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Absence of Capsule Reveals Glycan-Mediated Binding and Recognition of Salivary Mucin MUC7 by *Streptococcus pneumoniae*

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SUMMARY

Salivary proteins modulate bacterial colonization in the oral cavity and interact with systemic pathogens that pass through the oropharynx. An interesting example is the opportunistic respiratory pathogen *Streptococcus pneumoniae* that normally resides in the nasopharynx, but belongs to the greater Mitis group of streptococci, most of which colonize the oral cavity. *S. pneumoniae* also expresses a serine-rich repeat (SRR) adhesin, PsrP, that is a homologue to oral Mitis group SRR adhesins, such as Hsa of *S. gordonii* and SrpA of *S. sanguinis*. Since the latter bind to salivary glycoproteins through recognition of terminal sialic acids, we wanted to determine whether *S. pneumoniae* also binds to salivary proteins through possibly the same mechanism. We found that only a capsule-free mutant of *S. pneumoniae* TIGR4 binds to salivary proteins, most prominently to mucin MUC7, but that this binding was not mediated through PsrP or recognition of sialic acid. We also found, however, that PsrP is involved in agglutination of human red blood cells (RBCs). After removal of PsrP, an additional previously masked lectin-like adhesin activity mediating agglutination of sialidase-treated RBCs becomes revealed. Using a custom-spotted glycoprotein and neoglycoprotein dot blot array, we identify candidate glycan motifs recognized by PsrP and by the putative *S. pneumoniae* adhesin that could perhaps be responsible for pneumococcal binding to salivary MUC7 and glycoproteins on RBCs.

Keywords

Saliva; salivary proteins; serine-rich repeat adhesins; bacterial lectins

INTRODUCTION

Saliva helps to maintain an ecological balance within the diverse oral biofilm microbiota through clearance of pathogens and fostering colonization of the mouth by a physiological

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commensal microflora (Ruhl, 2012, Scannapieco, 1994, Nobbs *et al*., 2011). Oral Mitis group streptococci are frequently found among the earliest colonizers of human teeth (Frandsen *et al*., 1991). Binding of these bacteria to the surface of teeth requires specific biochemical interactions of protein adhesins expressed on the bacteria with cognate receptor motifs on salivary proteins and glycoproteins that are adsorbed to the mineralized tooth surface as a thin film, the so-called acquired enamel pellicle (Lendenmann *et al*., 2000, Nobbs *et al*., 2011). On salivary glycoproteins, terminal sialic acids serve as important glycan receptor motifs recognized by corresponding sialic acid-binding adhesins of oral commensal Mitis group streptococci (Levine *et al*., 1978, Murray *et al*., 1992, Murray *et al*., 1982, Deng *et al*., 2014). One example for such an adhesin is Hsa, expressed on the surface of *Streptococcus gordonii* DL1 (Challis), that preferentially binds sialic acids in a terminal position α2-3-linked to *O*-linked glycan chains (Takahashi *et al*., 1997, Takahashi *et al*., 2002a, Deng *et al*., 2014). Major sialoglycoproteins in saliva that serve as counter-receptors for Hsa-mediated bacterial adhesion are the low-molecular weight salivary mucin-7 (MUC7) (Ruhl *et al*., 2004, Takamatsu *et al*., 2006) and the heavy-chain of secretory immunoglobulin A1 (Ruhl *et al*., 1996). Hsa structurally and genetically belongs to a wider family of bacterial serine-rich repeat protein (SRR) adhesins that all participate in bacterial adhesion, colonization, and opportunistic virulence (Lizcano *et al*., 2012, Löfling *et al*., 2011, Pyburn *et al*., 2011, Zhou & Wu, 2009, Deng *et al*., 2014, Turner *et al*., 2009). Homologues of SRR adhesins are expressed by different bacterial genera including a number of other streptococcal species (Wu *et al*., 1998, Bensing *et al*., 2004, Plummer *et al*., 2005, Obert *et al*., 2006). However, besides Hsa, binding to sialic acid on salivary glycoproteins by SRR adhesins has only been shown for GspB (Takamatsu *et al*., 2006, Deng *et al*., 2014), a close homologue of Hsa in *S. gordonii* M99, and SrpA of *S. sanguinis* SK36 (Plummer & Douglas, 2006, Deng *et al*., 2014).

One prominent non-oral Mitis group streptococcal species that also expresses an SRR adhesin is the opportunistic respiratory pathogen *Streptococcus pneumoniae* (Löfling *et al*., 2011, Rose *et al*., 2008, Obert *et al*., 2006, Kawamura *et al*., 1995, Kilian *et al*., 2014, Johnston *et al*., 2010). The SRR adhesin expressed by *S. pneumoniae* was identified on strain TIGR4 (Hava & Camilli, 2002, Tettelin *et al*., 2001) and termed pneumococcal serine-rich repeat protein (PsrP) (Obert *et al*., 2006). PsrP is not required for nasal colonization, but is rather a lung-specific virulence factor (Obert *et al*., 2006, Rose *et al*., 2008), which also mediates pneumococcal intra-species aggregation and biofilm formation (Sanchez *et al*., 2010, Blanchette-Cain *et al*., 2013). It has been shown that PsrP binds to keratin 10 present on human lung cells (Shivshankar *et al*., 2009) through a binding domain that resembles microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (Schulte *et al*., 2014). There exist, however, earlier reports suggesting a role of glycan binding for adherence of *S. pneumoniae* (Barthelson *et al*., 1998, Idänpään-Heikkila *et al*., 1997, Andersson *et al*., 1983, Cundell & Tuomanen, 1994, Krivan *et al*., 1988). Even though the binding of PsrP to keratin 10 was proven to be independent of lectin-carbohydrate interaction (Shivshankar *et al*., 2009), the possible binding of the PsrP adhesin to glycans on other host proteins has not been fully excluded and deserves further study, particularly in view of its close homology with the glycan-binding adhesins Hsa and SrpA. In this study, we aimed to determine by using the bacterial overlay technique (Deng *et al*., 2014, Walz *et*

