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Growth of *Streptococcus pneumoniae* on Human Glycoconjugates Is Dependent upon the Sequential Activity of Bacterial Exoglycosidases[▽]

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In the human host, *Streptococcus pneumoniae* encounters a variety of glycoconjugates, including mucin, host defense molecules, and glycans associated with the epithelial surface. *S. pneumoniae* is known to encode a number of glycosidases that may modify these glycoconjugates in vivo. Three exoglycosidases, a neuraminidase (NanA), β-galactosidase (BgaA), and *N*-acetylglucosaminidase (StrH), have been previously demonstrated to sequentially deglycosylate N-linked glycans on host defense molecules, which coat the pneumococcal surface in vivo. This cleavage is proposed to alter the clearance function of these molecules, allowing pneumococci to persist in the airway. However, we propose that the exoglycosidase-dependent liberation of monosaccharides from these glycoconjugates in close proximity to the pneumococcal surface provides *S. pneumoniae* with a convenient source of fermentable carbohydrate in vivo. In this study, we demonstrate that *S. pneumoniae* is able to utilize complex N-linked human glycoconjugates as a sole source of carbon to sustain growth and that efficient growth is dependent upon the sequential deglycosylation of the glycoconjugate substrate by pneumococcal exoglycosidases. In addition to demonstrating a role for NanA, BgaA, and StrH, we have identified a function for the second pneumococcal neuraminidase, NanB, in the deglycosylation of host glycoconjugates and have demonstrated that NanB activity can partially compensate for the loss or dysfunction of NanA. To date, all known functions of pneumococcal neuraminidase have been attributed to NanA. Thus, this study describes the first proposed role for NanB by which it may contribute to *S. pneumoniae* colonization and pathogenesis.

Streptococcus pneumoniae is an important human pathogen and a leading cause of morbidity and mortality worldwide. Pneumococcal infections result in more than 1 million deaths worldwide each year, primarily due to cases of community-acquired pneumonia; *S. pneumoniae* is also a major cause of bacterial meningitis, septicemia, and otitis media (8, 22, 30).

Although renowned as a pathogen, *S. pneumoniae* more commonly colonizes the human oronasopharynx asymptomatically, with colonization rates exceeding 50% in some populations (7, 40). While colonization is often cleared by the host, it is a necessary precursor to disease and, as such, is a critical first step in pneumococcal pathogenesis. Despite this importance, very little is known about the mechanisms of colonization employed by *S. pneumoniae* that allow the organism to persist in the airway, including its means for nutrient acquisition in vivo.

Sugars are fundamental to the lifestyle of *S. pneumoniae*, as is reflected in its genome. Thirty percent of all transporters encoded by pneumococci are predicted to be sugar transporters, a greater proportion than is found in any other sequenced bacteria occupying the same niche (35). While *S. pneumoniae* is therefore capable of utilizing a variety of sugars, the concentration of free sugar in the human upper airway is generally low (29). However, both O- and N-linked glycans are abundant in airway secretions and on the surfaces of both epithelial cells and other bacteria.

S. pneumoniae encodes both secreted and surface-associated glycosidases that may modify glycoconjugates present in the host environment (2, 3, 5, 6, 24, 39, 42). We have previously demonstrated that *S. pneumoniae* deglycosylates host defense molecules, which coat the pneumococcal surface in vivo, through the sequential activity of a neuraminidase (NanA), β-galactosidase (BgaA), and *N*-acetylglucosaminidase (StrH), which cleave sialic acid (Neu5Ac), galactose (Gal), and *N*-acetylglucosamine (GlcNAc), respectively, when present as the terminal sugar on a glycan (19). Such exoglycosidase activity is proposed to alter the clearance function of host defense molecules, expose binding sites on the epithelial surface, and modify the surfaces of competing bacteria in the same niche (19, 26, 32, 37). However, the pneumococcal genome encodes predicted transporters and includes utilization genes for all three released monosaccharides, suggesting that these monosaccharides may serve as a source of fermentable carbon in vivo. In this study, we demonstrated that *S. pneumoniae* was able to utilize monosaccharides liberated from human glycoconjugates to sustain growth and that efficient growth was dependent upon the sequential deglycosylation of host glycoconjugates via the activity of four exoglycosidases. In addition to demonstrating a role for NanA, BgaA, and StrH in growth on human α-1 acid glycoprotein (AGP), we have identified a role for the second pneumococcal neuraminidase, NanB, by which it may contribute to pneumococcal colonization and pathogenesis.

