A*, SDS-PAGE: Equivalent amounts (4.0 $\mu$g) of purified OprD$^{+Sias}$ or OprD$^{-Sias}$ before and after sialidase treatment were run in SDS-PAGE (7.5%), siained with Coomassie brilient blue as described under "Experimental Procedures." *B,* Fluorimetric-HPLC: A representative profile of DMB derivatized Sias from OprD$^{+Sias}$ and OprD$^{-Sias}$. In parallel, liberated Sias from BSM was also DMB derivatized, analyzed which served as standard. *C,* MALDI-TOF-MS: HPLC fraction was lyophilized and dissolved in water (2 $\mu$l), processed by dried-droplet procedure using DHB (10 $\mu$g/$\mu$l) in ethanol (60%) as matrix and analyzed. Mass spectra were recorded in the positive ion mode as described under "Experimental Procedures." *D,* Fluorimetric acetyl acetone method: Total Sias present in equal amount (50 $\mu$g) of OprD$^{+Sias}$ (□) or OprD$^{-Sias}$ (■) purified from four different clinical isolates of PA were determined by mild oxidation with sodium metaperiodate as described under "Experimental Procedures." The results all four clinical isolated were shown. Mean ± S.D. are obtained from four independent experiments performed in duplicates. *p* values (**$<$0.005) are from one-tailed t tests comparing sialic acid content in OprD$^{+Sias}$ with OprD$^{-Sias}$.

PA and sialic acid contents were showed similar results like OprD$^{+Sias}$/OprD$^{-Sias}$ from PA14.

*High Mannose Type Carbohydrates Along with Sialylated Branched N-andO-linked Glycans in OprD$^{+Sias}$*—Functions of sialoglycoproteins of pathogens are very much dependent on the carbohydrate moieties. Accordingly, type of carbohydrate structure present in the proteins is fundamental requirement to understand their biological role. Presence of enhanced amount of sialic acid in OprD protein purified from PA$^{+Sias}$ as compared with OprD$^{-Sias}$ was demonstrated by different analytical as well as biochemical methods (Figs. 2–5). These observations were further corroborated by structural analysis of *N*- & *O*-linked glycans by mass spectra and high performance anion exchange chromatography (HPAEC) methods.

Occurrence of Neu5Ac showed 23-fold higher in sialylated OprD as compared with non-sialylated analog. However, total amount of monosaccharide (excluding sialic acid) as determined by HPAEC-PAD present was comparable within the range of 30.19–36.21 ng/$\mu$g of OprD$^{+Sias}$ and OprD$^{-Sias}$.

Predicted *N*-glycan structures from their respective masses were shown in the profiles which revealed the presence of high mannose, complex bi-antennary and different extent of sialylation on tri-antennary type of glycans on OprD$^{+Sias}$ (Fig. 6*A*). On the other hand *N*-glycans isolated from OprD$^{-Sias}$ is devoid of sialylation (except a minor contribution from a mono-sialylated glycan) (Fig. 6*B*). The presence of high mannose and sialylated branched *N*-glycans were also confirmed by HPAEC-PAD of 2-AB tagged *N*-glycans isolated from

