Lectin activity of the pneumococcal pilin proteins

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Streptococcus pneumoniae is a leading cause of morbidity and mortality globally. The Pilus-1 proteins, RrgA, RrgB and RrgC of S. pneumoniae have been previously assessed for their role in infection, invasive disease and as possible vaccine candidates. In this study we have investigated the glycan binding repertoire of all three Pilus-1 proteins, identifying that the tip adhesin RrgA has the broadest glycan recognition of the three proteins, binding to maltose/cellobiose, α/β-linked galactose and blood group A and H antigens. RrgB only bound mannose, while RrgC bound a subset of glycans also recognized by RrgA. Adherence of S. pneumoniae TIGR4 to epithelial cells was tested using four of the oligosaccharides identified through the glycan array analysis as competitive inhibitors. The blood group H trisaccharide provided the best blocking of S. pneumoniae TIGR4 adherence. Adherence is the first step in disease, and host glycoconjugates are a common target for many adhesins. This study has identified Pilus-1 proteins as new lectins involved in the targeting of host glycosylation by S. pneumoniae.

Streptococcus pneumoniae is one of the leading causes of morbidity and mortality worldwide 1–3. S. pneumoniae causes a range of diseases including pneumonia, meningitis, septicemia and otitis media, and produces a range of virulence factors including the toxin pneumolysin, pneumococcal surface protein A and pilus 4–6. The current S. pneumoniae vaccines target the capsular polysaccharide, but cover only a subset of the 97 known capsular serotypes. Differences in serotype distribution between developed and developing countries, and serotype replacement in response to widespread use of the vaccines, are reducing the overall impact of the vaccines on the burden of pneumococcal disease 1–3.

Expression of the pneumococcal Pilus-1, composed of three proteins RrgA, RrgB and RrgC, has been linked to pneumococcal meningitis in mouse infection models 5–7. Pilus-1 was found to be required for the bacteria to breach the blood brain barrier 5. The Pilus-1 protein complex consists of RrgB as the shaft protein, with RrgA as the tip adhesin and RrgC, which serves as a pilus anchor at the cell surface 6–8. The S. pneumoniae Pilus-1 protein complex has been proposed as a novel vaccine target 9,10. The RrgA and RrgB proteins were found to produce cross-protecting antibodies that led to blocking of adherence of S. pneumoniae to cells in culture and resulted in the opsonophagocytosis of S. pneumoniae 9,11. RrgA is also involved in regulation of the host immune response to S. pneumoniae by binding to both MAC-1 (complement receptor 3, CD11b/CD18) 12 and Toll-like receptor 2 13. RrgA has also been shown to interact directly with cultured epithelial cells and extracellular matrix components including fibronectin and collagen 10.

All of the known proteins that interact with RrgA are glycoproteins, indicating a potential role of the oligosaccharides in the interactions. In this study we aim to identify glycan targets of the Pilus-1 protein complex of S. pneumoniae.

Results

RrgA, RrgB and RrgC proteins bind glycans. Glycan array analysis of the three Pilus-1 proteins from S. pneumoniae TIGR4 and RrgA from strain SPEC6B revealed differential glycan recognition between the four proteins tested. S. pneumoniae TIGR4 RrgB bound the least number of glycans, with only an α-mannobiose recognized (Table 1 and Dataset S1). RrgA from S. pneumoniae TIGR4 and RrgA from S. pneumoniae

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| No. | Structure | RrgA T4 | RrgA SPEC6B | RrgB T4 | RrgC T4 |
|-----|-----------|---------|-------------|---------|---------|
| 5   | Gal3-s3  | 1587 ± 308 | 369 ± 188 | 108.8 ± 88.9 | 2165 ± 450 |
| 14  | GlcN(Ge)3-s4 | 157 ± 171 | 2112 ± 110 | 37.5 ± 63.5 | 191.3 ± 124 |
| 18  | Man3-s4  | 166.3 ± 198 | 2532 ± 436 | 1135.3 ± 68.8 | 600.5 ± 650 |
| 22  | GlcNAc3-s4 | 1140 ± 302 | 55.5 ± 413 | 227 ± 190 | 57.5 ± 182 |

