molecular oral microbiology

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Pneumococcal neuraminidase A: an essential upper airway colonization factor for *Streptococcus pneumoniae*

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SUMMARY

Streptococcus pneumoniae **colonizes the upper respiratory tract from where the organisms may disseminate systemically to cause life threatening infections. The mechanisms by which pneumococci colonize epithelia are not understood, but neuraminidase A (NanA) has a major role in promoting growth and survival in the upper respiratory tract. In this article we show that mutants of *S. pneumoniae* D39 deficient in NanA or neuraminidase B (NanB) are abrogated in adherence to three epithelial cell lines, and to primary nasopharyngeal cells. Adherence levels were partly restored by *nanA* complementation *in trans.* Enzymic activity of NanA was shown to be necessary for pneumococcal adherence to epithelial cells, and adherence of the *nanA* mutant was restored to wild-type level by pre-incubation of epithelial cells with *Lactococcus lactis* cells expressing NanA. Pneumococcal *nanA* or *nanB* mutants were deficient in biofilm formation, while expression of NanA on *L. lactis* or *Streptococcus gordonii* promoted biofilm formation by these heterologous host organisms. The results suggest that NanA is an enzymic factor mediating adherence to epithelial cells by decrypting receptors for adhesion, and functions at least in part as an adhesin in biofilm formation. Neuraminidase A thus appears to play multiple temporal roles in pneumococcal infection, from adherence to host tissues, colonization, and community development, to systemic spread and crossing of the blood-brain barrier.**

INTRODUCTION

Streptococcus pneumoniae is a major human pathogen associated with a wide range of disease conditions including otitis media, sinusitis, pneumonia and meningitis (Mitchell, 2003). Pneumococci frequently colonize the nasopharynx (Watt *et al.*, 2004; King, 2010), and from there they may disseminate to the lungs, or haematogenously, and cause disease. The pneumococcus produces a number of well-characterized virulence factors important in pathogenesis of infections (Bergmann & Hammerschmidt, 2006; Löfling *et al.*, 2011). These include the capsule (Cps), pore-forming toxin pneumolysin (Ply), surface proteins PsaA, PspA, PspC, PhtD, PavA, and PsrP (Paterson & Orihuela, 2010), hyaluronidase, and neuraminidases (sialidases) (Mitchell, 2003). Colonization is intimately related to the mechanisms of pathogenesis of disease, but rela-

tively less is understood about the processes of initial establishment and subsequent microbial community development. In particular, the mechanisms by which pneumococci adhere to epithelial cells are relatively uncharted, and are suggested to include recognition of surface bound matrix proteins, such as fibronectin (van der Flier *et al.*, 1995 Holmes *et al.*, 2001), and glycoconjugate receptors (King, 2010; Limoli *et al.*, 2011).

Streptococcus pneumoniae produces at least three sialidases (Xu *et al.*, 2011) with NanA and NanB proteins being the most well-characterized, and NanC present in only about 50% of strains (Pettigrew *et al.*, 2006). Both NanA and NanB are secreted proteins (Berry *et al.*, 1999) but only NanA carries a carboxy-terminal anchorage motif to secure it to the cell wall (Camara *et al.*, 1994). The two enzymes have different pH optima (NanA pH 6.5, NanB pH 4.5), and while NanA catalyzes the removal of Neu5Ac from a range of substrates, NanB is an intramolecular *trans*-sialidase selective for α2,3-sialosides (Xu *et al.*, 2011). The sialidases each have a carbohydrate-binding module in addition to the catalytic domain that cleaves sialoglycosides to release N-acetylneuraminic acid (Neu5Ac or NANA) (Uchiyama *et al.*, 2009).

Neuraminidase A is most highly expressed at the transcriptional level and is present in all strains (King *et al.*, 2005). Production of NanA is up-regulated by interaction of pneumococci with host cells (LeMessurier *et al.*, 2006) and by free sialic acid (Yesilkawa *et al.*, 2008). In a number of studies, NanA has been shown to contribute to nasopharyngeal colonization, spread of pneumococci to the lungs (Orihuela *et al.*, 2004), sepsis (Manco *et al.*, 2006), development of otitis media (Tong *et al.*, 2000), and passage across the blood-brain barrier (Uchiyama *et al.*, 2009). By contrast, other studies have shown no effect of *nanA* knockout on the ability of encapsulated strain D39 to colonize mice (King *et al.*, 2006), and no attenuation in an infant rat model of colonization (King *et al.*, 2004). In addition, D39Δ*nanA* mutants were unaffected in adherence levels to two epithelial cell lines (King *et al.*, 2006). However, it was also demonstrated that a Δ*nanA* mutant of strain R6, which is an avirulent non-encapsulated derivative of strain D39, was impaired in adherence to epithelial cells compared with parental strain R6 (King *et al.*, 2006). There is, therefore, conflicting evidence regarding potential roles for NanA in pneumococcal adherence to mucosa and in pneumococcal colonization.

Manipulation of complex carbohydrates by pneumococci has at least two functions. Firstly, deglycosylation of host tissue receptors reveals different glycan structures for adhesins on the pneumococcal cell surface to interact with. Secondly, sequential deglycosylation of complex carbohydrates, e.g. mucins, releases sugars as carbon and energy sources for growth. In these respects, NanA, and surface protein BagA (encoding a β-galactosidase), have been shown to deglycosylate structures on host cell surfaces to reveal cryptic receptors for adherence (King *et al.*, 2006; Limoli *et al.*, 2011). In confirmation, *S. pneumoniae* cells show increased binding to sialidase-treated chinchilla trachea cells in an otitis media model (Tong *et al.*, 2002). It is suggested that sialic acid itself has a direct role in promoting pneumococcal biofilm formation (Trappetti *et al.*, 2009), nasopharyngeal colonization, and translocation to the lungs (Trappetti *et al.*, 2009).

Several studies have shown specifically that NanA-deficient pneumococci are abrogated in colonizing the nasopharynx, trachea, and lungs of rodents following intranasal challenge (Tong *et al.*, 2000; Orihuela *et al.*, 2004; Manco *et al.*, 2006). In addition, NanB deficient pneumococci are impaired in colonization and are unable to cause sepsis following intranasal infection (Manco *et al.*, 2006). In this article we have further developed the hypothesis that NanA and NanB might be essential for pneumococcal colonization of nasopharyngeal and respiratory tract epithelia, processes defined *in vitro* by adherence and community development. In these studies we have utilized encapsulated strain D39, in contrast to many previous investigations that have employed non-encapsulated (non-pathogenic) derivatives, such as strain R6. We demonstrate that NanA activity is essential for strain D39 adherence to four epithelial cell types and that NanA behaves as a biofilm determinant. It is also shown that adherence and colonization deficiencies of a *nanB* mutant may result, at least in part, from decreased expression of NanA.

METHODS

Bacterial strains and growth conditions

Streptococci were grown in Todd-Hewitt broth containing 0.5% yeast extract, in sealed tubes at 37°C without shaking. *Lactococcus lactis* MG1363 was

grown in M17 medium containing 0.5% glucose. Antibiotic concentrations were 5 μg ml^{-1} erythromycin and 10 μg ml^{-1} spectinomycin. *Streptococcus pneumoniae* D39 and *nanA* (Berry & Payton, 2000) and *nanB* (Berry *et al.*, 1999) mutants were kindly provided by J. Paton. Strains 25925 (serotype 23F) and 45567 (serotype 14) were kindly provided by M. Smith (HPA, Taunton, UK).