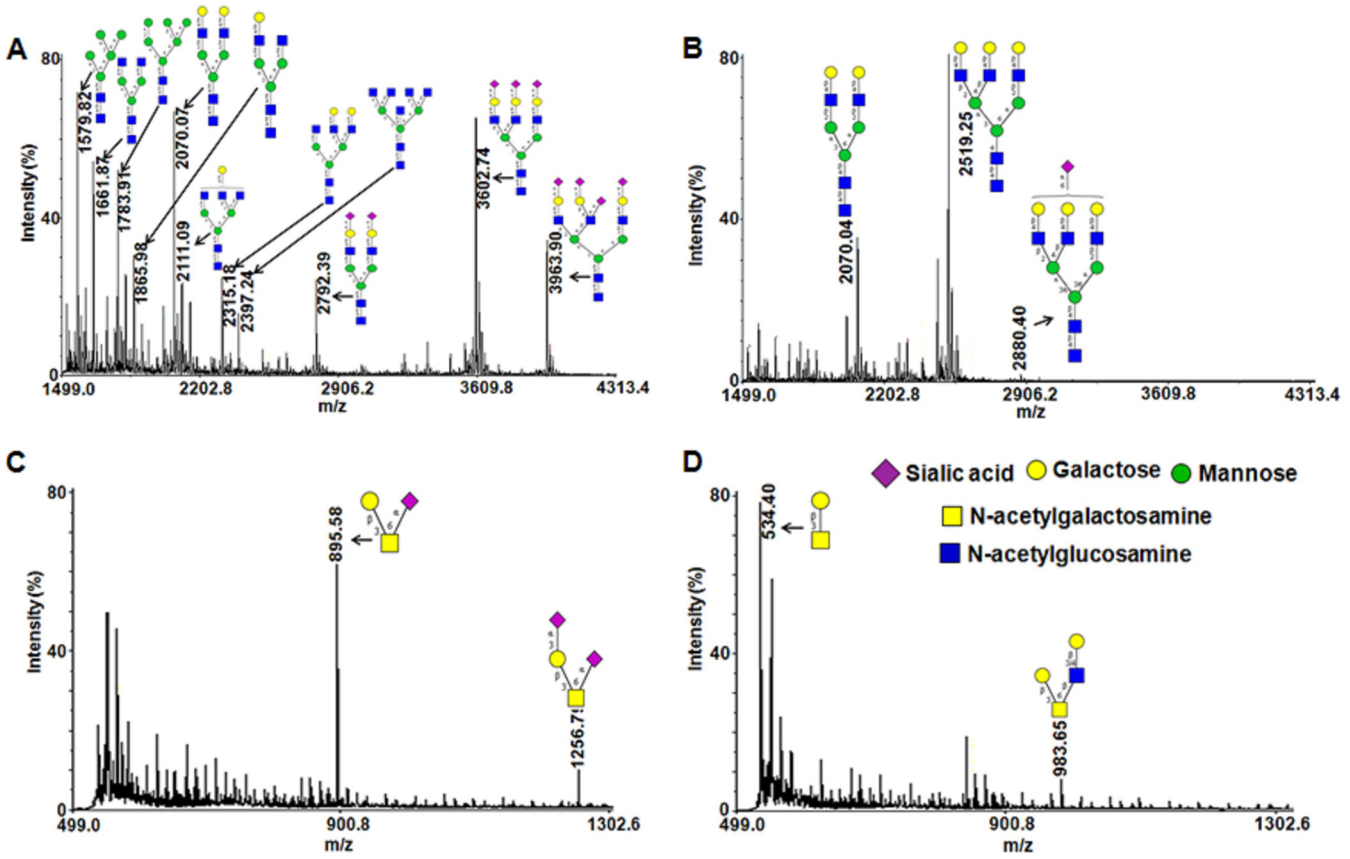



FIG. 6. **N-linked and *O*-linked profiling by MALDI-TOF-MS and HPAEC.** Glycans were isolated from purified OprD$^{+Sias}$ or OprD$^{-Sias}$, analyzed by MALDI-TOF-MS as well as HPAEC coupled with an online fluorescent detector as described in materials and method. *N*-linked (*A*) and *O*-linked (*C*) glycans of OprD$^{+Sias}$ were assigned from the MALDI-TOF spectrum depending upon masses. Similarly, *N*-linked (*B*) and *O*-linked (*D*) analogs of OprD$^{-Sias}$ were also analyzed. Spectrum of standards from RNase B and fetuin by HPAEC were correlated with the major structures observed in the MALDI-TOF spectrum.

standard RNase B and fetuin samples and compared with the structures observed by mass spectroscopy.

Three sialylated (*m/z* 2792.39, 3602.74, and 3963.90) *N*-glycan structures of OprD$^{+Sias}$ were observed (Fig. 6*A*). However, OprD$^{-Sias}$ showed only one sialylated *N*-glycans signal corresponding m/z 2880.40 (Fig. 6*B*).