**Terminal Galactose**

76 Gal3-1-Gal3-s3 1918.3 ± 154
80 Gal3-1-GlcNAc3-s3 90.75 ± 196
88 Gal3-1-3GlcNAc3-s3 1013 ± 101
89 Gal3-1-3GlcNAc-s3 1098 ± 308
94 Gal3-1-4Glc-s4 1218 ± 458
100 Gal3-1-6Gal-s4 2197 ± 486
220 Gal3-1-4Glc-glc3-s2 1429 ± 204
222 Gal3-1-4Gal3-1-4GlcNAc1-s3 990 ± 237
373 Gal3-1-4GlcNAc-s3 1802.5 ± 276
381 Gal3-1-3GlcNAc1-6Gal31-1-6GlcNAc3-s2 1985.4 ± 280
383 Gal3-1-4GlcNAc1-6Gal31-1-6Glc3-s2 1260.0 ± 279
489 Gal3-1-4GlcNAc3-1-3(GlcNAc1-6)Gal31-1-4GlcNAc-s2 782.5 ± 96.3
501 Gal3-1-3GlcNAc3-1-3Gal31-1-4Glc3-s4 116.0 ± 166
504 (A-GN-M0)-3,6-M-GN-GN3-s4 197.8 ± 201
1 G Gal3-1-3GlcNAc3-s3 4650 ± 1751
2 G Gal3-1-3GlcNAc3-1-3Gal31-1-6GlcNAc3-1-3Gal31-1-4Glc 1344 ± 583

**Terminal N-Acetylgalactosamine**

2 F GalNAc1-3-Gal31-1-4Glc 1221.5 ± 204
392 Fuco1-2-GalNAc31-3-Gal31-1-3GlcNAc-s3 1366 ± 296
480 Fuco1-2-Gal31-1-3GlcNAc-s3 315.25 ± 272.0421
483 Fuco1-3(Fuco1-2Gal31-1-3Gal31-1-3GlcNAc-s3 327.3 ± 438
496 Fuco1-2Gal31-1-3(Fuco1-4)GlcNAc31-3-Gal31-1-4Glc3-s4 274.5 ± 331
538 Le1-6(Lc1-3)Lac-s4 1261.5 ± 346
539 LacNAc1-6(Lc1-3)Lac-s4 206.8 ± 210
7 A Fuco1-2Gal31-1-3GlcNAc31-3-Gal31-1-4Glc 1897.3 ± 285
7 K GalNAc1-3(Fuco1-2)Gal 1159.0 ± 162
8 A SO31-3Gal31-1-3(Fuco1-4)GlcNAc 918.5 ± 84.0
8 I Fuco1-2Gal31-1-4(Fuco1-3)GlcNAc31-3(Fuco1-2)Gal31-1-4Glc 1692.3 ± 305
8 K Gal31-1-4(Fuco1-3)GlcNAc31-6(Gal31-1-4GlcNAc31-3)Gal31-1-4Glc 170.0 ± 177

**Mannose**

120 Man3-s4 18.0 ± 406

**Terminal N-Acetylgalactosamine**

117 GlcNAc31-1-3GlcNAc3-s4 145.6 ± 238
118 GlcNAc31-6GalNAc-s3 1322.5 ± 366
253 GlcNAc31-6Gal31-1-4GlcNAc3-s2 54.75 ± 212
505 (GN-M0)-3,6-M-GN-GN3-s4 244.5 ± 183

**Glucose**

112 Glc1-6Glc-s4 1284.8 ± 445
390 (Glc1-4)2-s3 930.5 ± 94.7

**High molecular weight Carageenan and Glycoaminoglycans (GAGS)**

141 HA 1600000 da 2.5 mg/ml 985 ± 884

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**Table 1.** Glycans bound by Rrg proteins in glycan array analysis. Values are global background subtracted. Red indicates binding. Binding is determined by positive interaction in three replicate array experiments. Positive interactions are determined by a background subtracted fluorescence value significantly above background subtracted fluorescence of negative control spots (average background fluorescence from 20 spots ± 3 standard deviations).

SPEC6B bound to a set of overlapping glycans including terminal galactose structures with both α and β linkages, glucose/maltose-related structures and blood group A (7 K and 392) and blood group H(O) (7 A) antigens (Table 1 and Dataset S1). No interactions with terminal galactose structures were observed when galactose was
Surface plasmon resonance was performed between free oligosaccharides and captured RrgA proteins (Table 2).