Cloning and expression of *nanA* gene

The *nanA* gene was PCR amplified with high-fidelity polymerase (Phusion, New England Biolabs) from strain D39 DNA using primers NanAF2 GGA**GGATCC**AAATGTCTTATTTCAGAAATCGGGATATA and NanAR2 GAT**CTGCAG**TTA TTCTTGTC TCTTTTTCCCTAGCGT (BamH1 and PstI sites in bold). The fragment was ligated into plasmid pKS80 (5.24 kb; Hartford *et al.*, 2001), or pKS80 *aad9*, digested with BamH1 and PstI, such that *nanA* expression was under transcriptional and translational control by endogenous vector sequences. *Streptococcus gordonii* was then transformed as previously described (Haisman & Jenkinson, 1991) with selection for erythromycin resistance (Em^R, 5 μg ml^{-1}). Plasmid pKS80 *aad9* was generated as follows: the *aad9* gene encoding spectinomycin resistance (Sm^R) was cut from pFW5 (Podbielski *et al.*, 1996) with a combination of SalI/XmaI and cloned into pUC19 similarly digested with SalI/XmaI. The *aad9* gene was then removed from pUC19 by digestion with PstI, ligated into the unique PstI site of pKS80 *nanA*$^+$, and transformed into *S. gordonii* DL1 with selection for Em^R and Sm^R. Plasmids were extracted from *S. gordonii* and transformed into *L. lactis* MG1363 by electroporation as previously described (Jakubovics *et al.*, 2005b).

Neuraminidase activity

To determine neuraminidase activity bacterial cells were grown to late exponential phase, harvested at 5000 ***g***, washed twice in PBS and frozen at −70 °C for 16 h. Cells were then suspended in 40 mM tris-HCl (pH 7.4), disrupted by shaking with glass beads and the supernatant was removed and stored at −70 °C for 24 h. Portions of cell-free supernatants were assayed for neuraminidase activity with 2-*O*-(*o*-nitrophenyl)-a-D-*N*-acetylneuraminic acid (*o*NP-NANA) (Manco *et al.*, 2006). Briefly, 25 μl aliquots of cell supernatants were added in triplicate to the wells of a microtitre plate and an equal volume of 0.3 mM *o*NP-NANA in dilution buffer (100 mM citric-acid phosphate, 25 mg ml^{-1} bovine serum albumin, 10% sodium azide; pH 6.6) was added. Samples were incubated at 37 ° for 2 h when the reaction was stopped by addition of 100 μl ice-cold 0.5 M Na_2CO_3. Once the solution had reached room temperature the absorbance at 405 nm was read. Standard curves were constructed with clostridial neuraminidase (Sigma, Poole, UK) and known concentrations of *p*-nitrophenol.

Cell culture

A549 cells (type II pneumocytes) and HEp-2 cells (derived from human larynx carcinoma) were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal calf serum, 2 mM L-glutamine and 0.5 μg ml^{-1} hydrocortisone. Detroit 562 cells (human pharyngeal carcinoma) were maintained in Eagle's minimum essential medium (EMEM) with 10% FCS, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. Cultured cells were maintained in humidified air (5% CO_2 at 37 °C) and were split twice weekly or as required by trypsinization. Primary cells (nasal brushings from a healthy child) were grown in bronchial epithelial cell basal medium (BEBM) with bullet-kit supplements (bovine pituitary extract, 2 ml; hydrocortisone, 0.5 ml; hEGF, 0.5 ml; epinephrine, 0.5 ml; transferrin, 0.5 ml; insulin, 0.5 ml; retinoic acid, 0.5 ml; triiodothyronine, 0.5 ml; GA-1000, 0.5 ml). Cells were grown to 80% confluence in 75 cm^2 culture flasks (Corning) and collected every 3–4 days (7 days for Detroit 562 cells) by washing with Dulbecco's phosphate-buffered saline (DPBS) and treating with trypsin-EDTA. Detached cells were centrifuged for 10 min (900 rpm), suspended in 5 ml appropriate growth medium and then seeded into fresh flasks using a volume equivalent to 0.5×10^6 cells ml^{-1}. Flasks were maintained in a humidified atmosphere (5% CO_2 at 37 °C). All solutions were from Cambrex unless otherwise stated.

Bacterial adherence to epithelial cells

Cultured cells were seeded into 24-well plates at a density of 1×10^4 cells per well in DMEM as above

and grown to 80% confluence (48–72 h) either directly on the plastic wells, or on IMS treated glass cover slips. To encourage attachment, these cover slips had been washed in industrial methylated spirit prior to use, and in the case of primary cells, incubated for 1 h with 0.5 ml 0.3% bovine dermal collagen (VWR International). Cover slips were transferred to a fresh well plate that had been blocked with 1% BSA overnight and then rinsed with PBS before fresh DMEM (or BEBM for primary cells) was added. To assay bacterial adherence, early stationary phase cells of *S. pneumoniae* were collected by centrifugation (9000 × ***g***, 10 min), washed once with DMEM, and then suspended in DMEM (OD_{600} = 0.6 ± 0.02). Wells were then inoculated with 0.2 ml pneumococcal cell suspension (1×10^8 bacterial cells). Plates were routinely incubated for 45 min at 37 °C in 5% CO_2. Numbers of cells adhered were determined by direct microscopic count, following Gram-stain, with an Olympus CX41 light microscope at 400× magnification. Fields of cells were selected randomly (on condition of >70% confluence and homogenous staining), and total numbers of pneumococci adhered to 500 human cells were counted. Cover slips were examined in triplicate for each treatment and adherence was expressed as numbers of pneumococci bound per 10 human cells.

In experiments utilizing the plastic wells directly, wells were rinsed with PBS and then inoculated with 1×10^8 bacterial cells (200 μl suspension OD_{600} = 0.6) in serum-free DMEM. Cells were incubated for up to 4 h at 37 °C in humidified 5% CO_2. The monolayers were washed with PBS, and the numbers of bacteria associated with the epithelial cells were determined as colony forming units (CFU) from dilution counts on Todd Hewitt Agar.

In some experiments, cultured cells were incubated at 37 °C with purified clostridial sialidase (Sigma), or with *L. lactis* cells, washed five times with DMEM, and then assayed for pneumococcal cell adherence by methods described above.

Biofilm formation

Biofilms were produced on saliva-pellicle coated glass cover slips following 18 h incubation at 37 °C. Biofilms were stained with FITC for microscopic imaging, or biomass quantified by crystal violet assay as previously described (Maddocks *et al.*, 2011). Pneumococcal biofilms were grown in THY medium, while *S. gordonii* and *L. lactis* biofilms were produced by growth in modified C medium (Maddocks *et al.*, 2011).

Statistics

Results were analysed using GraphPad Prism software version 4 and are shown as mean ± SD. Two tailed Student's *t*-test analyses were performed on data sets and differences were considered significant when $P < 0.05$.

RESULTS

Time course of pneumococcal adherence to epithelial cells

In initial studies we established conditions for determining adherence of *S. pneumoniae* to three epithelial cell lines and to primary human nasopharyngeal cells. One of the difficulties in these assays was that the production of pneumolysin affected epithelial cell integrity, a problem that we addressed previously by utilizing a pneumolysin-deficient mutant (Pracht *et al.*, 2005). However, to address the contextual role of NanA we did not adopt this route, rather we designed assays such that the lytic effects of pneumolysin might be minimized. We measured the time course of adherence of encapsulated *S. pneumoniae* D39 cells, and it was clear that lysis of epithelial cells started to become apparent around 60 min after infection. In these studies the assay cultures were stained by Gram's method to reveal dark purple pneumococci on a pink background of epithelial cells, with the nuclei staining darker pink. The kinetics of interaction of *S. pneumoniae* D39 with A549 epithelial cells is shown in Fig. 1. There was an approximately linear increase in the numbers of bacterial cells attaching per epithelial cell that began to level off between 45 and 60 min. The numbers of pneumococci then began to increase sharply concomitant with decrease in epithelial cell integrity (Fig. 1B). By 2 h there was marked increase in pneumococcal cell numbers (Fig. 1A) and by 4 h there was evidence for extensive pneumocccal cell multiplication promoted by lytic products from the epithelial cells (Fig. 1B, panels e and f). Similar results were obtained with a Hep-2 cell