O-Glycan data also confirmed the presence of mono (m/z 895.58) and di-sialylated Core-1 type structures (*m/z* 1256.79) in OprD$^{+Sias}$. However such sialylated *O*-linked glycans were absent in OprD$^{-Sias}$ (Fig. 6*C*–6*D*). Therefore, we may suggest that one of the sialylglycoprotein, OprD, may have mammalian *N*- and *O*-glycan structures.

Interestingly, a few non-sialylated *N*-glycans (m/z 1579.82, 1661.87, 1783.91, 1865.98, 2070.07, 2111.09, 2315.18, and 2397.24) were found in OprD$^{+Sias}$ (Fig. 6*A*) as compared with only two such signals (*m/z* 2070.04 and 2519.25) in OprD$^{-Sias}$ (Fig. 6*B*) with different intensities.

*OprD$^{+Sias}$ Showed Reduced Association with β-lactam Antibiotics*—To investigate whether these enhanced sialylation of OprD porin play any role in the binding with β-lactam antibiotics, we initially checked the association of two representative drugs *e.g.* piperacillin (Fig. 7*A*–7*B*) or ceftazidime (Fig. 7*C*–7*D*) with OprD$^{+Sias}$ proteins by SPR analysis. Proteins were separately immobilized on activated CM5 sensor chips to reach 6500 RU. To demonstrate the patterns and magnitude of binding, different amounts (10–100 μM) of piperacillin or ceftazidime were injected over OprD$^{+Sias}$ immobilized sensor chips separately. OprD$^{-Sias}$ was always used for comparison.

Piperacillin (10, 20, 50, and 100 μM) showed decreased association with OprD$^{+Sias}$ (17, 21, 23, and 27 RU, Fig. 7*A*) compared with OprD$^{-Sias}$ (45, 82, 101, and 114 RU respectively, Fig. 7*B*). Similarly, ceftazidime (10, 20, 50, and 100 μM) also showed reduced interaction with sialylated (17, 21, 23, and 27 RU, Fig. 7*C*) as compared with non-sialylated (45, 82, 101, and 114 RU respectively, Fig. 7*D*) analog.

The dissociation constants ($K_D$) for OprD$^{+Sias}$-piperacillin interaction were much higher ($7.60 \pm 0.13 \times 10^{-6}$ M) in contrast to the binding of OprD$^{-Sias}$-piperacillin ($0.58 \pm 0.09 \times 10^{-8}$ M). Similarly, greater $K_D$ values ($9.08 \pm 0.93 \times 10^{-6}$ M) of OprD$^{+Sias}$-ceftazidime compared with OprD$^{-Sias}$-ceftazidime ($0.55 \pm 0.01 \times 10^{-8}$ M) demonstrated less efficiency of sialylated analog of OprD toward the binding of two representative commonly used antibiotics.