**Table 2.** Surface plasmon resonance results for RrgA proteins and glycans.a Data are dissociation equilibrium constants (K_D) of the interactions between free oligosaccharides and captured RrgA proteins; NCDI: no concentration-dependent interaction detected.

| Oligosaccharide          | RrgA TIGR4 | RrgA SPEC6B |
|--------------------------|------------|-------------|
| Blood group A tris       | 916.5 nM ± 66.4 | 1.4 μM ± 0.29 |
| Blood group A type3/4    | 870.7 nM ± 286 | 643.6 nM ± 157 |
| Blood group H type 3/4   | 89.4 nM ± 16 | 558.1 nM ± 51 |
| Lewis X                  | 434.0 nM ± 118 | 363.3 nM ± 107 |
| Lacto-N-tetraose         | 1.03 μM ± 0.05 | 868.6 nM ± 454 |
| maltobiose               | 1.16 μM ± 0.09 | 964.0 nM ± 226 |
| cellobiose               | 1.48 μM ± 251 | 582.8 nM ± 155 |
| chitobiose               | 409.3 nM ± 160 | 426.2 nM ± 144 |
| lactose                  | NCDI        | NCDI        |

Table 3. Adherence of TIGR4 vs TIGR4ΔrrgA. Data are the average of two separate experiments consisting of 4 individual wells (experiment 1) and 5 individual wells (experiment 2) (n = 9). a100% = 1.97 × 10^7 CFU per well. b100% = 8.70 × 10^6 CFU per well.

|          | A549 adherence (% TIGR4 ± SEM)a | Detroit 562 (% TIGR4 ± SEM)b |
|----------|---------------------------------|-------------------------------|
| TIGR4    | 100 ± 5.4                       | 100 ± 19.8                   |
| TIGR4ΔrrgA | 52.7 ± 3.9                     | 27.1 ± 7.4                  |
| Significance | P < 0.001                      | P < 0.01                     |

Discussion

Adherence to host tissues is the first step in disease and host glycoconjugates are a common target for numerous bacterial adhesins14–16, including pili/fimbriae17,18. Pilus-1 of *S. pneumoniae* has been shown to interact with Toll-like receptor 2 and MAC-112,13 and epithelial cells and ECM components19, all of which are glycosylated.

The glycan array analysis of RrgA from *S. pneumoniae* TIGR4 and SPEC6B identified a cluster of oligosaccharide binding that was consistent between the two proteins. These proteins have previously been shown to bind to both cells and ECM components equally, indicating recognition of uncapped β-linked galactose. Binding of *S. pneumoniae* RrgA to β-linked galactose and blood group A glycan is consistent with interactions between *S. pneumoniae* and a wide variety of cell surfaces in the human host.
Rrg proteins to many non-human and non-mammalian glycans. Rrg proteins recognised maltose and cellulose may allow *S. pneumoniae* nature of with α1-3-linked galactose is only observed in humans that produce blood group B anti-

1-3-linked galactose structures present on the array, while RrgA only bound 1-2 terminal α of the 11 terminal

S. pneumoniae glycans as recombinant soluble proteins. Thus, we have identified three new lectins produced by nising a range of common host cell surface glycans. All three proteins that make up the Pilus-1 complex recognise

binding performance as replicates (n = 100). The significance of these interactions in terms of interaction with host surfaces was further confirmed by competitive inhibition of TIGR4 adherence to A549 cells by exogenous maltose, cellulbiose and GlcNAc were confirmed through SPR analysis, with the highest affinity interaction observed occurring between RrgA from TIGR4 with the H-type 3/4 oligosaccharide (Table 2). As observed in the array experiment, no concentration dependent interaction was observed using SPR for terminal galactose with underlying glucose (lactose; Table 2). The SPR analysis found high affinity binding between RrgA and Lewis X (K < 500 nM; Table 2).

The direct interaction between RrgA and Lewis X was not seen on the array, but Lewis X was a terminal group on several of the larger fucosylated structures identified (Table 1; 8 J and 8 K). This may indicate that the binding of Lewis X requires a specific presentation that is available in solution, but not available on the array except as a part of a larger structure. Lewis X is a member of the Lewis system histo-blood group antigens, which were originally identified on RBCs where they are acquired from the plasma as glycolipids and is expressed on most cell types but is over-expressed by human tumor cells from various sites.

*S. pneumoniae* is a host-adapted pathogen that infects humans so it is unexpected to observe the binding of the Rrg proteins to many non-human and non-mammalian glycans. Rrg proteins recognised maltose and cellulbiose saccharides, repeating units of glucose typically produced by plants, algae and some bacteria. This interaction may allow *S. pneumoniae* to interact with other bacterial species either directly or to the matrix of bacteria in biofilm communities.