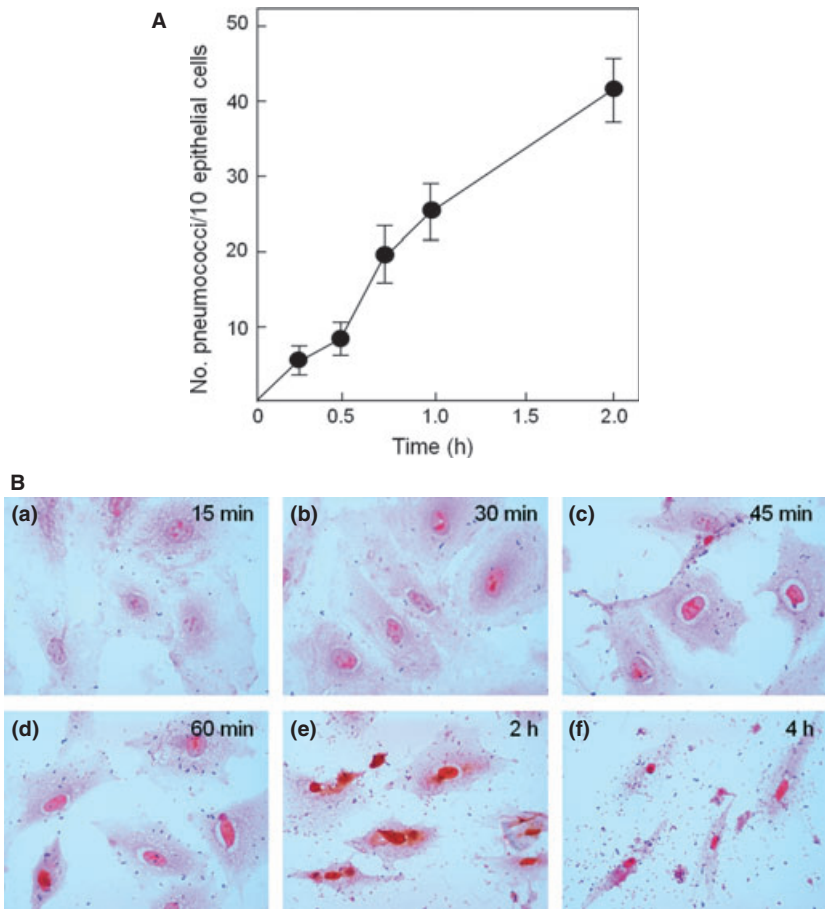
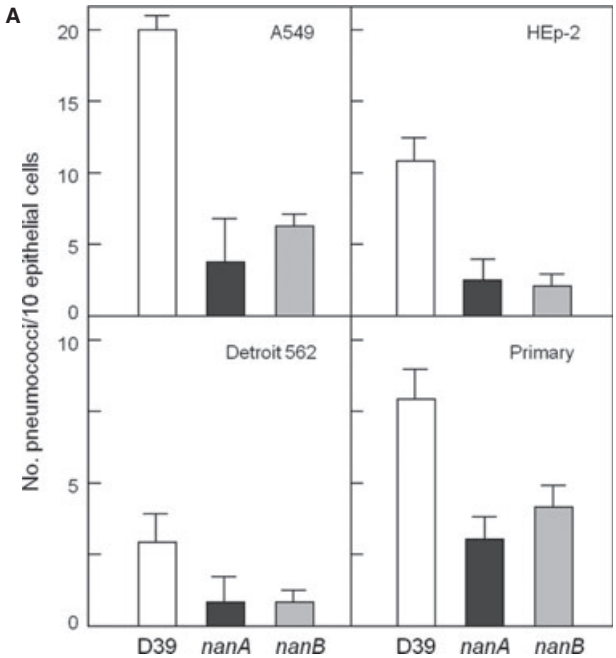


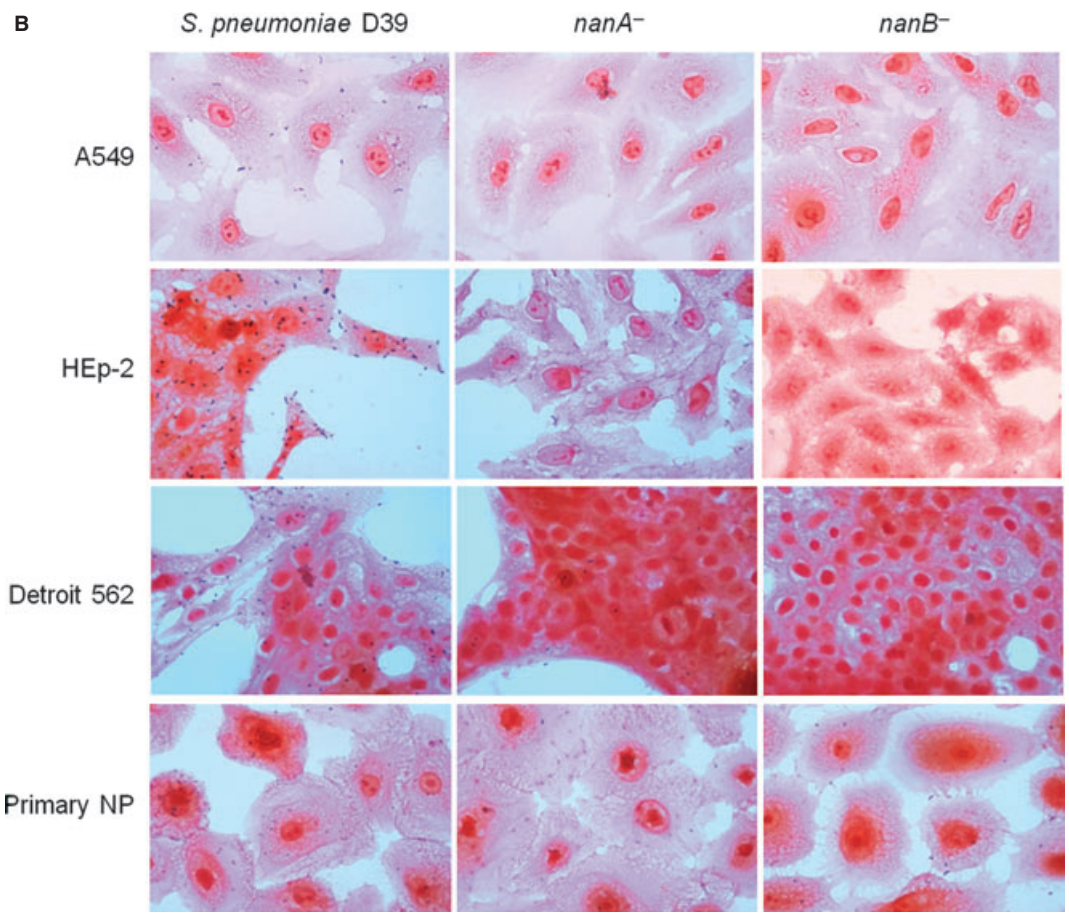
Figure 1 Time course of adherence of *Streptococcus pneumoniae* D39 to A549 cells. Panel A, numbers of *S. pneumoniae* cells attached to A549 epithelial cells (pneumocytes) were determined by microscopic count as described in Methods. Data points are means ± SD of triplicates from three experiments. Panel B, microscopic analysis of A549 cells infected with *S. pneumoniae* D39 (Brown-Brenn stained). Numbers of bacteria (purple) adhering can be seen to increase over the first 45–60 min (panels a-d). By 2 h (panel e) cell lysis was apparent, and at 4 h (panel f) the A549 cell monolayer was almost completely destroyed.

line, and accordingly, adherence assays were performed for 45 min.