al., 2009, Prakobphol *et al*., 1987) if *S. pneumoniae* TIGR4 and, specifically, PsrP can bind to salivary glycoproteins through a similar mechanism as Hsa and SrpA. Because *S. pneumoniae* differs from the other Mitis group streptococci by the expression of a polysaccharide capsule that can vary in its thickness (Kim & Weiser, 1998, Lizcano *et al*., 2012, Löfling *et al*., 2011), we have also investigated the influence of this polysaccharide layer on adhesin activity.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The bacterial strains and mutants used in this study are listed in Table 1. All streptococcal strains were cultured overnight at 37°C as stationary cultures in a 5% CO_2 environment. *S. gordonii* DL1 and isogenic Hsa-deficient mutant (kindly provided by Dr. Howard F. Jenkinson, University of Bristol, UK) as well as *S. sanguinis* SK36 and isogenic SrpA-deficient mutant (kindly provided by Dr. Hui Wu, University of Alabama at Birmingham) were grown in brain heart infusion (Becton Dickinson, Franklin Lakes, NJ). *S. pneumoniae* wild-type strain TIGR4, capsule-free mutant strain HR1001.1 and isogenic SRR adhesin-deficient mutants were grown as suspension cultures in Todd-Hewitt broth (Difco Laboratories, Detroit, MI) containing 0.5% yeast extract, or as plate cultures on tryptic soy agar supplemented with 5% sheep blood. Bacteria were washed three times in 0.02 M phosphate buffered saline containing 0.02% sodium azide (PBS, pH 7.2) before use in the assays. In assays involving *S. pneumoniae*, 2% choline chloride was included at all steps to prevent pneumococcal autolysis (Martner *et al*., 2009), which we found resulted in unreproducible bacterial binding results and also adhesin-independent agglutination of RBCs, as reported much earlier (Löffler, 1950). Antibiotic concentrations that were used for selection are as follows: kanamycin (Kan) was used at 250 μg/ml and 25 μg/ml for *S. gordonii* mutant strain UB1545 and *S. sanguinis* mutant strain VT1614, respectively; erythromycin (Erm) was used at 0.3 μg/ml and chloramphenicol (Cm) was used at 4 μg/ml for pneumococci where appropriate. All antibiotics were purchased from Sigma-Aldrich, St. Louis, MO.

Construction of capsule-free TIGR4 and *psrP*-deficient mutants

A capsule-free *S. pneumoniae* TIGR4 mutant was generated by transforming encapsulated TIGR4 bacteria with chromosomal DNA from strain JD908 (a capsule-free serotype 3 strain carrying an Erm cassette in the capsule synthase gene *wchE* (*cpsS*) required for capsule production and having homology to the TIGR4 capsule locus (Dillard & Yother, 1994)) in the presence of competence signal peptide 2 (CSP2). Unencapsulated clones were selected on blood agar plates containing 0.3 μg/ml erythromycin after overnight grown at 37°C in a 5% CO_2 environment. Two independent erythromycin-resistant clones were selected and were transformed with chromosomal DNA from the TIGR4 wild-type parent strain (back-crossed) three times and selected on erythromycin agar plates to retain the capsule mutation, yet ensure that the remaining genetic background consisted purely of TIGR4 chromosomal DNA, as described (Kelly *et al*., 1994). The final capsule-free *S. pneumoniae* TIGR4 mutant used in this study was named HR1001.1. This mutant was shown to lack capsule production by ELISA, according to the method of Bender and Yother (Bender & Yother, 2001). Briefly,

pneumococci were grown to an OD600 of 0.5, washed and resuspended in PBS and heat killed at 56°C. Bacterial suspension adjusted to an OD of 0.2 with PBS (100 μL) was added in a two-fold dilution series to ELISA plates and left at 4°C overnight. After washing the plates three times with PBS containing 0.05% Tween 20, a capsule type 4-specific rabbit anti-serum (Statens Serum Institute, Copenhagen, Denmark) was added as the primary antibody (1:10,000 dilution in PBS). After 1 h of incubation at room temperature, the plates were washed and HRP-conjugated goat anti-rabbit antibodies (1:1,000 in PBS-T) were added for 1 h. The plates were washed and developed using *o*-phenylenediamine dihydrochloride substrate, and the absorbance was read at 492 nm in a Multiskan MS microtitre plate reader (Labsystems). Results of the HR1001.1 mutant were compared to wild-type TIGR4 using JD908 as a negative control for background absorbance. The HR1001.1 showed no absorbance above the JD908 background.

For deletion of the *psrP* gene, HR1001.1 was transformed with chromosomal DNA from STM199, a chloramphenicol-resistant TIGR4 transposon-mutant lacking expression of the *psrP* gene (Hava & Camilli, 2002), kindly provided by Dr. Andrew Camilli, Tufts University School of Medicine, Boston, MA. Transformants were selected on blood agar plates containing chloramphenicol (4 μg/ml) and erythromycin (0.3 μg/ml). The resulting strain used in this study was named HR1001.1Δ*psrP*. All mutants were verified by PCR and sequencing of amplicons, using primers upstream of the insertion of each construct and from within the inserted resistance cassette.

