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 α2,3- and α2,6-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 β-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 α2,6-linked, α2,3-linked, and both α2,3- and α2,6-linked sialylated proteins in PA. The outer membrane porin protein D (OprD), a specialized channel-forming protein, responsible for uptake of β-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 β-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 β-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-suppressed 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 \( ^{\text{14S}} \)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, pipracillin, 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-\( \beta \)-glucose, 2-acetamido-2,6-dideoxy-\( \beta \)-galactose, and 5-acetamido-3,5,7,9-tetrahydroxy-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 PA12 (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—**Pseudomonas 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 strains of PA were isolated from urine (PA14\( \text{urine} \)), pus (PA14\( \text{pust} \)), and sputum (PA14\( \text{spu} \)) 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 \( \cdot \)-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\text{2}O\text{2}, uracil (10 ml, 2.0 mg/ml 0.1 N NaOH), and inosine (20 ml, 20 mg/ml H\text{2}O\text{2}), \( \text{pH} = 7.5 \) (PA \( ^{\text{Sias}} \)) or in presence of 10% heat inactivated normal human serum (HI-NHS) used as a source of sialic acids (PA \( ^{\text{Sias}} \)) (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 \( ^{\text{Sias}} \) of four clinical isolates including PA14 were measured by using two known \( \beta \)-lactam antibiotics such as pipracillin and cefazidime (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 (OD600 nm) = 0.3] separately and allowed to grow at 37 °C with shaking. Bacterial suspensions were counted by measuring OD600 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 described elsewhere (41). Typically, PA14, PA4214, PA6 and PA4214 nunn et al., (6 × 1014 cells) were washed in ice cold Tris-HCl (50 mM), magnesium chloride (MgCl2, 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), MgCl2 (1.0 mM), calcium chloride (0.1 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 0.1% 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- sialoglycoproteins 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 was activated using a mixture of (70 μl, 1:1 of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (200 mM) and N-hydroxysulfosuccinimide (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 μl, 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 Na2HPO4, 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 μl) 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 sensorsgrams. The dissociation constant (Kd) for antibiotic-OprD protein association was calculated as the ratio of the backward (k0) and forward rates (k3).

Purification of Sialoglycoproteins by Lectin Affinity Chromatography—Linkage-specific sialoglycoproteins of PA were purified using SNA-Sepharose-4B and MAA-Sepharose-4B column matrix. 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 mM), sodium chloride (0.5 mM), pH 8.5. CNBr-activated Sepharose 4B (0.6 g) was soaked 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 ml, 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 mM sodium acetate, 0.5 mM 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 spin 6 (GE healthcare, 5 KDa cut off). Purified sialoglycoproteins were 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 brilliant blue R 250 (Bio-Rad) and stained 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 mM/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 μl) 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 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 pl was determined from the pl 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 iodoacetic acid (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 Coo massie (45) and destained 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-
PA14, PAUrine, PApus, and PASputum membrane pellets were separated (p/H11006) 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 Calfins, 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: carboxamidomethylation; missed cleavages permitted: one missed cleavages; minimal 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: 5; 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 proteoglycans 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, PAUrine, PApus, and PASputum membrane pellets were separately mixed with Tris-HCI buffer (10 mM, pH 8, 5 ml) containing β-ocyt chloride (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. Diethylaminomethyl (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), β-ocyt chloride (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 of MALDI-TOF/TOF-MS. PMF and MS/MS combined spectra were compared with MSDB database sequences.