We then determined the numbers of pneumococci attached to monolayers of different epithelial cell types at approximately 10^5 epithelial cells per well. Microscopic counts of numbers of bacteria adhered to 100 epithelial cells over numerous fields of vision and triplicate wells were determined for four epithelial cell types: A459 human lung pneumocytes; Hep-2 human laryngeal carcinoma; Detroit 562 human pharyngeal carcinoma; and primary culture of nasopharyngeal cells. Actual numbers of pneumococci wild-type D39 attached varied considerably for each cell type, with A549 cells supporting the highest levels of adherence, and Detroit 562 cells showing the lowest (Fig. 2A). There were no obvious differences

Figure 2 Adherence of *Streptococcus pneumoniae* D39 wild-type and isogenic mutants to four types of cultured epithelial cells as indicated. Panel A, numbers of adherent pneumococci were determined by microscopic count as described in Methods. Values were obtained from three independent experiments and error bars are ±SD. $^*P \leq 0.05$ compared to D39. Panel B, microscopic images of adherence of *S. pneumoniae* D39 wild-type, UB1816 *nanA* or UB1817 *nanB* mutants to cultured epithelial cell types as indicated on the left sides of the panels. Adherence of pneumococci (purple) to the cultured cells (following Brown-Brenn staining) can be clearly visualized (arrows) for D39 wild-type much less so for *nanA* or *nanB* mutants (50–80% reduced levels of adherence).





in the patterns of pneumococcal cell adhesion to the epithelial cells, but there was noticeable adherence of bacteria to the regions of cytoplasmic extension for A549 and Hep-2 cells (Fig. 2B). Moreover, Detroit 562 cells were more rounded with less uniform coverage.

Neuraminidase activity is associated with adherence

To determine if enzymic activity was necessary for adherence of pneumococci to A549 cells, strains of pneumococci expressing a range of neuraminidase activities were tested for adherence in the presence or absence of the neuraminidase inhibitor Dehydro-NeuNAc (Kumar *et al.*, 1981). The assay conditions measure principally NanA activity (Xu *et al.*, 2011) as NanB is a trans-sialidase with pH optimum = 4.5 (Berry *et al.*, 1996). Neuraminidase activities in cell-free extracts of strain D39 were highly sensitive to inhibition by dehydro-NeuNAc (Fig. 3A), as was commercially available clostridial sialidase (Fig. 3A). Adhesion levels of three pneumococcal strains, representing low, medium and high producers, were reduced in the presence of Dehydro-NeuNAc (Fig. 3 B), with the high-producing strain 45567 (serotype 14) being ~80% inhibited. These data suggested that neuraminidase activity might be a major requirement for the adherence and colonization processes.

Effects of *nanA* or *nanB* mutations on neuraminidase activity and adherence

We then examined the effects of *nanA* or *nanB* mutations on neuraminidase activity and adherence to cultured epithelial cells. Inactivation of *nanA* reduced neuraminidase A activity by >99% (Table 1) and led to >70% reduction in adherence levels to each of the cell cultures (Fig. 2A). Mutation of *nanB* led to 52% reduction in total neuraminidase activity (Table 1). It is important to note that neuraminidase B activity is optimal at pH 4.5 and therefore does not contribute significantly to the cell-associated neuraminidase activity measured at pH 7.0 in the assays utilized here. The *nanB* mutant strain showed 50–80% reduced adherence levels to each of the cell cultures, compared to wild-type D39 levels (Fig. 2A). The microscopic images (Fig. 2B) show clearly the effects of *nanA* or *nanB* mutations on the numbers of pneumococcal cells adhered to each of the cultured epithelial cell types.

Previous work has demonstrated that mutation in the *pavA* gene, encoding a fibronectin-binding protein (Holmes *et al.*, 2001), led to reduced adherence to epithelial cells (Pracht *et al.*, 2005), and to deficiency in colonization of the respiratory tract (Kadioglu *et al.*, 2010). The adhesion and infection phenotype of the *pavA* mutant was thus very similar to that of the *nanA* mutant. However, the *pavA* mutant UB1341

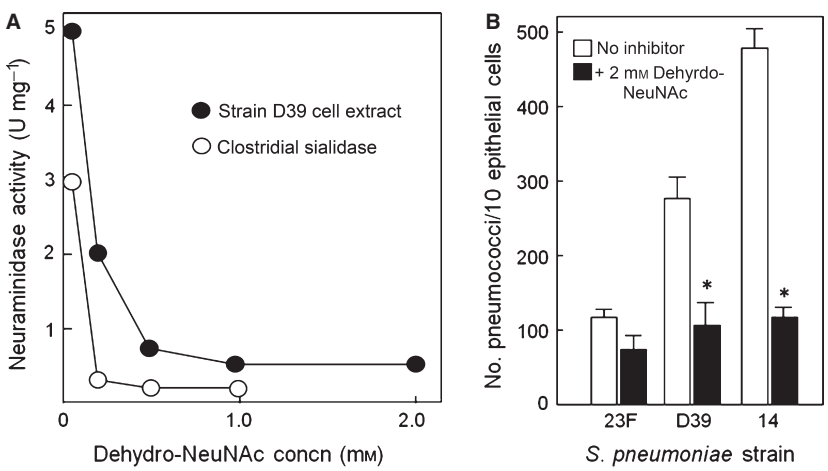


Figure 3 Inhibition of neuraminidase activity and effect on pneumococcal adherence to A549 cells. Panel A, dose responsive inhibition of neuramindase activity from *Clostridium* or *Streptococcus pneumoniae* D39 by 2,3-dehydro-2-deoxy-N-acetylneuraminic acid (dehydro-NeuNAc). Panel B, effect of pre-treatment of cells from low (UB979), medium (D39) or high (UB1050) level neuraminidase-producing strains of *S. pneumoniae* with 2 mM dehydro-NeuNAc on adherence levels to A549 cells. Error bars are ±SD from three independent experiments, $^*P \leq 0.05$.

Table 1 Bacterial strains utilized in this study and their neuraminidase activities

Strain	Genotype	Source/Reference	Neuraminidase activity ($U \times 10^{-3}/10^{10}$ cells) ± SD
UB 1830	*S. pneumoniae* D39 (wild-type) capsular serotype 2	J. Paton/laboratory stock	52.0 ± 6.30
UB 1816	D39 *nanA*::*ermAM*	Berry & Payton (2000)	4.10 ± 1.80
UB 1817	D39 *nanB*	Berry *et al.* (1999)	24.7 ± 4.90
UB 1912	UB 1830 (pKS80 *nanA*$^+$)	This study	91.6 ± 4.70
UB 1913	UB 1817 (pKS80 *nanA*$^+$)	This study	56.9 ± 5.40
UB 1915	UB 1816 (pKS80 *aad9 nanA*$^+$)	This study	44.6 ± 6.30
UB 1916	UB 1817 (pKS80 *aad9 nanA*$^+$)	This study	54.0 ± 7.10
UB 1341	*pavA1*::*cat*	Holmes *et al.*, 2001	46.0 ± 3.23
UB 1920	UB 1341 (pKS80 *nanA*$^+$)	This study	76.9 ± 4.50
UB979	*S. pneumoniae* 25925 serotype 23F	PHLS (meningitis) CSF	28.0 ± 2.0
UB1050	*Streptococcus pneumoniae* 45567 serotype 14	PHLS (pneumonia) blood	158 ± 24.7
UB 1504	*S. gordonii* DL1 (wild-type)	D. LeBlanc	<2.0
UB 1863	UB 1504 (pKS80 *nanA*$^+$)	This study	61.9 ± 3.71
UB 1545	*S. gordonii hsa*::*aphA-3*	Kerrigan *et al.*, 2007	<2.0
UB 1897	UB 1545 (pKS80 *nanA*$^+$)	This study	66.6 ± 7.41
UB 754	*L. lactis* MG1363 (wild-type)	M. Gasson	<2.0
UB 2132	*L. lactis* (pKS80)	This study	<2.0
UB 2138	*L. lactis* (pKS80 *nanA*$^+$)	This study	41.3 ± 12.0

A_{405} = 1.0 is equivalent to 0.2 *U* neuraminidase/10^{10} cells.