Bacterial-mediated hemagglutination

Human blood (group 0) was collected in Vacutainers containing 3.2% sodium citrate as anticoagulant (Becton Dickenson) according to guidelines approved by the Health Science Institutional Review Board (#ORB0511008E), University at Buffalo. Red blood cells (RBCs) were washed and resuspended to a final concentration of 0.5 % (v/v) in PBS containing 2 mg/ml bovine serum albumin (Sigma-Aldrich, St. Louis, MO) (PBS-BSA). For removal of sialic acids, RBCs were treated for 1 h at 37°C with sialidase from *Clostridium perfringens* (Type X, Sigma-Aldrich) at a final concentration of 0.05 U/ml in PBS and washed three times with PBS thereafter. To demonstrate glycan-dependency of binding, periodate treatment was performed with either 2 mM or 10 mM sodium periodate in PBS (pH 6.5) to oxidize sialic acids or general glycans, respectively (Fukuda, 2001). The reaction was carried out for 1 h at 4°C in the dark with constant agitation and was stopped thereafter by the addition of 100 mM sodium borohydride for 10 min at room temperature. The volume ratio of sodium periodate to sodium borohydride was 4 parts to 1 part. After treatment, RBCs were washed three times with sodium acetate buffer (50 mM sodium acetate, 100 mM sodium chloride, pH 5.5), followed by washing with PBS twice more before resuspension. Both sialidase- and periodate-treated RBCs were resuspended in PBS-BSA to a final concentration of 0.5% (v/v) for later use in the hemagglutination assay. Hemagglutination was performed as previously described (Takahashi *et al*., 1997). Two-fold serial dilutions of 75 μl bacterial suspensions at an optical density of 1.0 (~ 10^9 streptococci per ml) in PBS-BSA were mixed with 25 μl untreated, sialidase-treated, or periodate-treated RBCs in round-bottom 96-well microtiter plates (Costar, Corning Incorporated, Corning, NY). As a control for the presence of sialic acids on RBCs, serial two-fold dilutions of

Maackia amurensis lectin (MAL II, Vector Laboratories, Burlingame, CA) starting at 0.01 mg/ml were substituted for bacteria. Hemagglutination titers were scored after overnight stationary incubation of plates at 4°C and recorded using a digital camera.

Dot blot preparations, SDS PAGE, and electroblot transfer

Dot blots for bacterial overlay were prepared by immobilizing human salivary samples, naturally occurring glycoproteins (Table S1) and neoglycoproteins (Table S2) as dots containing 1 μg of protein on nitrocellulose (0.45 μm pore size, Whatman Protran BA 85, Fisher Scientific). Salivary samples that were collected on ice as previously described (Heo *et al*., 2013) according to the protocol approved by the University at Buffalo's Health Sciences Institutional Review Board (HSIRB #ORB0511008E) included whole saliva (WS), parotid saliva (PAR) and submandibular/sublingual saliva (SMSL). Unstimulated WS samples were centrifuged at 12,000 x g for 15 min at 4°C to remove unwanted particulates. The resultant clarified saliva supernatant was transferred into a separate polypropylene microtube and stored at −80°C until further use. Samples treated with sialidase from *Clostridium perfringens* (Type X, Sigma-Aldrich) at a final concentration of 0.05 U/ml at 37°C for 30 min were also included in the dot blots where indicated. Proteins and glycoproteins of WS, PAR and SMSL secretions, both untreated and sialidase-treated, were denatured under reducing conditions and separated by 8–16% gradient Tris-glycine mini gels (Novex, Invitrogen, Carlsbad, CA) as previously described (Walz *et al*., 2009). Separated salivary glycoproteins (15 μg of total protein per lane) were visualized in gels by periodic acid-Schiff (PAS) stain (Heo *et al*., 2013) and imaged using a flat-bed scanner (ImageScanner III, GE Healthcare, Piscataway, NJ) in the transparent mode. In parallel, proteins and glycoproteins (15 μg of total protein per lane) were transferred to nitrocellulose (0.45 μm pore size, Whatman Protran BA 85, Fisher Scientific, Fair Lawn, NJ) in a semi-dry transfer unit (Amersham Biosciences, GE Healthcare, Piscataway, NJ) for 3 h at room temperature using transfer buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol) under constant current of 45 mA per gel. Transfer membranes were briefly rinsed in Tris-buffered saline (0.15 M NaCl, 20 mM Tris HCl and 0.02% NaN_3) (TBS) prior to performing immunoblotting or bacterial overlay.

Immunoblotting

Immunoblotting was performed as previously described (Heo *et al*., 2013). In brief, membranes were blocked with 2% non-fat dry milk (Carnation, Nestlé, Solon, OH) in TBS containing 0.1% Tween-20 (TBST) and subsequently incubated with 1:2,000 diluted rabbit polyclonal anti-human mucin-7 (MUC7) antiserum (AB-3, a gift from Dr. Libuse A. Bobek at the University at Buffalo). After washing with TBST, bound antibodies were detected with 1:1,000 diluted AlexaFluor 488 IgG (H+L) secondary antibodies (Molecular Probes, Invitrogen). All incubations were done for 1 h at room temperature. Signals of bound antibodies were detected using a fluorescence laser scanner (Typhoon 9400, GE Healthcare).

Bacterial overlay

Fluorochrome-labeling of bacteria and overlay method were performed as previously described (Walz *et al*., 2005). In brief, dot blots or transfers were blocked in 5% BSA and

subsequently overlaid with suspensions of fluorescein-5-isothiocyanate (FITC)-labeled bacteria for 2 h to allow for binding. Membranes were then washed at 4°C to remove unbound bacteria and dried. Unless otherwise indicated, 1 mM $CaCl_2$ and 1 mM $MgCl_2$ were included in buffers, while 5 mM EDTA was used for conditions where the overlay was performed in the absence of divalent cations. Fluorescence signals of bound bacteria were detected using a fluorescence laser scanner (Typhoon 9400) and were analyzed by densitometry using the ImageQuant software (Version 5.2, Molecular Dynamics, Sunnyvale, CA).