Detection of Sialic Acids on OprD

Thin Layer Chromatography (TLC)—OprD was separated with 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 from bovine submandibular mucin (BSM) along with neuraminic acid (Neu5Ac, Sigma) were used as internal standards. Plates were developed by spraying orcinol/HCl/FeCl3 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 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 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 μl) and processed by dried-droplet procedure. An equal volume of 5-dihydroxybenzoic acid (DHB, 10 μg/μl) in ethanol (60%) as matrix was mixed with DMB-sialic acids. The mixture (0.50 μl) 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 was quantified using an acetyl acetone method (49) and compared with PA14, PAUrine, PApus, and PASputum was measured by mild oxidation with sodium metaperiodate separately using pure Neu5Ac as standard (51). The fluorometric 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-sialys were isolated from 200 μg 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 detecting the samples with 5 μM of PNGaseF (New England Biolabs) in enzyme reaction buffer at 37 °C for 20 h. N-Glycans were purified using sequential passage 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 (HPAECPAD) 20 μg 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, Sunnyvalle, CA). Sialic acid was done using an ultra
performance liquid chromatography with fluorescence (UPLC-FL, Agilent 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 fluorescence 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 in water 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-Sciei). 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 DMF 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 150 μl of sample was dried in speed vac and re-dissolved in 5 μl of methanol followed by addition of 1 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 mM NaH2PO4 at 45 °C for 16 h on stirring cum heating block (ReactiTherm, Pierce, Rockford, IL). The samples were cooled over ice-water bath, neutralized with ice-cold 30% acetic acid and de-salted using cation-exchange resin (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).

Sialylation of OprD in Antibiotic Resistance of PA

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% (MIC50, 8 μg/ml) of ceftazidime antibiotics, PA−Sias (5.92 ± 0.25 × 105) showed higher CFU compared with 1.43 ± 0.18 × 105 shown by PA−Sias (Fig. 1A). In parallel, PA−Sias with piperacillin (6.79 ± 0.34 × 105) also showed enhanced CFU as compared with PA−Sias with piperacillin (3.12 ± 0.21 × 105) in the vicinity of MIC50 (15 μg/ml) indicating Sias on PA−Sias may play some crucial role for their resistivity toward antibiotics (Fig. 1B). However, both PA−Sias and PA−Sias showed low but similar CFU value (0.26 ± 0.06 × 105) 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 PAUrine (Fig. 1C, 1D), PAPus (Fig. 1E, 1F), and PASputum (Fig. 1G, 1H) 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 carboxymide and hydroxysuccinimide buffers, used immediately after mixing.
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 μg/30 μl) 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. 1.** PA^Sias^ showed increased survival against antibiotics. PA14, PA_{	ext{urinary}}, PA_{	ext{putum}}, and PA_{	ext{putum}} 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^ and PA^Sias^ were measured. PA^Sias^ or PA^Sias^ were grown in presence of ceftazidime (0, 2, 4, 8, 10, 15 and 20 μg/ml) or piperacillin (0, 4, 8, 15, 30 and 50 μg/ml) at 37 °C overnight. PA was counted by turbidity method using OD600 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 μg/30 μl) 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
binding (Fig. 2A, 2B). Dose dependent increased association of PA membrane protein (10, 20, 30, 50, and 100 μg/30 μl) with SNA (648, 872, 1107, 1431, and 1675 RU) as well as MAA (755, 847, 1135, and 1342 RU) respectively was observed. The bacterial membrane-lectin interactions with both SNA and MAA reduced significantly after desialylation as compared with PA proteins (755, 847, 1135, and 1342 RU) respectively. The extent of binding was represented by a lower RU value indicating specificity of the binding (Inset, Fig. 2 A, B).

PA\textsuperscript{+Sias} Exhibited More Number of α2,6-linked Compared with α2,3-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 α2,6- and α2,3-linkages were purified from total membrane protein of PA\textsuperscript{+Sias} by using SNA and MAA as their respective affinity ligands. Approximately 8.9 ± 1.23% and 7.45 ± 1.38% of total membrane proteins are α2,6-linked and α2,3-linked of purified sialoglycoproteins respectively.