was unaltered in NanA activity (Table 1) and so the colonization-deficient phenotype of the *pavA* mutant remains unexplained for now (Kadioglu *et al.*, 2010).

nanA complements in *trans* the *nanA* and *nanB* mutant phenotypes

An explanation for the *nanB* mutant phenotype data above was that mutation of *nanB* affected expression of NanA. To test this possibility, and to also complement the *nanA* mutant, plasmids pKS80 (*nanA*$^+$) or pKS80 (*aad9 nanA*$^+$) were introduced into the *nanB* or *nanA* mutant strains, respectively. Complementation of the *nanA* mutant produced cells with almost fully restored neuraminidase activity (Table 1; Fig. 4 A), and increased levels of adherence to A549 cells, but only to approximately 50% of wild-type levels (Fig. 4B). Complementation of the *nanB* mutant also

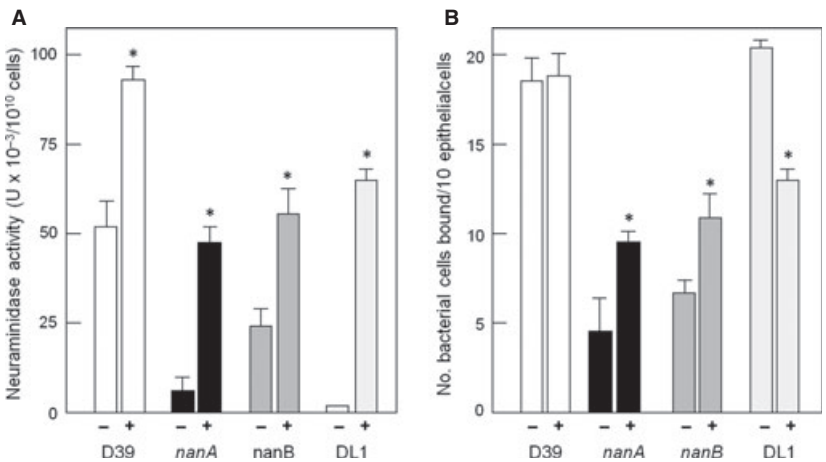


Figure 4 Effect of complementation with *nanA in trans* on (A) enzymic activity, or (B) adherence to A549 cells, of *Streptococcus pneumoniae* D39 wild-type, *nanA* mutant, *nanB* mutant and *S. gordonii* DL1. (–) control pKS80 vector only, (+) corresponding strain carrying plasmid pKS80 *aad9 nanA*+ expressing NanA (see Table 1). Values were obtained from three independent experiments and error bars are ±SD. *$P \leq 0.05$ relative to respective control.

led to wild-type levels of neuraminidase activity (Fig. 4A) and restored adherence to 58% of wild-type levels (Fig. 4B). This suggested that *nanA* activity *per se* was not the determinant of adherence. Overexpression of *nanA* in the wild-type strain D39 (Fig. 4 A) did not result in increased levels of adherence, suggesting that there may be a threshold of NanA activity above which adherence is limited by other factors.

We also expressed the *nanA* gene in the oral bacterium *S. gordonii DL1*, which is normally devoid of neuraminidase activity (Fig. 4A). *S. gordonii* adheres to sialic acid residues on the surfaces of epithelial cells, and platelets, through the activity of sialic acid-binding adhesin Hsa (Jakubovics *et al.*, 2005a,b; Kerrigan *et al.*, 2007; Jakubovics *et al.*, 2009). It was hypothesized therefore that removal of sialic acid residues by NanA would result in reduced levels of adherence. Indeed, *S. gordonii* DL1 expressing *nanA* was ~30% reduced in adherence levels to epithelial cells compared with wild-type (Fig. 4B). Moreover, expression of *nanA* in *hsa* mutant strain UB1897 had no effect on adhesion levels to epithelial cells (data not shown). These results demonstrated that neuraminidase activity, expressed on the bacterial cell surface, was able to modulate adherence levels by removing sialic acid receptors from the host cell surface.

In situ complementation of *nanA* mutant

To take advantage of the observation that NanA expressed on the surface of a heterologous cell was efficient at removing sialic acid molecules available for adhesion, we expressed *nanA* in *Lactococcus lactis*. This bacterium does not produce neuraminidase activity, and cells do not adhere to epithelial cells or to human tissue proteins (Jakubovics *et al.*, 2009). When A549 cells were pre-incubated with *L. lactis*, subsequent adherence levels of pneumococcal wild-type or *nanA* mutant strains were slightly reduced, although not statistically significantly (Fig. 5). However, when A549 cells were pre-incubated with *L. lactis* expressing NanA activity, adherence levels of the *nanA* mutant were restored to wild-type (Fig. 5). This suggested that enzymic activity, applied to the epithelial cells *in situ* prior to adherence assay, was sufficient to expose receptors necessary for adherence of the pneumococcal *nanA* mutant. However, the effect

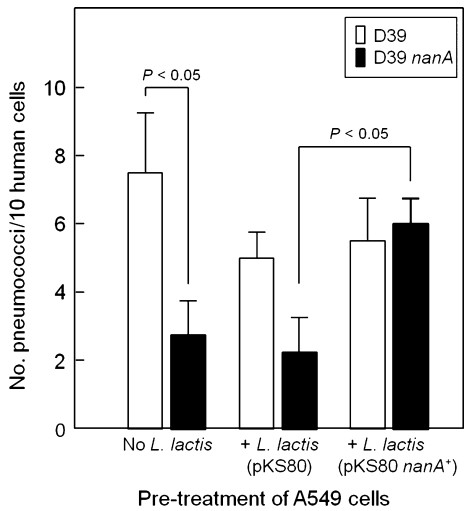


Figure 5 Effect of pre-incubating A549 cell monolayers with *L. lactis* MG1363 cells expressing NanA on subsequent adherence of *S. pneumoniae* D39 or *nanA* mutant. Cultured cells were incubated for 30 min at 37 °C with *L. lactis* pKS80 (UB2132) (vector control), *L. lactis* expressing NanA (UB2138), or no *L. lactis* cells. Epithelial cells were then washed and assayed for ability to support adherence of *S. pneumoniae* D39 or *nanA* mutant cells by viable plate count. Values were obtained with triplicate samples from two independent experiments and error bars are ±SD. *P* values are as indicated.

seemed to be specific to NanA, because incubation of epithelial cells with clostridial sialidase did not complement the *nanA* mutant (data not shown). Therefore it is concluded that the *nanA* mutation does not affect expression of adhesins, but affects the ability to specifically expose the necessary receptors for adherence.

NanA expression modulates biofilm formation

To test the role of NanA in biofilm formation, which results from initial adherence, we utilized saliva-coated glass cover slips for biofilm growth. The *nanA* and *nanB* mutants were both deficient in biofilm production, measured as biomass, compared with wild-type (Fig. 6A). The growth rates of the *nanA* or *nanB* mutant strains were similar to wild-type D39 in the medium (THY) utilized for biofilm formation. Complementation of the mutants with the corresponding *nanA*-expressing plasmids led to significantly increased biomass in each case, and restored biomass production by the *nanB* mutant to wild-type levels (Fig. 6A). Biofilm formation by *S. pneumoniae*