RESULTS

S. pneumoniae binds to salivary proteins only when unencapsulated

Binding of the streptococcal strains to saliva was first tested by overlaying dot blots of immobilized salivary secretions with suspensions of fluorochrome-labeled bacteria (Fig. 1). Wild-type strain *S. gordonii* DL1 bound strongly to whole saliva (WS) and submandibular-sublingual (SMSL) secretion, but only weakly to parotid (PAR) secretion. This binding was abrogated or, in the case of SMSL secretion, much reduced when the salivary samples were treated with sialidase prior to immobilizing them on the membrane. Together with the fact that strain DL1 bound to fetuin but not to its de-sialylated form, these findings confirm that binding of *S. gordonii* DL1 to these substrates is mediated by recognition of sialic acid. Furthermore, because the Hsa adhesin-deficient mutant of strain DL1 did not bind to the same substrates, it can be concluded that binding was mediated through the SRR adhesin Hsa, which is in agreement with previous studies (Takahashi *et al*., 1997, Deng *et al*., 2014). It has to be noted that the binding experiment was performed in the presence of EDTA to focus on the sialic acid binding activity and to abrogate other adhesin activities on strain DL1 (Takahashi *et al*., 2002b, Ruhl *et al*., 2004, Deng *et al*., 2014) that require the presence of divalent cations (Takahashi *et al*., 1997).

Wild-type strain *S. sanguinis* SK36 bound to WS and SMSL secretions similar to strain DL1, and no binding was seen to sialidase-treated samples. Binding of strain SK36 was clearly mediated by the SRR adhesin SrpA, since the adhesin-deficient mutant did not bind. This finding also agrees with previous reports (Plummer & Douglas, 2006). In contrast to DL1, strain SK36 did not bind to fetuin. This could be due to overall weaker binding of SK36 or to the fact that this strain requires a certain subtype, linkage, or sterical presentation of sialic acid for binding that is present on salivary proteins, but not on fetuin (Deng *et al*., 2014).

Neither *S. pneumoniae* TIGR4 wild-type nor the PsrP-deficient mutant strain bound to the immobilized saliva samples. However, the capsule-free mutant strain HR1001.1 showed binding. Interestingly, strain HR1001.1 also bound to fetuin. However, neither treatment of the samples by sialidase nor removal of PsrP affected binding of strain HR1001.1. Notably, in the case of fetuin and to a certain degree also for SMSL-secretion, binding by the PsrP-deficient HR1001.1 mutant strain was stronger to the sialidase-treated samples. These findings show that after removal of their capsule, pneumococci are able to bind to salivary compounds but suggest that neither the recognition of sialic acid nor the adhesin PsrP play a dominant role in binding to saliva.

Unencapsulated *S. pneumoniae* binds to salivary mucin MUC7

To find out which salivary proteins serve as counter-receptors for lectin-like bacterial adhesin-mediated binding, transfer blots of SDS-PAGE-separated salivary secretions were overlaid with fluorochrome-labeled streptococcal strains and respective SRR adhesin-deficient mutants (Fig. 2). Both *S. gordonii* DL1 and *S. sanguinis* SK36 bound predominantly to a glycosylated protein band of ~150 kDa, present in WS and SMSL secretion, which was identified by periodic acid-Schiff glycan stain (Fig. 2 A) and immunoblotting (Fig. 2 B) as the low-molecular weight salivary mucin-7 (MUC7). Binding of DL1 occurred only to the sialylated form of MUC7. When sialic acid was removed by treating the samples with sialidase prior to electrophoretic separation, binding was abolished (Fig. 2 C). The loss of sialic acids on MUC7 became also evident from the glycoprotein stain and the immunoblot (Fig. 2 A, B) in that the sialylated form of MUC7 migrated faster in the gel than the de-sialylated form due to the overall loss of negative charge of the molecule after removal of sialic acids (Kirkbride *et al*., 2001). Binding of both strains to MUC7 was also clearly SRR adhesin-dependent, as seen with loss of binding in the adhesin-deficient mutants. For DL1 and to a lesser degree for SK36, binding to a lower 130 kDa band was observed. This band represents most likely a subtype of MUC7 that can be detected by various MUC7-specific antibodies (Soares *et al*., 2012), but was apparently not recognized by the anti-MUC7 antibody used in this study.

For *S. pneumoniae*, encapsulated strain TIGR4 did not bind to any secretions and only weak binding was observed to a triplet of bands ranging between ~100 – 150 kDa in sialidase-treated SMSL secretion that could not be identified. However, the capsule-deficient mutant strain HR1001.1 bound to MUC7, but binding was neither dependent on the presence of sialic acid nor on the expression of the PsrP adhesin on the pneumococci (Fig. 2 C). This shows that the PsrP adhesin, similar to the results obtained in the dot blot experiment (Fig. 1), is not required for binding to salivary proteins and suggests that another putative adhesin might possibly be responsible for binding to MUC7.

Unencapsulated *S. pneumoniae* agglutinates RBCs in a glycan-dependent but sialic acid-independent manner

To further scrutinize whether *S. pneumoniae* can bind to sialic acids, we tested for hemagglutination ability of these bacteria using untreated and sialidase-treated human RBCs (Fig. 3 A). *S. gordonii* DL1 hemagglutinated human RBCs to a similar extent as lectin *Maackia amurensis* (MAL II), which specifically binds to α2,3-linked sialic acids. Hemagglutination caused by DL1 was sialic acid-dependent since it could be abolished by sialidase treatment of the RBCs. Hemagglutination activity was also entirely dependent on the presence of the Hsa adhesin on strain DL1. *S. sanguinis* SK36 displayed much weaker hemagglutination activity than *S. gordonii* DL1, but its activity was clearly sialic acid- and SrpA adhesin-dependent.