RrgC and RrgA also bound to the non-human terminal α1-3-linked galactose structures. RrgC bound four of the 11 terminal α1-3-linked galactose structures present on the array, while RrgA only bound 1-2 terminal α1-3-linked galactose structures on the array (Table 1). This binding does not correlate with the host adapted nature of *S. pneumoniae* with α1-3-linked galactose widely expressed in non-human mammals but not in humans. The expression of α1-3-linked galactose is only observed in humans that produce blood group B antigens, structures not recognised on the array by the Rrg proteins.

The Pilus-1 complex of *S. pneumoniae* is known to be an important mediator of adherence of *S. pneumoniae* to host epithelial cells. This binding appears to be glycan related, as RrgA was found to have lectin activity, recognising a range of common host cell surface glycans. All three proteins that make up the Pilus-1 complex recognise glycans as recombinant soluble proteins. Thus, we have identified three new lectins produced by *S. pneumoniae*, with the pilus tip protein RrgA directly involved in interactions between pathogen and host.

**Materials and Methods**

**Cloning, expression and purification of Rrg proteins.** Genes were cloned into pET protein expression vectors and proteins were expressed and purified as previously described.

**Glycan Array.** Glycan array slides were printed using SuperEpoxy 3 activated substrates as previously described using an ArrayIt Spotbot Extreme 3 contact printer with solid metal pins. The Glycan array binding experiments were performed and analysed as previously described. Briefly 1 μg of protein in 1xPBS containing 1 mM MgCl₂ and 1 mM CaCl₂ was pre-complexed with mouse anti-His antibody (Cell signaling), Alexa555 rabbit anti-mouse IgG and Alexa555 goat anti-rabbit IgG and allowed to bind to a pre-blocked (1% bovine serum albumin in PBS) for 15 minutes. Slides were washed three times for 2 minutes in 1xPBS, dried by centrifugation and scanned and analysed using the Scan Array Express software package (Perkin Elmer) and Microsoft Excel for statistical analysis (Student's unpaired t-test of fluorescence of background spots vs fluorescence of glycan printed spots).

| Strain/glycan | A549 adherence (% control ± SEM) | Significance vs untreated |
|---------------|----------------------------------|--------------------------|
| TIGR4         | 100 ± 9.2                        | —                        |
| TIGR4 + 200 μM lactose | 85.5 ± 11.0                  | Not significant          |
| TIGR4 + 200 μM maltose     | 43.7 ± 9.2                     | P < 0.001                |
| TIGR4 + 200 μM cellobiose  | 44.6 ± 3.1                     | P < 0.001                |
| TIGR4 + 200 μM BGA type 3/4 | 44.0 ± 2.5                 | P < 0.001                |
| TIGR4 + 200 μM BGH type 3/4 | 32.3 ± 2.7                 | P < 0.001                |
| TIGR4ΔrrgA         | 100 ± 3.9                      | —                        |
| TIGR4ΔrrgA + 200 μM cellobiose | 62.4 ± 4.2              | P < 0.001                |
| TIGR4ΔrrgA + 200 μM BGA type 3/4 | 69.6 ± 2.1             | P < 0.001                |
| TIGR4ΔrrgA + 200 μM BGH type 3/4 | 53.5 ± 1.6             | P < 0.001                |

Table 4. Effect of glycans on adherence of TIGR4 to A549 cells. Data are the average from four individual wells performed as replicates (n = 4). *100% = 3.50 × 10⁵ CFU per well. *4100% = 1.97 × 10⁵ CFU per well. BGA: Blood group A, BGH: Blood group H.
Surface Plasmon Resonance analysis. The interactions between the Rrg proteins and test glycans were analysed using surface plasmon resonance ( SPR) using a Biacore T100 system as described Shewell et al.46 with the following modifications. Proteins were immobilised onto a CM5 chip at pH 4.5, flow rate of 10 μL/min for 420 seconds with an ethanolamine blank flow cell as a control. Glycans were tested between 160 nM and 100 μM. All data was double reference subtracted.