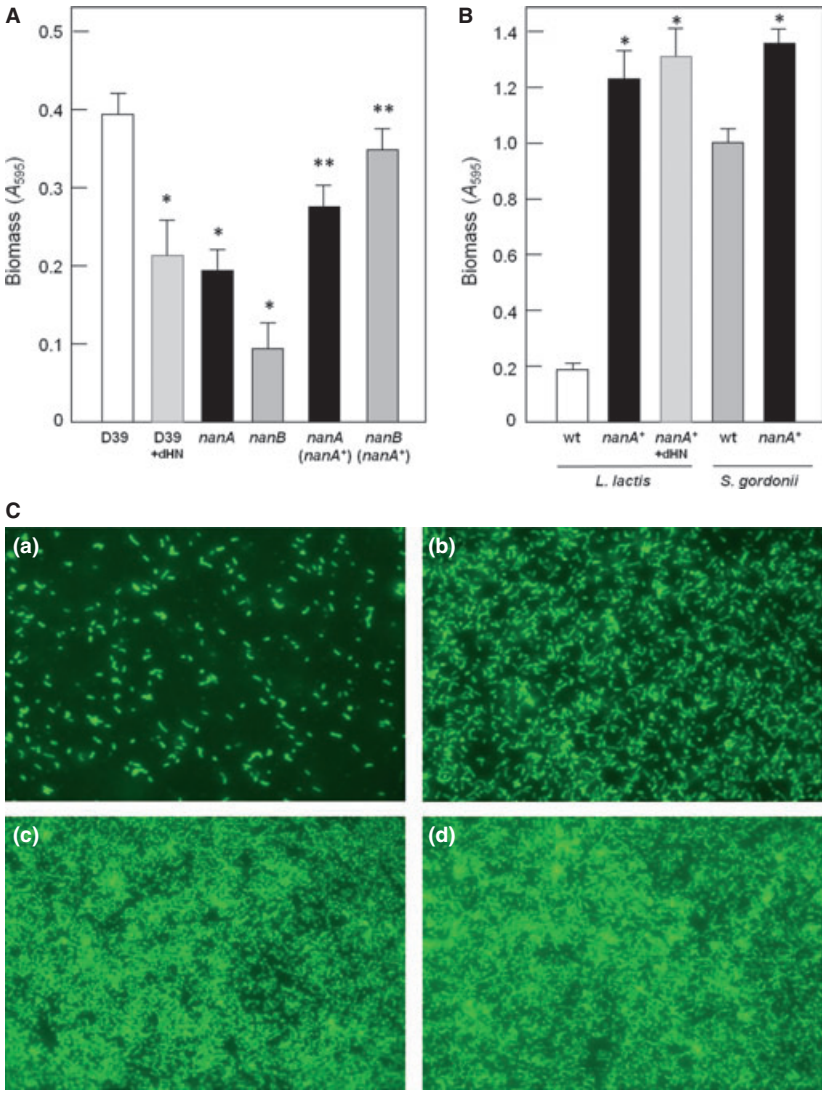


Figure 6 NanA expression promotes biofilm formation. Panel A, effects of *nanA* or *nanB* mutations, or 10 mM deydro-NeuNAC (dHN) on *Streptococcus pneumoniae* biofilm formation (biomass), and of complementation of mutants with NanA$^+$. *$P \leq 0.05$ relative to D39, **$P \leq 0.05$ relative to respective non-complemented mutant. Panel B, effects of over-expression of NanA from pKS80 *nanA*$^+$ on biofilm formation by *L. lactis* MG1363 (UB2138), in the presence or absence of 10 mM dHN, or by *S. gordonii* DL1 (UB1897). Results were obtained from three independent experiments and error bars are ±SD. *$P \leq 0.05$ relative to respective wild-type (wt). Panel C, fluorescence microscopy images of biofilms formed on saliva-coated glass surfaces by (a) *L. lactis* MG1363 (pKS80), (b) *L. lactis* NanA$^+$ (UB2138), (c) *S. gordonii* DL1, and (d) *S. gordonii* Nan$^+$ (UB1863).

D39, measured as biomass, was 45% reduced in the presence of neuraminidase activity inhibitor 10 mM-NeuNAc (Fig. 6A). This is in keeping with previous observations of the inhibitory effects of neuraminidase enzyme inhibitors on pneumococcal biofilm formation (Parker *et al.*, 2009).

To then test the direct role of *nanA* in biofilm formation, *L. lactis* or *S. gordonii* strains expressing *nanA* were compared with corresponding wild type strains in respect of biofilm biomass and architecture. Expression of *nanA* conferred on *L. lactis* the ability to form a biofilm (Fig. 6B), without which the organism formed only a sparse covering of the substratum (Fig. 6C, panel a). Expression of *nanA* clearly promoted formation of a dense layer of *L. lactis* cells that tended to form clusters (Fig. 6C, panel b). In contrast to the results for *S. pneumoniae* D39, biofilm formation by *L. lactis* expressing NanA was unaf-

fected in the presence of 10 mM dehydro-NeuNAc (Fig. 6B), suggesting that enzymatic activity was not the main reason for promotion of biofilm formation. In addition, biofilm formation by *S. gordonii* was significantly enhanced by expression of *nanA* (Fig. 6B, Fig. 6C, panels c and d), with dense clusters of cells appearing to be contained within a matrix. These observations generally are in contrast to those obtained for adherence, where *nanA* expression did not confer epithelial cell adherence properties upon *L. lactis*, and resulted in decreased levels of *S. gordonii* adherence (Fig. 4B). This suggests that under the conditions employed for biofilm formation by NanA-expressing surrogate hosts, NanA acts as an adhesin promoting formation of the microbial community. Overall, these data support the evidence that NanA contains at least two functional domains, one for enzymic activity and another for adherence.

DISCUSSION

This study was undertaken to provide *in vitro* data to account for, or otherwise, the defects of *S. pneumoniae nanA* and *nanB* mutants in their abilities to colonize the murine upper respiratory tract and to translocate to the lungs (Manco *et al.*, 2006). We show here that under our assay conditions the *nanA* and nanB mutants are deficient in adherence to four epithelial cell types. This would tend to support the hypothesis that one of the reasons that the *nanA* and *nanB* mutants were colonization deficient *in vivo* was because of attenuation in ability to adhere to epithelial cells. Our observations are in contrast to those of King *et al.* (2004, 2006) who reported that their strain D39Δ*nanA* mutant was unaffected in ability to colonize rodent models or adhere to two human epithelial cell lines. The conflicting results with respect to *in vivo* studies may be related to the use of different inocula sizes and animal backgrounds (Manco *et al.*, 2006). With regard to the discrepancies between our adherence data and those of King *et al.* (2004), these could be due to our different assay techniques (Gould & Weiser, 2002). In addition to suggesting a role for *nanA* in adherence, we have shown that *nanA* is necessary for *in vitro* microbial community development. Thus, *nanA* may impact on the ability of pneumococci to form biofilms or aggregates for efficient colonization of the nasopharynx, a prerequisite for translocation to the lungs (Orihuela *et al.*, 2004; Oggioni *et al.*, 2006). An analogous effect was recently demonstrated for surface protein PsrP, which appears to induce aggregation of pneumococcal cells and enhance colonization of the lungs (Sanchez *et al.*, 2010). In addition, it seems likely that the inability of the *nanB* mutant to colonize efficiently *in vivo* might be related, at least in part, to reduced expression of NanA activity.

There have been a relatively large number of studies on pneumococcus NanA because of its proposed association with infection, and the ability of a NanA vaccine to protect against pneumococcal challenge (Long *et al.*, 2004). However, the role of NanA in epithelial cell interactions has not been fully understood. The enzyme has been shown to possess a catalytic domain, within the C-terminal region, and an N-terminal adhesion domain that is proposed to be lectin-like in binding endothelial cell surface receptors (Uchiyama *et al.*, 2009). In studies with brain microvascular endothelial cells it was shown that the NanA lectin-like domain promoted chemokine induction and pneumococcal invasion (Banerjee *et al.*, 2010). However, in our experiments, recombinant *L. lactis* expressing NanA were not enhanced in adherence levels to epithelial cells, suggesting a fundamental difference in functioning of NanA in mediating epithelial vs. endothelial cell adherence. Incubation of Detroit 562 epithelial cells with *L. lactis* expressing NanA led to modification of the epithelial cell surface such that subsequent adherence levels of the *nanA* mutant were restored to wild-type. These observations are therefore in keeping with the notion that epithelial cell adherence requires NanA enzymic activity in order to unmask receptors to which pneumococci are able to adhere. In addition, it would appear that epithelial cell adherence by pneumococcus is not driven by the lectin-like domain of NanA, as has been shown for adherence to endothelial cells (Uchiyama *et al.*, 2009).