For *S. pneumoniae*, hemagglutination occurred only with the capsule-free mutant strain HR1001.1, which agrees with its ability to bind salivary proteins (Fig. 2). Sialidase treatment of RBCs did not reduce, but rather improved hemagglutination by strain HR1001.1. This indicates that sialic acid may not be a receptor on RBCs but that

subterminal glycans, revealed after removal of terminal sialic acids, may serve as receptor motifs for pneumococcal binding. Remarkably, agglutinating activity was abolished in the PsrP adhesin-deficient mutant of strain HR1001.1 which suggests that PsrP may exhibit lectin-like binding. However, the PsrP adhesin-deficient mutant still agglutinated sialidase-treated RBCs. Taken together, these observations support the assumption that *S. pneumoniae* may express two glycan-binding adhesins.

To determine glycan dependency of *S. pneumoniae* binding to RBCs, we performed periodate treatment of both untreated and sialidase-treated RBCs prior to hemagglutination with 2 mM or 10 mM sodium periodate to oxidize sialic acids or general glycans on the RBCs, respectively (Fig. 3 B). Treatment with 2 mM sodium periodate (mild periodate treatment) oxidizes vicinal hydroxyl groups of terminal sialic acid into permanent aldehydes, thereby “destroying” terminal sialic acid. Dissimilar to the treatment with sialidase, where terminal-end sialic acids are completely cleaved off, mild periodate treatment allows for the terminal oxidized sialic acid moiety to be retained on the glycan chain. Treatment with 10 mM sodium periodate (strong periodate treatment) can oxidize all vicinal hydroxyl groups of monosaccharide moieties within the glycan chain, thereby “destroying” the entire glycan chain without cleaving it from the surface of RBCs (Fukuda, 2001). As expected, sialic acid-dependent hemagglutination by *S. gordonii* DL1 was markedly reduced by mild periodate treatment of RBCs (Fig. 3 B) confirming that the binding of *S. gordonii* DL1 to RBCs is mediated by the recognition of sialic acid. For *S. pneumoniae* HR1001.1, only a partial reduction in hemagglutination titer was seen after periodate treatment of RBCs. Interestingly, when the PsrP-deficient mutant of HR1001.1 was tested, only strong periodate treatment of RBCs completely abolished hemagglutination activity. Taken together, these results suggest that *S. pneumoniae* strain HR1001.1 possesses an additional adhesin besides PsrP which binds to glycans other than sialic acids, and that its glycan-mediated binding activity might be partially masked by the presence of PsrP.

Unencapsulated *S. pneumoniae* binds to glycans via PsrP and another lectin-like adhesin

To further assess the specificity of glycan-mediated binding of capsule-free *S. pneumoniae* strain HR1001.1, custom-spotted glycan array dot blots containing immobilized salivary proteins, naturally occurring glycoproteins, and related neoglycoproteins were overlaid with fluorochrome-labeled bacteria (Fig. 4). Wild-type *S. gordonii* strain DL1 bound strongly to SMSL (A3) and considerably to both WS (A1) and PAR (A5) secretions, but not to their corresponding de-sialylated salivary samples (A2, A4 and A6). Strain DL1 also bound to a number of naturally occurring sialylated glycoproteins, which include salivary mucins MUC5B (B1) and MUC7 (B3), red blood cell membrane-associated glycoprotein glycophorin A (C1) and blood glycoprotein fetuin (C3). Binding activities to these substrates were abrogated when these samples were de-sialylated (B2, B4, C2 and C4). Strain DL1 bound explicitly to neoglycoconjugates that presented sialylated glycans (D1, E1, E5, and F1), but not to the desialylated glycans (D2, D3, E2, E6, and F2). Taken together, these findings confirmed that the binding of *S. gordonii* DL1 to these immobilized substrates was solely mediated by the recognition of sialic acid. Furthermore, all mentioned binding activities were no longer observed when the adhesin Hsa-deficient mutant strain of DL1 was used under the same conditions, signifying that binding activity was mediated through the

SRR adhesin Hsa. These observations are in agreement with previous results in the study and previous studies (Deng *et al*., 2014, Takahashi *et al*., 1997, Takamatsu *et al*., 2005).

Capsule-free *S. pneumoniae* HR1001.1 displayed a much broader binding profile. Strain HR1001.1 bound to all immobilized saliva samples (A1, A3 and A5) with no influence of sialidase treatment (A2, A4 and A6). In the case of MUC7 (B3), glycophorin A (C1) and fetuin (C3), binding was even stronger to the sialidase-treated samples (B4, C2 and C4), supporting our previous observations (see Fig. 1). Binding to glycophorin and fetuin was markedly reduced or abrogated when these glycoproteins were treated with 10 mM periodate prior to immobilization on the blot (data not shown), further consolidating the glycan-mediated mechanism of binding. Strain HR1001.1 bound to neoglycoproteins carrying Galβ/α1-3GalNAc-(D2, D3 and D4), lacto-*N*-tetraose (E4), *N*-acetyllactosamine (E6), lacto-*N*-neotetraose (F4), lactose (F5), globo-*N*-tetrasose (G1), gangliotetraose (G2), and to a lesser extent to GalNAcβ1-3/4-Galα-(D5 and D6). Binding to these glycans was not lost when the PsrP-deficient mutant strain of HR1001.1 was used. The differences in pneumococcal binding to glycoconjugates on the dot blot array were quantified by densitometry (Figure S1). In summary, these results provide strong evidence for the existence of an additional lectin-like adhesin on *S. pneumoniae* other than PsrP that is involved in the recognition of these Gal- or GalNAc containing glycans, and perhaps salivary secretions as well.