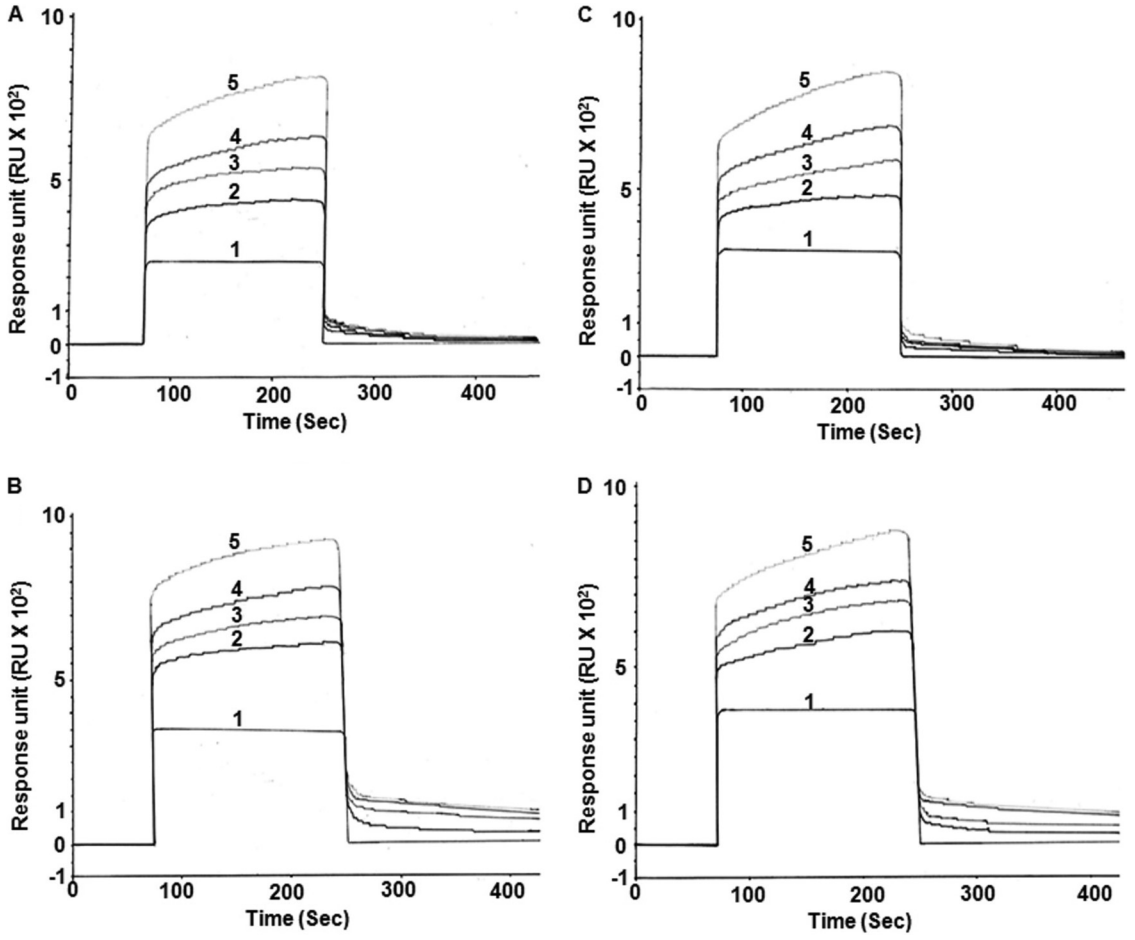



FIG. 7. **OprD$^{+Sias}$ showed decreased interaction with $\beta$-lactam antibiotics.** Association of OprD$^{+Sias}$ with piperacillin (*A*) and OprD$^{+Sias}$ with ceftazidime (*C*) or OprD$^{-Sias}$ with piperacillin (*B*) or OprD$^{-Sias}$ with ceftazidime (*D*) was examined by SPR as described in materials and methods. OprD$^{+Sias}$ or OprD$^{-Sias}$ (200 $\mu$g/ml) were immobilized in CM5 sensor chip. Several doses (0, 10, 20, 50, and 100 $\mu$g/ml labeled as 1, 2, 3, 4, and 5 respectively) of antibiotics were allowed to interact with OprD$^{+Sias}$- and OprD$^{-Sias}$-immobilized chip at a flow rate of 10 $\mu$l/min. The sensorgram was evaluated by the BiaEvaluation 3.0 software.