Other results presented here suggest that NanA functions as an epithelial adherence factor for pneumococcus as a result of enzymatic activity. Thus, over-expression of NanA in *nanA* and *nanB* mutant backgrounds led to increased adherence levels, and adherence to epithelial cells was blocked by pretreatment of pneumococcal cell extracts with enzyme inhibitor Dehydro-NeuNAc. Furthermore, expression of NanA in *S. gordonii*, which produces a sialic acid-binding adhesin Hsa (Kerrigan *et al.*, 2007), led to

30% reduced adherence levels to epithelial cells. Taken together, these observations suggest that sialidase activity may be important for survival of *S. pneumoniae* within the niche. *Streptococcus pneumoniae*, unlike *S. gordonii*, does not express a sialic acid-binding adhesin but, as discussed above, may rely upon decryption of sialic acid glycoconjugates by NanA to expose novel receptors. Concomitantly, the sialidase activity would remove sialic acid receptors utilized by other bacteria for adhesion, such as *S. gordonii* (Bensing *et al.*, 2004; Takamatsu *et al.*, 2006) and *Streptococcus sanguinis* (Cowan *et al.*, 1987; Neeser *et al.*, 1995; Plummer *et al.*, 2005), thus potentially helping to relieve competition in colonization of upper respiratory tract surfaces by commensal streptococci.

A summary of the multi-functional and temporal roles of NanA in pneumococcal infection is as follows. Activity of NanA is suggested from our results as necessary for exposure of cryptic receptors for adherence of *S. pneumoniae* to epithelia, and then subsequently for community (biofilm) formation (Soong *et al.*, 2006). This process could be promoted by continued release of free sialic acid (Trappetti *et al.*, 2009) and up-regulation of NanA production (LeMessurier *et al.*, 2006; Oggioni *et al.*, 2006; Williamson *et al.*, 2008). The role of NanA in biofilm formation has been previously noted (Parker *et al.*, 2009) where *nanA* mutants were unable to form cellular aggregates. Since inhibition of NanA enzymatic activity does not affect biofilm formation by *L. lactis* expressing NanA, it is proposed that NanA functions principally as an adhesin under these conditions. The lectin-like domain of NanA may then promote internalization of pneumococci by endothelial cells, systemic spread, induction of pro-inflammatory responses by leukocytes (Chang *et al.*, 2012) and crossing of the blood brain barrier (Uchiyama *et al.*, 2009). Such a multi-faceted protein would clearly be a highly desirable component of an effective vaccine.

An unexplained observation following from this study is that inactivation of *nanB* resulted in approximately 50% reduction in NanA activity. Previous data have shown that inactivation of *nanB* did not affect transcription levels of *nanA*, and *vice versa* (Manco *et al.*, 2006). Therefore, to explain the reduction in NanA activity in *nanB* mutants we hypothesize that there must be some form of control operating at the translational level on NanA expression. Complementation of the *nanA* mutant with plasmid pKS80 *nanA*$^+$, where *nanA* expression is under plasmid-based control sequences, including the ribosome binding site, would naturally circumvent any translational controls operating on *nanA*. This might explain why NanA activity could be restored in the *nanB* mutant complemented *in trans* with pKS80 expressing NanA. It should be noted that the assay utilized for NanA activity would not detect NanB (Xu *et al.*, 2011).

In conclusion, these data provide a clearer understanding of multiple activities of NanA in pneumococcal infections. The experiments were conducted with encapsulated strain D39 cells, suggesting that NanA activity must be projected at the capsule periphery in order that enzymic activity can access the substrate. Moreover, the adhesins that come into play for receptor recognition following desialylation must also be surface exposed. The precise nature of these adhesins is not known and is currently under investigation. These might provide additional targets for vaccines or pharmaceuticals to control epithelial cell colonization by *S. pneumoniae*.

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REFERENCES

Banerjee, V., Van Sorge, N.M., Sheen, T.R. *et al.* (2010) Activation of brain endothelium by pneumococcal neuraminidase NanA promotes bacterial internalization. *Cell Microbiol* **12**: 1576–1588.

Bensing, B.A., Lopez, J.A. and Sullam, P.M. (2004) The *Streptococcus gordonii* surface proteins GspB and Hsa mediate binding to sialylated carbohydrate epitopes on the platelet membrane glycoprotein Ibα. *Infect Immun* **72**: 6528–6537.

Bergmann, S. and Hammerschmidt, S. (2006) Versatility of pneumococcal surface proteins. *Microbiology* **152**: 295–303.

Berry, A.M. and Payton, J.C. (2000) Additive attenuation of virulence of *Streptococcus pneumoniae* by mutation

of the genes encoding pneumolysin and other putative pneumococcal virulence proteins. *Infect Immun* **68**: 133–140.

Berry, A.M., Lock, R. and Payton, J. (1996) Cloning and characterization of *nanB*, a second *Streptococcus pneumoniae* neuraminidase gene, and purification of the NanB enzyme from recombinant *Escherichia coli*. *J Bacteriol* **178**: 4854–4860.

Berry, A.M., Ogunniyi, A.D., Miller, D.C. and Paton, J.C. (1999) Comparative virulence of *Streptococcus pneumoniae* strains with insertion-duplication, point, and deletion mutations in the pneumolysin gene. *Infect Immun* **67**: 981–985.

Camara, M., Boulnois, G.J., Andrew, P.W. and Mitchell, T.J. (1994) A neuraminidase from *Streptococcus pneumoniae* has features of a surface protein. *Infect Immun* **62**: 3688–3695.

Chang, Y.-C., Uchiyama, S., Varki, A. and Nizet, V. (2012) Leukocyte inflammatory responses provoked by pneumococcal sialidase. *MBio* **3**: e00220–11.

Cowan, M.M., Taylor, K.G. and Doyle, R.J. (1987) Role of sialic acid in the kinetics of *Streptococcus sanguis* adhesion to artificial pellicle. *Infect Immun* **55**: 1552–1557.

van der Flier, M., Chhun, N., Wizemann, T.M. *et al.* (1995) Adherence of *Streptococcus pneumoniae* to immobilized fibronectin. *Infect Immun* **63**: 4317–4322.

Gould, J.M. and Weiser, J.N. (2002) The inhibitory effect of C-reactive protein on bacterial phosphorylcholine platelet-activating factor receptor-mediated adherence is blocked by surfactant. *J Infect Dis* **186**: 36–371.

Haisman, R.J. and Jenkinson, H.F. (1991) Mutants of *Streptococcus gordonii* Challis over-producing glucosyltransferase. *J Gen Microbiol* **137**: 483–489.

Hartford, O., O'Brien, L., Schofield, K. *et al.* (2001) The Fbe (SdrG) protein of *Staphylococcus epidermidis* HB promotes bacterial adherence to fibrinogen. *Microbiology* **147**: 2545–2552.

Holmes, A.R., McNab, R., Millsap, K.W. *et al.* (2001) The *pavA* gene of *Streptococcus pneumoniae* encodes a fibronectin-binding protein that is essential for virulence. *Mol Microbiol* **41**: 1395–1408.

Jakubovics, N.S., Kerrigan, S.W., Nobbs, A.H. *et al.* (2005a) Functions of cell surface-anchored antigen I/II family and Hsa polypeptides in interactions of *Streptococcus gordonii* with host receptors. *Infect Immun* **73**: 6629–6638.

Jakubovics, N.S., Stromberg, N., van Dolleweerd, C.J. *et al.* (2005b) Differential binding specificities of oral streptococcal antigen I/II family adhesins for human or bacterial ligands. *Mol Microbiol* **55**: 1591–1605.

Jakubovics, N.S., Brittan, J.L., Dutton, L.C. and Jenkinson, H.F. (2009) Multiple adhesin proteins on the cell surface of *Streptococcus gordonii* are involved in adhesion to human fibronectin. *Microbiology* **155**: 3572–3580.