Equal amount (10 μg) of total membrane proteins from PA\textsuperscript{+Sias}, purified α2,6- and α2,3-linked sialylated glycoproteins as well as unbound proteins from affinity column were separated by SDS-PAGE (Fig. 3A). 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 μg) were separately analyzed by 2D-SDS-PAGE and images were taken. There were 22 spots corresponding to α2,6-linked sialoglycoproteins (Fig. 3B) whereas α2,3-linked sialoglycoproteins showed only 14 spots (Fig. 3C) indicating PA membrane fractions contained more number of α2,6-linked compared with α2,3-linked sialylated glycoproteins.

Linkage-specific Sialoglycoproteins Identified on PA\textsuperscript{+Sias}—Molecular identification of these linkage-specific sialoglycoproteins is necessary to understand their role in PA\textsuperscript{+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] spectrum was compared with the MSDB sequence database. A few α2,6-linked, α2,3-linked (Table I) and both α2,3- and α2,6-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–S83. 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\textsuperscript{+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\textsuperscript{+Sias}, we have selected OprD porin proteins, having both α2,3- and α2,6-linked sialic acids, to understand the detailed functional role of such acquired sialic acids with respect to the entry of β-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\textsuperscript{+Sias} (OprD\textsuperscript{+Sias}) and PA\textsuperscript{−Sias} (OprD\textsuperscript{−Sias})
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/H11001 Sias and OprD/H11002 Sias was 0.045/0.08 mg and 0.056/0.10 mg from 6×10^14 cells, corresponding to 2.25/0.07% and 2.80/0.08% respectively. Coomassie brilliant blue stained SDS-PAGE (10%) of purified OprD/H11001 Sias and OprD/H11002 Sias appeared as a single band (supplemental Fig S2A). Different amounts of purified protein also showed single band on SDS-PAGE (data not shown). These proteins exhibited single band in IEF (supplemental Fig S2B).

For further confirmation, gel band (lane 4, Fig. 4A) was excised, digested with trypsin and identified by MALDI-TOF/TOF-MS. Combined PMF (Fig. 4B) 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. 4C, shown in red color) and protein identity is S23771. Matched peptide sequences are also shown in Fig. 4D.

**OprD**/**H11001** Sias is Highly Sialylated—Before and after neuraminidase treatment, OprD/**H11001** Sias showed variation in mobility when analyzed on 10% SDS-PAGE (supplemental Fig S2A). Difference in molecular mass (1.25 kDa) were more prominent in 7.5% gel (Fig. 5A). On contrary, OprD/**H11002** Sias showed negligible difference in molecular mass upon neuraminidase treatment indicating presence of less Sias. Such dissimilarity in mobility between OprD/**H11001** Sias and OprD/**H11002** Sias further suggested that they may differ also in charge and/or structure.
Charge heterogeneity of OprD/H11001 Sias and OprD/H11002 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 S2B).

Interestingly, neuraminidase-treated OprD/H11001 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/H11002 Sias is only 6.10, which did not change appreciably after neuraminidase treatment suggesting minimal presence of Sias (supplemental Fig. S2B, lane 5).

Presence of Enhanced Sias on OprD/H11001 Sias was Confirmed by Different Analytical Methods—To check the status of glycosidically bound Sias, OprD/H11001 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. 5B). The chromatogram of DMB-Sias of OprD/H11001 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 (Neu5,9Ac2) derived from BSM and used as internal standards. In parallel OprD/H11002 Sias was similarly processed. As expected, DMB-Sias from OprD/H11002 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. 5C) and yielded the expected signal for the sodium cationized molecular ion having \( m/z \) at 448.7 convincingly demonstrated their occurrence on OprD/H11001 Sias. In parallel, Sias on OprD/H11001 Sias were shown using orcinol-stained TLC plates, further demonstrating the presence of Neu5Ac (supplemental Fig. S2C). The \( R_f \) values corresponded to standard Neu5Ac and free Sias purified from BSM. No such spot was observed with OprD/H11002 Sias.