Notably however, after deletion of PsrP, binding to the Lewis blood group-related glycans (E1 – E3 and F1 – F3) was lost. Furthermore, binding to GalNAcβ1-3/4-Galα- (D5 and D6), lacto-*N*-tetraose (E4), lacto-*N*-neotetraose (F4), globo-*N*-tetraose (G1), gangliotetraose (G2), *N*-acetyllactosamine (E6) and lactose (F5), as well as all monosaccharide-containing neoglycoconjugates (G3 – G6 and H1 – H5) was reduced when PsrP was not expressed. Taken together, these results suggest that PsrP might exhibit an as of yet undetermined, but sialic acid independent, glycan binding activity. It is perplexing that binding of strain HR1001.1 was equally strong to all the monosaccharide conjugates (G3 – G6 and H1 – H5), and was attenuated after removal of PsrP. One possible explanation could be that all the monosaccharides used for this array were linked to the carrier protein by the chemical spacer arm *p*-aminophenyl (PAP), whereas all other neoglycoproteins on this array were linked by different spacers (Table S2). Thus, the binding results to the monosaccharide units have to interpreted with reservation, because of possible sterical interference of the chemical linker. Another explanation could be that binding by PsrP is more complex and may require additional unknown structural motifs. Perhaps the true high-affinity counter-receptor for PsrP was not included among the neoglycoconjugates on the present array.

DISCUSSION

Here we showed that, when its capsule is absent, *S. pneumoniae* binds to low molecular weight salivary mucin MUC7 through presumably a glycan-mediated mechanism. Different to related oral Mitis group streptococci, binding of MUC7 by the pneumococci was not dependent on the expression of the SRR adhesin PsrP and recognition of sialic acid. The adhesin PsrP, however, was involved in agglutination of RBCs. Using a dot blot glycan array, we obtained evidence for glycan binding by PsrP and also showed that an additional

glycan-binding adhesin exists on *S. pneumoniae* that becomes exposed only after the removal of the polysaccharide capsule and requires the removal of terminal sialic acids for its counter-receptors to become revealed.

We observed that *S. pneumoniae* TIGR4 bound to salivary MUC7 and hemagglutinated RBCs only when its capsule was absent. This suggests that the capsule hinders adhesive interactions with host receptors. Others have also shown that the presence of a capsular polysaccharide layer prevents pneumococcal biofilm formation (Muñoz-Elías *et al*., 2008) and hinders adherence to epithelial cells (Hammerschmidt *et al*., 2005). In fact, it was proposed that *S. pneumoniae* down-regulate their capsule expression in the presence of host mucosal surfaces thereby exposing previously buried surface adhesins (Hammerschmidt *et al*., 2005, Sanchez *et al*., 2011, Marks *et al*., 2012). *S. pneumoniae* are also known to switch from a highly encapsulated phenotype (opaque colonies) to a reduced capsule-expressing phenotype (transparent colonies) as a response to environmental cues (Kim & Weiser, 1998). Although exposure of the keratin 10 binding region of PsrP was shown not to be hindered by the capsule, and adherence to lung epithelial cells was not inhibited by the presence of a capsule (Shivshankar *et al*., 2009, Rose *et al*., 2008), it still could be that the putative domain responsible for binding to MUC7 and RBCs remains buried in the capsular polysaccharide layer and becomes exposed only when the capsule is removed.

Deletion of the PsrP adhesin in the unencapsulated mutant strain abolished hemagglutination of untreated RBCs. This opens up the possibility that PsrP possesses a lectin activity recognizing a glycan counter-receptor on the RBCs. There are earlier reports on sialic acid recognition by *S. pneumoniae* (Barthelson *et al*., 1998, Idänpään-Heikkila *et al*., 1997), but in our study neither hemagglutination nor binding to MUC7, glycophorin A and fetuin by *S. pneumoniae* were reduced after sialidase treatment. Even though this clearly speaks against recognition of sialic acids by PsrP, the possibility that this adhesin might bind to an unusual sialidase-resistant subtype of sialic acid (Powell & Varki, 2001) still remains. A more likely explanation, however, is that PsrP binds to glycans other than sialic acids. By using a custom-spotted array of natural glycoproteins and neoglycoproteins, evidence was obtained that PsrP could possibly recognize Lewis blood group-related glycans containing Galβ1-3/4GlcNAc. Binding of *S. pneumoniae* to Galβ1-3/4GlcNAc-containing glycan motifs, has been reported in earlier studies (Barthelson *et al*., 1998, Idänpään-Heikkila *et al*., 1997). Further binding studies involving larger glycan arrays and sugar inhibition experiments will have to narrow down on the preferred glycan motif recognized by PsrP.

When PsrP was deleted, unencapsulated *S. pneumoniae* still retained its binding to sialidase-treated RBCs, MUC7, glycophorin A, fetuin, as well as GalNAcβ1-3/4-Galα-, lacto-*N*-tetraose, *N*-acetyllactosamine, lacto-*N*-neotetraose, lactose, globo-*N*-tetraose, and gangliotetraose. This suggests that *S. pneumoniae* must possess at least one additional lectin-like adhesin besides PsrP. There are earlier reports by different groups demonstrating evidence for lectin-mediated binding by *S. pneumoniae* to GlcNAc-, GalNAc-, or Gal-containing glycans (Andersson *et al*., 1983, Krivan *et al*., 1988, Cundell & Tuomanen, 1994, Barthelson *et al*., 1998). Presently, lectin-mediated binding by *S. pneumoniae* is still poorly understood (Voß *et al*., 2012), although some glycan-binding proteins on *S. pneumoniae* have been described (Limoli *et al*., 2011, King *et al*., 2006, Dalia *et al*., 2010, King, 2010).