Mutagenesis of rrgA in S. pneumoniae TIGR4. The rrgA gene in TIGR4 was deleted in-frame and replaced with an erythromycin resistance cassette (erm) by direct transformation with a linear DNA fragment constructed by overlap-extension PCR, essentially as previously described24. This involved using primer pairs rrgAF/rrgAermR and rrgAermF/rrgAR to amplify 5′ and 3′ regions flanking rrgA from TIGR4 template DNA, and J214/J215 to amplify erm from pVA838 (Table 5). Primers included overlapping sequences such that the three PCR products could be fused in a second round of PCR using primer pair rrgAF/rrgAR Erythromycin-resistant transformants were screened by PCR for deletion of rrgA, confirmed by DNA sequencing and designated TIGR4ΔrrgA.

Competitive inhibition of adherence of S. pneumoniae with free glycans. Adherence assays on A549 (human type II pneumocyte) and Detroit 562 (human nasopharyngeal carcinoma) cells were carried out as previously described29. A549 and Detroit 562 cells were grown in Dulbecco’s Modified Eagle Medium (DMEM; Gibco), or a 1:1 mix of DMEM and Ham’s F-12 Nutrient Mixture (Gibco), respectively, supplemented in both cases with 5% fetal bovine serum, 2 mM L-glutamine, 50 IU of penicillin and 50 μg/ml streptomycin. Confluent monolayers in 24-well plates were washed with PBS and infected with pneumococci (approximately 5 × 10^6 CFU per well) in a 1:1 mixture of the respective culture medium (without antibiotics) and C + Y medium, pH 7.425. Plates were centrifuged at 500 × g for 5 min, and then incubated at 37 °C in 5% CO2 for 2.5 h. Monolayers were washed 3 times in PBS, and adherent bacteria were released by treatment with 100 μl trypsin/EDTA, followed by 400 μl 0.025% Triton X-100. Lysates were serially diluted and plated on blood agar to enumerate adherent bacteria. Total adherence (mean ± SEM for 4–9 replicates) was expressed as a percentage of that for TIGR4 (or TIGR4ΔrrgA where appropriate) without additives. For inhibition assays, free oligosaccharides were used at a final concentration of 200 μM ( > 100 × Kd) throughout the adherence assay. Data were analysed using Student’s un-paired t-test (two-tailed).

Lectin array analysis of Detroit 562 and A549 cells. The two cell lines used for adherence assays, A549 and Detroit 562, were analysed for surface glycans using lectin arrays. Lectin arrays were printed using an Arrayjet Argus Marathon Inkjet Bio-Printing System on Arrayit SME3 substrates. The lectins are immobilized to the epoxy activated substrate through non-specific amine coupling through free amines on the lectin proteins and the epoxide groups on the glass. Arrays were neutralized and performed as previously described26, with the exception that Bodipy 558/568 succinimidyl ester (Thermo Scientific) was used in place of CFDA-SE. Briefly, cells were epoxided, washed three times with PBS, and resuspended in PBS containing 1 mM MgCl_2 and 1 mM CaCl_2 for 10^6 cells/mL and 300 μL was applied to the array in a 125 μL frame without a coverslip. Cells were allowed to interact with the lectins on the array for 30 minutes and then the slides were washed three times with PBS, fixed in 4% formaldehyde and dried by centrifugation. Slides were scanned on an Innopsys InnoScan 1100AL to acquire the data of which lectins bound to the cells and analysed using Innopsys Mapix data acquisition and analysis software and Microsoft Excel for statistical analysis (Student’s unpaired t-test of fluorescence of background spots vs fluorescence of lectin printed spots).

Table 5. Oligonucleotide primers.

| Primer        | Sequence 5′-3′                     |
|---------------|------------------------------------|
| rrgAF         | CAGAAACTAGAGACAGAAGTG              |
| rrgAR         | CTGACCAGCCTAGTAGTAAGCC             |
| rrgAermR      | TGTTCTCATGAAATCCTCTACCTGTAGAGAACA |
| rrgAermF      | CGGGGAAAGAATATCTCTGAGTGAAATAATGAT |
| J214          | GAAGGAGTATTACATGAAACAA             |
| J215          | CTTCATAGAATATTTCCTCCCCG            |

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Author Contributions

Conceived and designed the experiments: L.E.H., A.W.P., J.C.P., J.T., M.P.J., C.J.D., K.L.S. Performed the experiments: S.S., V.M., A.W.P., L.E.H., C.J.D., N.B., R.M.H. Analyzed the data: L.E.H., A.W.P., C.J.D., J.C.P., M.P.J. Wrote the paper: C.J.D., A.W.P., R.M.H., L.E.H., K.L.S., J.T., N.B., S.S., V.M., J.C.P., M.P.J.

Additional Information

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