### Table I

| Spot no. | Linkage specificity | NCBI accession number | **Protein name** | Mol wt (Da) | pl | Mascot Score | Sequence Coverage (%) | FDR values (%) |
|----------|---------------------|-----------------------|-----------------|-------------|----|--------------|----------------------|---------------|
| 1        | 2,6-                | 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 | 41 | 0.00 |
| 7        |                     | C83316                | NADH-dehydrogenase beta-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       | Q9AG13_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 alpha 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 beta chain | 49,526 | 4.98 | 111 | 47 | 0.00 |
| 22       |                     | Q83WT8_PSEAE         | Flic-pseudomonas aeruginosa | 39,408 | 4.78 | 96 | 12 | 50.00 |
| 25       | 2,3-                | 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                | Bacterial ferritin | 17,986 | 5.01 | 249 | 70 | 0.00 |
| 34       |                     | A83000                | Conserved hypothetical protein | 15,451 | 5.45 | 216 | 57 | 0.00 |

### Table II

| Spot no. | NCBI accession number | *Protein name* | Mol wt (Da) | pl | 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 |

*Identified peptides with their score were given in supplemental Figs. S32 to S57.

### Table III

Identified sialoglycoproteins having both 2,6- and 2,3-linkages

| Spot no. | NCBI accession number | *Protein name* | Mol wt (Da) | pl | 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 |
Presence of Sias on OprD was quantitated by fluorimetric acetyl acetone method (Fig. 5D). OprD showed significantly ($p < 0.0001$) higher amount of Sias ($1.98 \pm 0.15 \mu g$) compared with OprD (0.15 ± 0.02 $\mu g$). Additionally, we have also purified sialylated and non-sialylated OprD proteins from three more clinical isolates of PA.
PA and sialic acid contents were showed similar results like OprD/Sias from PA14.

**High Mannose Type Carbohydrates Along with Sialylated Branched N- and O-linked Glycans in OprD**

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 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- and 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/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 and sialylated branched N-glycans were also confirmed by HPAEC-PAD of 2-AB tagged N-glycans isolated from PA14.

**Fig. 5. Identification of Sias on OprD**

A, SDS-PAGE: Equivalent amounts (4.0 μg) of purified OprD/Sias or OprD/Sias before and after sialidase treatment were run in SDS-PAGE (7.5%), stained with Coomassie brilliant 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 μl), processed by dried-droplet procedure using DHB (10 μg/μl) 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 μg) 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.
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/H11001 Sias were observed (Fig. 6A). However, OprD/H11002 Sias showed only one sialylated N-glycans signal corresponding m/z 2880.40 (Fig. 6B).

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/H11001 Sias. However such sialylated O-linked glycans were absent in OprD/H11002 Sias (Fig. 6C–6D). 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/H11001 Sias (Fig. 6A) as compared with only two such signals (m/z 2070.04 and 2519.25) in OprD/H11002 Sias (Fig. 6B) with different intensities.

**OprD+H11001 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. 7A–7B) or ceftazidime (Fig. 7C–7D) with OprD+H11001 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+H11001 Sias immobilized sensor chips separately. OprD−H11002 Sias was always used for comparison.

Piperacillin (10, 20, 50, and 100 μM) showed decreased association with OprD+H11001 Sias (17, 21, 23, and 27 RU, Fig. 7A) compared with OprD+H11002 Sias (45, 82, 101, and 114 RU respectively, Fig. 7B). Similarly, ceftazidime (10, 20, 50, and 100 μM) also showed reduced interaction with sialylated (17, 21, 23, and 27 RU, Fig. 7C) as compared with non-sialylated (45, 82, 101, and 114 RU respectively, Fig. 7D) analog.