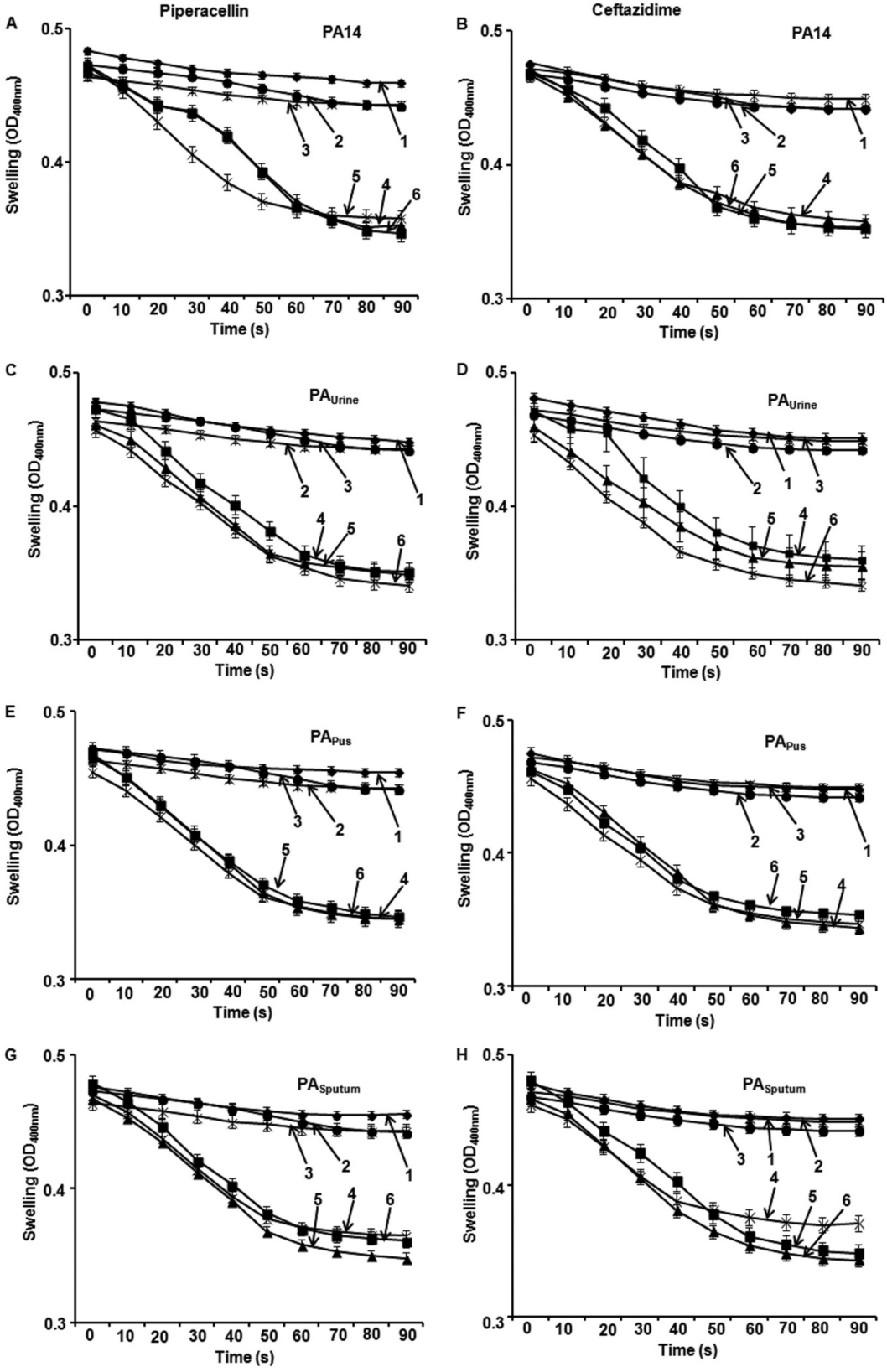
*Permeability of Piperacillin or Ceftazidime Decreased Through OprD$^{+Sias}$*—Searching for the crucial role of sialic acids found on this porin protein (OprD$^{+Sias}$) from PA14 in the penetration of antibiotics inside the bacteria, we checked permeabilization of piperacillin (Fig. 8*A*) or ceftazidime (Fig. 8*B*) through artificially formed liposome membrane with OprD$^{+Sias}$ by liposome swelling assay. Decrease in OD was observed upon the dilution of OprD$^{-Sias}$ or sialidase treated-OprD$^{-Sias}$ or sialidase treated-OprD$^{+Sias}$ protein-containing liposomes in either piperacillin or ceftazidime. In contrast, little decrease of absorbance was noticed upon the dilution of OprD$^{+Sias}$ or BSA containing liposomes upon addition of antibiotics. Moreover, membrane permeability of antibiotics through liposome using sialylated and non-sialylated OprDs from other three clinical isolates of PA $PA_{Urine}$, $PA_{Pus}$, and $PA_{Sputum}$ (Fig. 8*C*, 8*E*, 8*G*) demonstrated similar results with piperacillin. These three strains also exhibited comparable results with ceftazidime (Fig. 8*D*, 8*F*, 8*H*).