Kadioglu, A., Brewin, H., Hartel, T. *et al.* (2010) Pneumococcal protein PavA is important for nasopharyngeal carriage and development of sepsis. *Mol Oral Microbiol* **25**: 50–60.

Kerrigan, S.W., Jakubovics, N.S., Keane, C. *et al.* (2007) Role of *Streptococcus gordonii* surface proteins SspA/SspB and Hsa in platelet function. *Infect Immun* **75**: 5740–5747.

King, S.J. (2010) Pneumococcal modification of host sugars: a major contributor to colonization of the human airway? *Mol Oral Microbiol* **25**: 15–24.

King, S.J., Hippe, K.R., Gould, J.M. *et al.* (2004) Phase variable desialylation of host proteins that bind to *Streptococcus pneumoniae* in vivo and protect the airway. *Mol Microbiol* **54**: 159–171.

King, S.J., Whatmore, A.M. and Dowson, C.G. (2005) NanA, a neuraminidase from *Streptococcus pneumoniae*, shows high levels of sequence diversity, at least in part through recombination with *Streptococcus oralis*. *J Bacteriol* **187**: 5376–5386.

King, S.J., Hippe, K.R. and Weiser, J.N. (2006) Deglycosylation of human glycoconjugates by the sequential activities of exoglycosidases expressed by *Streptococcus pneumoniae*. *Mol Microbiol* **59**: 961–974.

Kumar, V., Kessier, J., Scott, M.E. *et al.* (1981) 2,3-Dehydro-4-epi-N-acetylneuraminic acid: a neuraminidase inhibitor. *Carbohydr Res* **94**: 123–130.

LeMessurier, K.S., Ogunniyi, A.D. and Paton, J.C. (2006) Differential expression of key pneumococcal genes in vivo. *Microbiology* **152**: 305–311.

Limoli, D.H., Sladek, J.A., Fuller, L.A. *et al.* (2011) BgaA acts as an adhesin to mediate attachment of some pneumococcal strains to human epithelial cells. *Microbiology* **157**: 2369–2381.

Löfling, J., Vimberg, V., Battig, P. and Henriques-Normark, B. (2011) Cellular interactions by LPxTG-anchored pneumococcal adhesins and their streptococcal homologues. *Cell Microbiol* **13**: 186–197.

Long, J.P., Tong, H.H. and DeMaria, T.F. (2004) Immunization with native or recombinant *Streptococcus pneumoniae* neuraminidase afford protection in the chinchilla otitis media model. *Infect Immun* **72**: 4309–4313.

Maddocks, S.E., Wright, C.J., Nobbs, A.H. *et al.* (2011) *Streptococcus pyogenes* antigen I/II-family polypeptide AspA shows differential ligand-binding properties and mediates biofilm formation. *Mol Microbiol* **81**: 1034–1049.

Manco, S., Hernon, F., Yesilkaya, H. *et al.* (2006) Pneumococcal neuraminidases A and B both have essential roles during infection of the respiratory tract and sepsis. *Infect Immun* **74**: 4014–4020.

Mitchell, T.J. (2003) The pathogenesis of streptococcal infections: from tooth decay to meningitis. *Nat Rev Microbiol* **1**: 219–230.

Neeser, J.R., Grafstrom, R.C., Woltz, A. *et al.* (1995) A 23 kDa membrane glycoprotein bearing NeuNAc α2-3Galβ1-3GalNAc O-linked carbohydrate chains acts as a receptor for *Streptococcus sanguis* OMZ 9 on human buccal epithelial cells. *Glycobiology* **5**: 97–104.

Oggioni, M.R., Trappetti, C., Kadioglu, A. *et al.* (2006) Switch from planktonic to sessile life: a major event in pneumococcal pathogenesis. *Mo Microbiol* **61**: 1196–1210.

Orihuela, C.J., Gao, G., Francis, J. *et al.* (2004) Tissue-specific contributions of pneumococcal virulence factors to pathogenesis. *J Infect Dis* **190**: 1661–1669.

Parker, D., Soong, G., Planet, P. *et al.* (2009) The NanA neuraminidase of *Streptococcus pneumoniae* is involved in biofilm formation. *Infect Immun* **77**: 3722–3730.

Paterson, G.K. and Orihuela, C.J. (2010) Pneumococcal surface components recognizing adhesive matrix molecules targeting of the extracellular matrix. *Mol Microbiol* **77**: 1–5.

Pettigrew, M.M., Fennie, K.P., York, M.P. *et al.* (2006) Variation in the presence o of neuraminidase genes among *Streptococcus pneumoniae* isolates with identical sequence types. *Infect Immun* **74**: 3360–3365.

Plummer, C., Wu, H., Kerrigan, S.W. *et al.* (2005) A serine-rich glycoprotein of *Streptococcus sanguis* mediates adhesion to platelets via GPIb. *Br J Haematol* **129**: 101–109.

Podbielski, A., Spellerberg, B., Woischnik, M. *et al.* (1996) Novel series of plasmid vectors for gene inactivation and expression analysis in group A streptococci (GAS). *Gene* **177**: 137–147.

Pracht, D., Elm, C., Gerber, J. *et al.* (2005) PavA of *Streptococcus pneumoniae* modulates adherence, invasion, and meningeal inflammation. *Infect Immun* **73**: 2680–2689.

Sanchez, C.J., Shivshankar, P., Stol, K. *et al.* (2010) The pneumococcal serine-rich repeat protein is an intra-species bacterial adhesion that promotes bacterial aggregation in vivo and in biofilms. *PLoS Pathog* **6**: e1001044.

Soong, G., Muir, A., Gomez, M.I. *et al.* (2006) Bacterial neuraminidase facilitates mucosal infection by participating in biofilm production. *J Clin Invest* **116**: 2297–2305.

Takamatsu, D., Bensing, B.A., Prakobphol, A. *et al.* (2006) Binding of the streptococcal surface glycoproteins GspB and Hsa to human salivary proteins. *Infect Immun* **74**: 1933–1940.

Tong, H.H., Blue, L.E., James, M.A. and DeMaria, T.F. (2000) Evaluation of the virulence of a *Streptococcus pneumoniae* neuraminidase-deficient mutant in nasopharyngeal colonization and development of otitis media in the chinchilla model. *Infect Immun* **68**: 921–924.

Tong, H.H., Liu, X., Chen, Y. *et al.* (2002) Effect of neuraminidase on receptor-mediated adherence of *Streptococcus pneumoniae* to chinchilla tracheal epithelium. *Acta Otolaryngol* **122**: 413–419.

Trappetti, C., Kadioglu, A., Carter, M. *et al.* (2009) Sialic acid: a preventable signal for pneumococcal biofilm formation, colonization, and invasion of the host. *J Infect Dis* **199**: 1497–1505.

Uchiyama, S., Carlin, A.F., Khosravi, A. *et al.* (2009) The surface-anchored NanA protein promotes pneumococcal brain endothelial cell invasion. *J Exp Med* **206**: 1845–1852.

Watt, J.P., O'Brien, K.L., Katz, S. *et al.* (2004) Nasopharyngeal versus oropharyngeal sampling for detection of pneumococcal carriage in adults. *J Clin Microbiol* **42**: 4974–4976.

Williamson, Y.M., Gowrisankar, R., Longo, D.L. *et al.* (2008) Adherence of nontypeable *Streptococcus pneumoniae* to human conjunctival epithelial cells. *Microb Pathog* **44**: 175–185.

Xu, G., Kiefel, M.J., Wilson, J.C. *et al.* (2011) Three *Streptococcus pneumoniae* sialidases: three different products. *J Amer Chem Soc* **133**: 1718–1721.

Yesilkawa, H.S., Manco, A., Kadioglu, A. *et al.* (2008) The ability to utilize mucin affects the regulation of virulence gene expression in *Streptococcus pneumoniae*. *FEMS Microbiol Lett* **278**: 231–235.