The dissociation constants (K_D) for OprD+H11001 Sias-piperacillin interaction were much higher (7.60 ± 0.13 × 10^-6 M) in contrast to the binding of OprD−H11001 Sias-piperacillin (0.58 ± 0.09 × 10^-8 M). Similarly, greater K_D values (9.08 ± 0.93 × 10^-6 M) of OprD+H11001 Sias-ceftazidime compared with OprD−H11001 Sias-ceftazidime (0.55 ± 0.01 × 10^-8 M) demonstrated less efficiency of sialylated analog of OprD toward the binding of two representative commonly used antibiotics.
Permeability of Piperacillin or Ceftazidime Decreased
Through OprD-Sias—Searching for the crucial role of sialic
acids found on this porin protein (OprD\textsuperscript{-Sias}) from PA14 in the
penetration of antibiotics inside the bacteria, we checked
permeabilization of piperacillin (Fig. 8\textsuperscript{A}) or ceftazidime (Fig.
8\textsuperscript{B}) through artificially formed liposome membrane with
OprD\textsuperscript{-Sias} by liposome swelling assay. Decrease in OD was
observed upon the dilution of OprD\textsuperscript{-Sias} or sialidase treated-
OprD\textsuperscript{-Sias} protein-containing
liposomes in either piperacillin or ceftazidime. In contrast, little
decrease of absorbance was noticed upon the dilution of
OprD\textsuperscript{+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\textsubscript{Urine}, PA\textsubscript{Pus}, and
PA\textsubscript{Urine} (Fig. 8C, 8E, 8G) demonstrated similar results with
piperacillin. These three strains also exhibited comparable
results with ceftazidime (Fig. 8D, 8F, 8H).

This result indicated that non-sialylated porin is highly capa-
bile to permeabilize antibiotics through them. Because of pres-
ence of sialic acid on the porin protein, it hindered to bind
antibiotics leading to inefficient uptake of antibiotics which pos-
sibly 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 depo-
sition (19). Correlation of sialylation of glycoproteins in PA
and their survival in host is relatively untouched field. There-
fore, we considered it may be essential to identify these
sialylated glycoproteins that might play an important molecu-
lar determinant on PA for their survival.

The main achievements of the current investigations in-
clude purification of a few sialoglycoproteins of PA, their
molecular identification, characterization and more impor-
Sialylation of OprD in Antibiotic Resistance of PA

Siantly established the role of one such sialoglycoprotein (OprD) on PA. Treatments of PA−Sias as well as purified OprD−Sias with two commonly used β-lactam antibiotics (cef-tazidime 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 sialoglyco- proteins 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, β-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 (69). OprD is specific protein through which PA up-takes basic amino acids, peptides and β-lactam antibiotics (60). PA can colonize in the various surfaces to form biofilm with the help of flagellin type B protein and becomes imper- vious 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, ceftriax-one, cefoperazone, cefpirome, and cepfime 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 cepofera-zone. 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-resis-tance, 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 α2,6- and α2,3-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 sta-bility 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 β-lactam antibiotics (ceftazidime and piperacillin). SPR analysis showed that ceftazidime-OprD−Sias and piperacillin-OprD−Sias interaction 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 permeabi-lization in PA.

This was further corroborated in live cell experiment. Near the MIC90 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 hinder-ing 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.](image-url) 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 μg, 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 μM 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 β-lactam antibiotics. This is because of lower penetration capability of drugs through this highly sialylated porin protein of PA. 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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The references have been omitted from this text.
Sialylation of OprD in Antibiotic Resistance of PA

J. K. (2009) Pmp2p is a channel-forming protein in Mammalian peroxisomal membrane. PLoS One 4, e5030

27. Hancock, R. E. (1998) Resistance mechanisms in Pseudomonas aeruginosa and other nonfermentative gram-negative bacteria. Clin. Infect. Dis. 27, Suppl. 1, S93–S99

28. Gong, F., Zhan, W., Wang, L., Song, Y., Xing, M., and Song, J. (2012) Role of MexA-MexB-OprM efflux pump system in chronic Pseudomonas aeruginosa pulmonary infection in mice. J. Huazhong Univ. Sci. Technol. Med. Sci. 32, 546–551

29. Sánchez, P., Rojo, F., and Martínez, J. L. (2002) Transcriptional regulation of mexR, the repressor of Pseudomonas aeruginosa mexAB-oprM multidrug efflux pump. FEMS Microbiol. Lett. 207, 63–68