This result indicated that non-sialylated porin is highly capable to permeabilize antibiotics through them. Because of presence of sialic acid on the porin protein, it hindered to bind antibiotics leading to inefficient uptake of antibiotics which possibly responsible for bacterial resistivity toward $\beta$-lactam antibiotics.

## DISCUSSION

Both $\alpha$2,3- and $\alpha$2,6-linked sialic acids found on the PA's surface help them to resist from the serum complement deposition (19). Correlation of sialylation of glycoproteins in PA and their survival in host is relatively untouched field. Therefore, we considered it may be essential to identify these sialylated glycoproteins that might play an important molecular determinant on PA for their survival.

The main achievements of the current investigations include purification of a few sialoglycoproteins of PA, their molecular identification, characterization and more impor-


A
Piperacellin
PA14
B
Ceftazidime
PA14
C
PA Urine
D
PA Urine
E
PA Pus
F
PA Pus
G
PA Sputum
H
PA Sputum
Swelling (OD400nm)
Time (s)
0.5
0.4
0.3
0 10 20 30 40 50 60 70 80 90

tantly established the role of one such sialoglycoprotein (OprD) on PA. Treatments of $PA^{+Sias}$ as well as purified $OprD^{+Sias}$ with two commonly used $\beta$-lactam antibiotics (ceftazidime and piperacillin), demonstrated that Sias might play an essential responsibility for their uptake during infection and thereby implement a new strategy for the successful survival of PA within host.

The PMF spectrum of the purified linkage-specific sialoglycoproteins is compared with the MSDB sequence database and the sequence homology matched with PA origin. A few such identified sialoglycoproteins are outer membrane protein OprD, OprF, OprG, flagellin type B etc. are known to execute different survival strategies of PA. In general, $\beta$-barrel shaped, channel forming porins (OprD and OprF) play the central role in outer membrane permeability of drug (57). OprF plays the crucial role to maintain structural integrity of PA (58), whereas OprG protein is tightly regulated by anaerobiosis and contributes to the cytotoxicity of this bacterium during the early infection (59). OprD is specific protein through which PA uptakes basic amino acids, peptides and $\beta$-lactam antibiotics (60). PA can colonize in the various surfaces to form biofilm with the help of flagellin type B protein and becomes impervious to therapeutic concentrations of many antibiotics (61). We have demonstrated sialylation in all these vital proteins. Therefore, it is expected that Sias must play some key role to carry out these fundamental functions of PA.

Other major important outer membrane proteins are OprB, OprE, OprI, OprL, OprP, and iron repressible outer membrane proteins already reported in PA (62). Interestingly, under the experimental conditions, they did not show any sialylation. Glycosylation of these proteins demands future investigation.

PA, in general, shows very poor diffusion rate of many drugs like cephalosporins, cefotaxime, ceftazidime, ceftriaxone, cefoperazone, cefpirome, and cefepime etc. through outer membrane. It showed resistance possibly because of the presence of MexA-MexB-OprM efflux pump (32). Similarly efflux in *E. coli* also showed less susceptibility to cefoperazone. Another efflux pump (MexX-MexY-OprM) of PA can efficiently extrude quinolones (30). Efflux pump, MexC-MexD-OprJ, effectively remove several molecules including erythromycin, azithromycin, norfloxacin, crystal violet, and SDS (32). MexE-MexF-OprN is active against quinolones, chloramphenicol and trimethoprim (31). Whether glycosylation of these efflux pumps contribute any role is currently unknown.

Among these different mechanisms of antibiotics-resistance, we investigated the involvement of Sias of OprD porin protein to find out the possible role of Sias toward the uptake of drug in this bacterium. Sequence study revealed that OprD porin is a highly sialylated glycoprotein having both $\alpha$2,6- and $\alpha$2,3-linked Sias.