From our current hemagglutination data using periodate-treated RBCs as well as the binding results with our custom-spotted glycan array, it can now be concluded that *S. pneumoniae* TIGR4 possesses an additional glycan-binding adhesin, other than PsrP with a specificity for Gal/GalNAc-containing glycan motifs. It is likely that related glycan motifs are present among the highly diverse oligosaccharides found on salivary mucin MUC7 (Karlsson & Thomsson, 2009, Prakobphol *et al*., 1982) and may explain *S. pneumoniae* binding to this molecule.

At this stage, we can only speculate what the binding of unencapsulated pneumococci to salivary MUC7 could mean in terms of physiology or pathology. On the one hand, binding to this mucin could mediate adhesion of pneumococci to saliva-coated surfaces in the oral cavity. However, *S. pneumoniae* is rarely found as an early colonizer in the oral cavity, much in contrast to other members of the Mitis group of streptococci (Whitmore & Lamont, 2011, Li *et al*., 2004). On the other hand, binding to MUC7 could agglutinate pneumococci and, thus, constitute a mechanism of clearance of these bacteria from the mouth. Notably, MUC7 is also produced by the mucous glands lining the nasopharynx and upper respiratory tract (Sharma *et al*., 1998), the natural habitat of the pneumococcus (Kadioglu *et al*., 2010, Kilian *et al*., 2014, Marks *et al*., 2012). As such, MUC7 could either mediate pneumococcal binding or contribute to the clearance of these bacteria from respiratory mucous surfaces (King *et al*., 2004). Alternative explanations are that the pneumococci bind MUC7 for nutritional purposes (Yesilkaya *et al*., 2008) or as a stealth strategy to evade mucosal host defense through decoration with host-derived molecules (Heo *et al*., 2013). As all these hypothetical scenarios cause quite opposing biological implications, further work will be required to better understand the role of MUC7 and glycan-mediated binding in pneumococcal colonization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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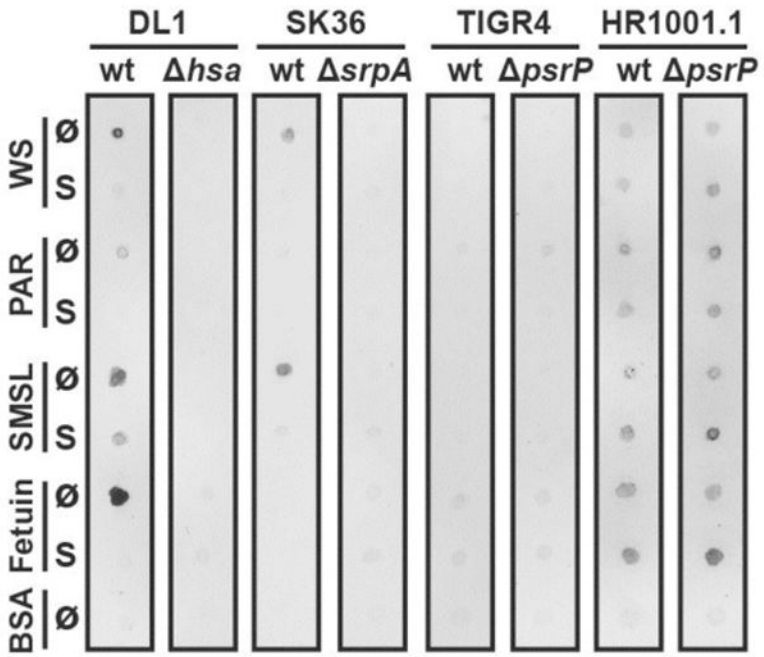

Fig. 1.

Streptococcal adhesion to immobilized whole saliva, parotid saliva and submandibular/sublingual saliva. Suspensions of fluorochrome-labeled *S. gordonii* DL1, *S. sanguinis* SK36, *S. pneumoniae* wild-type strain TIGR4, capsule-free mutant strain HR1001.1, and the respective isogenic SRR adhesin-deficient mutants were overlaid on nitrocellulose membranes carrying immobilized untreated (Ø) or sialidase-treated (S) dots (1 μg of total protein per dot) of whole saliva (WS), parotid saliva (PAR), and submandibular/sublingual saliva (SMSL). Fetuin and bovine serum albumin (BSA) were included as controls. Fluorescent signals of bound bacteria were detected by use of a fluorescence scanner.

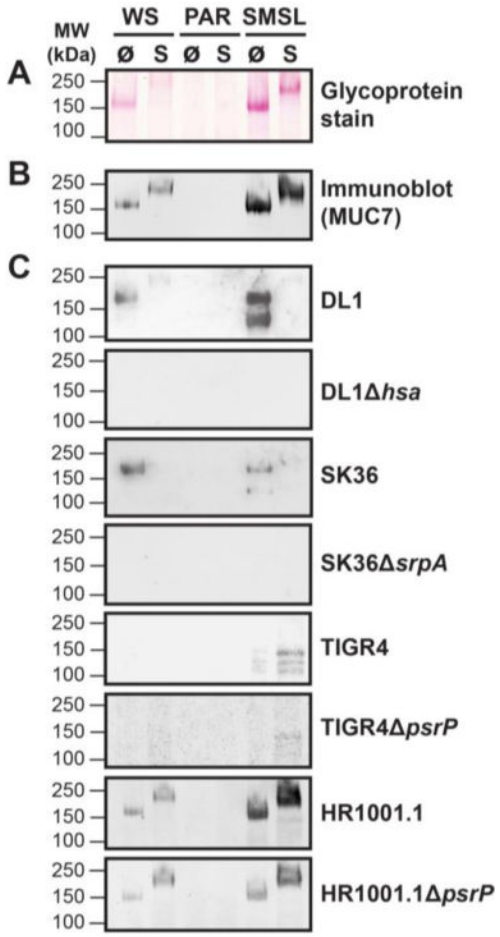

Fig. 2.