30. Masuda, N., Sakagawa, E., Ohya, S., Gotoh, N., Tsujimoto, H., and Nishino, T. (2000) Contribution of the MexM-MexY-oprM efflux system to intrinsic resistance in Pseudomonas aeruginosa. Antimicrob. Agents. Chemother. 44, 2242–2246

31. Köhler, T., Michéa-Hamzehpour, M., Henze, U., Gotth, N., Curty, L. K., and Pechère, J. C. (1997) Characterization of MexE-MexF-OprN, a positively regulated multidrug efflux system of Pseudomonas aeruginosa. Mol. Microbiol. 23, 345–354

32. Srikumar, R., Kon, T., Gotth, N., and Poole, K. (1998) Expression of Pseudomonas aeruginosa multidrug efflux pumps MexA-MexB-OprM and MexC-MexD-OprJ in a multidrug-sensitive Escherichia coli strain. Antimicrob. Agents. Chemother. 42, 65–71

33. Wang, J., and Liu, J. (2004) Directly fishing out subtle mutations in genomic DNA with histidine-tagged Thermus thermophilus Mut. Mutat. Res. 547, 41–47

34. Dubois, V., Arpin, C., Dupart, V., Scavelli, A., Coulange, L., André, C., Fischer, I., Grobst, F., Brochet, J. P., Lagrange, I., Dutilh, B., Jullin, J., Noury, P., Larbret, G., and Quentin, C. (2008) Beta-lactam and antibiotic-glycoside resistance rates and mechanisms among Pseudomonas aeruginosa in a general practice community and private healthcare centres). J. Antimicrob. Chemother. 62, 316–323

35. Dean, C. R., Datta, A., Carlson, R. W., and Goldberg, J. B. (2002) WbjA adds sialic acid to epithelial cells. J. Bacteriol. 184, 323–326

36. Horzempa, J., Dean, C. R., Goldberg, J. B., and Castric, P. (2006) Contribution of the MexM-MexY-oprM efflux system to intrinsic resistance in Pseudomonas aeruginosa. Antimicrob. Agents. Chemother. 49, 3304–3309

37. Ghoshal, A., Mukhopadhyay, S., Demine, R., Forgber, M., Jarmalavicius, S., Saha, B., Sundar, S., Mandal, C., and Mandal, C. (2009) Detection and characterization of a sialoglycosylated bacterial ABC-type phosphate transporter protein from patients with visceral leishmaniasis. Glycoconj. J. 26, 675–689

38. Yoshihara, E., and Nakae, T. (1989) Identification of porins in the outer membrane of Pseudomonas aeruginosa that form small diffusion pores. J. Biol. Chem. 264, 6297–6301

39. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254

40. Pal, S., Ghosh, S., Mandal, C., Kohla, G., Brossmer, R., Isecke, R., Merling, A., Schauer, R., Schwartz-Albiez, R., Bhattacharya, D. K., and Mandal, C. (2004) Purification and characterization of a 9-O-acetylated sialo-glycoproteins from leukemic cells and their potential as immunological tool for monitoring childhood acute lymphoblastic leukemia. Glycobiology 14, 859–870

41. Mandal, C., and Mandal, C. (2013) Identification and analysis of O-acetylated sialylglycopeptides. Methods Mol. Biol. 871, 57–93

42. Sharma, V., Chatterjee, M., Mandal, C., Sen, S., and Basu, D. (1998) Rapid isolation of Pseudomonas aeruginosa virulence factors in Escherichia coli from patients with visceral leishmaniasis using AchatinINH, a 9-O-acetylated sialic acid binding lectin. J. Trop. Med. Hyg. 95, 551–554

43. Ansar, W., Habib, S. K., Roy, S., Mandal, C., and Mandal, C. (2009) Combining results from lectin affinity chromatography and glycopeptide capture approaches substantially improves the coverage of the glycome from leukemia cells and their potential as immunological tool for monitoring childhood acute lymphoblastic leukemia. Glycobiology 14, 859–870