*N*- and *O*-linked glycosylation are the two most common forms of glycosylation in proteins. Glycoproteomic analysis of $OprD^{+Sias}$/$OprD^{-Sias}$ reveals that they may have mammalian like *N*- and *O*-glycan structures. Though comparable amount of total carbohydrates were found in $OprD^{+Sias}$ and $OprD^{-Sias}$, a 23-fold higher Sias was observed only in $OprD^{+Sias}$. Such a huge amount of Sias may be responsible for vast modulation of its structure. The enhanced presence of high-mannose contained *N*-glycans in $OprD^{+Sias}$ possibly playing an additional role for their proper folding and dynamic stability through hydrogen bonds. Literature search reveals that mammalian like *N*-glycans are also present on *E. coli* (63). Such high-mannose containing *N*-glycans are also been reported on *influenza virus* (64).

Increased presence of Sias in $OprD^{+Sias}$ has further been convincingly demonstrated by TLC, fluorimetric-HPLC and MALDI-TOF-MS. Isoelectric point of $OprD^{+Sias}$ has been revealed in acidic region and a huge shift of pI after removal of Sias established enhanced sialylation compared with $OprD^{-Sias}$. Increased sialylation in $OprD^{+Sias}$ possibly causes structural modification of this protein.

Structural characterization revealed higher glycans in $OprD^{+Sias}$ compared with $OprD^{-Sias}$. It may be envisaged that all the OprD proteins are not glycosylated in similar extent, however it needs further detailed investigation.

Next we addressed whether sialylation of OprD play any role in resistance of two commonly prescribed $\beta$-lactam antibiotics (ceftazidime and piperacillin). SPR analysis showed that ceftazidime-$OprD^{+Sias}$ and piperacillin-$OprD^{+Sias}$ interactions are relatively weak compared with $OprD^{-Sias}$ suggesting Sias are possibly creating some problem for antibiotics to interact with porin protein for their entry. Both the antibiotics also showed higher membrane permeability through liposome containing only $OprD^{-Sias}$. In contrast, lower capability of $OprD^{+Sias}$ revealed important role of Sias in drug permeabilization in PA.

This was further corroborated in live cell experiment. Near the $MIC_{50}$ values of both the drugs, antibiotics-treated $PA^{+Sias}$ showed higher CFU count with respect to $PA^{-Sias}$. The main deference between $PA^{+Sias}$ and $PA^{-Sias}$ is sialic acids, therefore Sias definitely play some crucial role for hindering antibiotics uptake in PA that leads to resistance against drugs.

We have used four clinical isolates of PA to show the relationship between sialylation of protein and the antibiotic resistance. All four clinical isolates showed comparable rate

FIG. 8. **Decreased permeability of piperacillin or ceftazidime through $OprD^{+Sias}$.** Liposome membrane were prepared using purified $OprD^{+Sias}$ (line 3), $OprD^{-Sias}$ (line 4), sialidase-$OprD^{+Sias}$ (line 5), sialidase-$OprD^{-Sias}$ (line 6), BSA (10 $\mu$g, line 2) proteins and without protein (line 1) separately as described under "Experimental Procedures." Liposome-membrane suspensions from all four clinical isolates were mixed with 20 mM of piperacillin (*A, C, E, G*) or ceftazidime (*B, D, F, H*) solution separately and the optical density was monitored at 400 nm with a spectrophotometer for 90 s.

of antibiotic resistance. Additionally, sialylation of purified OprDs from these four isolates exhibited similar degree of inhibition of drug permeabilization. These observations indicated a possible link between sialylation of OprD and the antibiotic confrontation in PA. This may be a general trend as all four clinical isolates showed comparable degree of resistance against antibiotics.

In conclusion, extensive glycoproteomic analysis gave us some information on sialylated *N*- and *O*-glycan compositions of purified OprD porin protein which may be responsible for their structural modulation; hence functional impairment. Both sialylated PA and purified OprD showed more resistance toward $\beta$-lactam antibiotics. This is because of lower penetration capability of drugs through this highly sialylated porin protein of $PA^{+Sias}$. This may be an alternative drug resistance mechanism of PA.

To the best of our knowledge, this is the first report where sialic acids acquired by OprD protein have been assigned a vital function for antibiotics uptake in four different clinical isolates of PA. The findings may help to design newer drug which can enter cells freely even in presence of Sias or block sialylation or cleave sialic acids. However consequence of such study needs further in depth investigation.

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S This article contains supplemental Figs. S1 to S83.

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