Streptococcal binding to salivary mucin MUC7. Untreated (Ø) or sialidase-treated (S) whole saliva (WS) as well as parotid (PAR), and submandibular/sublingual glandular secretions (SMSL) were separated by SDS-PAGE under reducing conditions, transferred to nitrocellulose and overlaid with fluorochrome-labeled bacteria. **A**, Periodic acid-Schiff stain to reveal the glycosylated mucin bands. **B**, Immunoblot with anti-MUC7 antibody. **C**, Bacterial overlay with fluorochrome-labeled *S. gordonii* DL1, *S. sanguinis* SK36, encapsulated *S. pneumoniae* wild-type strain TIGR4, capsule-free mutant strain HR1001.1, and respective isogenic SRR adhesin-deficient mutants. Fluorescent signals of bound antibodies or bound bacteria were detected using a fluorescence scanner.

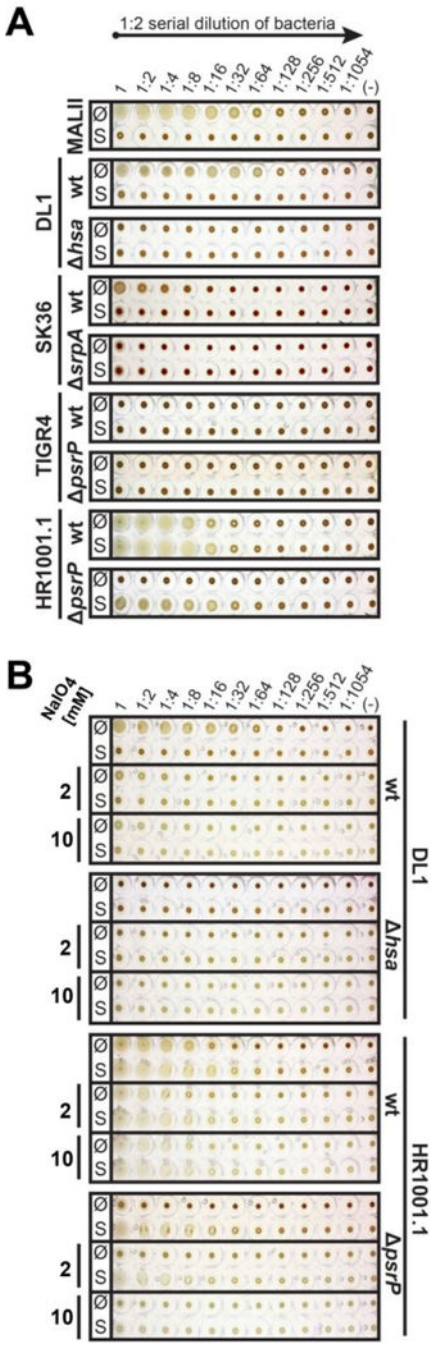

Fig. 3.

Streptococcal-mediated hemagglutination of human red blood cells. Two-fold serial dilutions of *S. gordonii* DL1, *S. sanguinis* SK36, *S. pneumoniae* wild-type strain TIGR4, capsule-free mutant strain HR1001.1, and respective isogenic SRR adhesin-deficient mutants were mixed and incubated with human red blood cells. The sialic acid specific lectin from *Maackia amurensis* (MAL II) was used as a control for the presence or loss of sialic acid residues. **A**, Hemagglutination done with untreated (Ø) or sialidase-treated (S)

RBCs. **B**, Treatment of RBCs with 2 mM or 10 mM sodium periodate to oxidize sialic acids or general glycans, respectively, prior to hemagglutination.

Fig. 4.

Streptococcal adhesion to immobilized WS and ductal glandular secretions (SMSL, PAR), naturally occurring glycoproteins and related neoglycoproteins. Suspensions of fluorochrome-labeled *S. gordonii* DL1, capsule-free *S. pneumoniae* TIGR4 mutant strain HR1001.1, and the respective isogenic SRR adhesin-deficient mutants were overlaid on dot blots of immobilized naturally occurring glycoproteins (Table S1) and related neoglycoproteins (Table S2). Fluorescent signals of bound bacteria were detected by use of a fluorescence scanner.

Table 1

Bacterial strains used in this study.

Strain	Relevant characteristics	References
DL1 (Challis)	*Streptococcus gordonii* blood isolate	(Kilian *et al*., 1989)
UB1545	DL1, *hsa::aphA3*; Kan^r	(Jakubovics *et al*., 2005)
SK36	*S. sanguinis* human oral plaque isolate	(Plummer *et al*., 2005)
VT1614	SK36, *srpA::aphA3*; Kan^r	(Plummer *et al*., 2005)
TIGR4	*S. pneumoniae* serotype 4 clinical isolate	(Tettelin *et al*., 2001)
STM199	Signature-tagged mutagenesis of *psrP* gene done in TIGR4; Cm^r	(Hava & Camilli, 2002)
HR1001.1	Capsule-free TIGR4	This study
HR1001.1Δ*psrP*	Capsule-free and Δ*psrP*	This study

[a] Kan^r, Kanamycin resistant; Cm^r, Chloramphenicol resistant.