44. Sarkar, S., Dutta, D., Ghoshal, A., Mukhopadhyay, S., Saha, B., Sundar, S., Jarmalavicius, S., Forberg, M., Mandal, C., and Mandal, C. (2011) Glycoproteomics of milk: differences in sugar epitopes on human and bovine milk fat globule membranes. J. Proteome Res. 7, 1650–1659

45. Wilson, N. L., Robinson, L. J., Donnet, A., Bovetto, L., Packer, N. H., and Karlsson, N. G. (2008) Glycoproteomics of milk: differences in sugar epitopes on human and bovine milk fat globule membranes. J. Proteome Res. 7, 3687–3696

46. McPhee, J. B., Tamber, S., Bains, M., Maier, E., Gellatly, S., Lo, A., Benz, R., and Hancock, R. E. (2009) The major outer membrane protein OprG of Pseudomonas aeruginosa contributes to cytotoxicity and forms an anaerobiologically regulated, cation-selective channel. FEMS Microbiol. Lett. 296, 241–247

47. Huang, H., and Hancock, R. E. W. (1993) Glycoproteomics of milk: differences in sugar epitopes on human and bovine milk fat globule membranes. J. Proteome Res. 7, 1650–1659

48. Costerton, J. W., Steward, P. S., and Greenberg, E. P. (1999) Bacterial biofilms: a common cause of persistent infections. Science 284, 1318–1322

49. Hancock, R. E. W., Siehnen, R., and Martin, N. (1996) Outer membrane proteins of Pseudomonas. Mol. Microbiol. 4, 1069–1075

50. Valderrama-Rincon, J. D., Fisher, A. C., Merritt, J. H., Fan, Y. Y., Reading, J. G., Montgomery, K., Kucherlapati, R., Rahme, L. G., and Ausubel, F. M. (2000) Characterization of membrane-associated porins of Pseudomonas aeruginosa. J. Bacteriol. 182, 6195–6206

51. Ansar, W., Habib, S. K., Roy, S., Mandal, C., and Mandal, C. (2009) Unraveling the C-reactive protein complement-cascade in destruction of red blood cells: potential pathological implications in Plasmodium falciparum malaria. Cell. Physiol. Biochem. 23, 175–190

52. Sarkar, S., Dutta, D., Samanta, S. K., Bhattacharya, K., Pal, B., C., Li., J., Datta, K., Mandal, C., and Mandal, C. (2013) Oxidative inhibition of Hsp90 disrupts the super-chaperone complex and attenuates pancreatic adenocarcinoma in vivo and in vivo. Int. J. Cancer 132, 696–706

53. McDonald, C. A., Yang, J. Y., Marathe, V., Yen, T. Y., and Machler, B. A. (2009) Combining results from lectin affinity chromatography and glyco-capture approaches substantially improves the coverage of the glyco-proteome. Mol. Cell. Proteomics 8, 287–301

54. Samanta, S., Dutta, D., Ghoshal, A., Mukhopadhyay, S., Saha, B., Sundar, S., Jarmalavicius, S., Forberg, M., Mandal, C., and Mandal, C. (2011) Glycosylation of erythrocyte spectrin and its modification in visceral leishmaniasis. PLoS One 6, e28169

55. Ghoshal, A., Mukhopadhyay, S., Demine, R., Forberg, M., Jarmalavicius, S., Saha, B., Sundar, S., Walden, P., Mandal, C., and Mandal, C. (2009) Unraveling the C-reactive protein complement-cascade in destruction of red blood cells: potential pathological implications in Plasmodium falciparum malaria. Cell. Physiol. Biochem. 23, 175–190

56. Jitsuhara, Y., Toyoda, T., Itai, T., and Yamaguchi, H. (2002) Chaperone-like proteins of functional roles in the formation of the super-chaperone complex and attenuation of pancreatic adenocarcinoma in vivo and in vivo. Int. J. Cancer 132, 696–706