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TABLE 1. *S. pneumoniae* strains used in this study

Strain name	Serotype	Description[a]	Source or reference
6A-T	6A	Transparent variant of clinical isolate P303	16
6A-T Sm^r	6A	Lys56→Thr in RpsL [*rpsL*(*K56T*)] conferring Sm^r	This study
6A-TΔ*nanA*	6A	Δ*nanA* (Cm^r)	18
6A-TΔ*nanB*	6A	Δ*nanB rpsL*(*K56T*) (Sm^r)	This study
6A-TΔ*nanA*Δ*nanB*	6A	Δ*nanA* (Cm^r) Δ*nanB rpsL*(*K56T*) (Sm^r)	This study
6A-TΔ*bgaA*	6A	Δ*bgaA* (Em^r)	This study
6A-TΔ*strH*	6A	Δ*strH* (Sp^r)	This study
6A-TΔ*nanA*Δ*nanB*Δ*bgaA*Δ*strH*	6A	Δ*nanA* (Cm^r) Δ*nanB rpsL*(*K56T*) (Sm^r) Δ*bgaA* (Em^r) Δ*strH* (Sp^r)	This study
TIGR4	4	Clinical isolate	35
TIGR4 Sm^r	4	Lys56→Thr in RpsL [*rpsL*(*K56T*)] conferring Sm^r	1
TIGR4Δ*nanA*	4	Δ*nanA* (Cm^r)	This study
TIGR4Δ*nanB*	4	Δ*nanB rpsL*(*K56T*) (Sm^r)	This study
TIGR4Δ*nanA*Δ*nanB*	4	Δ*nanA* (Cm^r) Δ*nanB rpsL*(*K56T*) (Sm^r)	This study
TIGR4Δ*nanA*Δ*nanB*Δ*bgaA*Δ*strH*	4	Δ*nanA* (Cm^r) Δ*nanB rpsL*(*K56T*) (Sm^r) Δ*bgaA* (Em^r) Δ*strH* (Sp^r)	This study
C06_18	22F	Clinical isolate	This study
C06_29	15B/C	Clinical isolate	This study
C06_31	23F	Clinical isolate	This study
C06_39	35F	Clinical isolate	This study
C06_57	6A/B	Clinical isolate	This study
C06_58	19A	Clinical isolate	This study

[a] Cm^r, resistant to chloramphenicol; Sm^r, resistant to streptomycin; Em^r, resistant to erythromycin; Sp^r, resistant to spectinomycin.

MATERIALS AND METHODS

Bacterial strains, culture media, and chemicals. Wild-type and genetically modified strains of *S. pneumoniae* and the six pneumococcal clinical isolates utilized in this study are described in Table 1. Broth cultures were routinely grown at 37°C in Todd-Hewitt broth (Becton, Dickinson and Co., Sparks, MD) supplemented with 0.2% (wt/vol) yeast extract (Becton, Dickinson and Co.). C medium with 5% yeast extract (C+Y medium), pH 8, was used for transformations (21). *S. pneumoniae* was also grown at 37°C and 5% CO_2 overnight on tryptic soy (TS; Becton, Dickinson and Co.) plates with 1.5% agar that were spread with 5,000 U of catalase (Worthington Biochemical Corporation, Lakewood, NJ) prior to the plating of bacteria. Mutants were selected on TS plates that contained either streptomycin (200 μg/ml), kanamycin (500 μg/ml), chloramphenicol (2.5 μg/ml), erythromycin (1 μg/ml), or spectinomycin (200 μg/ml), as appropriate.

Unless otherwise specified, all chemicals, substrates, and enzymes were purchased from Sigma Chemicals (St. Louis, MO).

Mutation of glycosidases. Genes encoding the pneumococcal exoglycosidases NanA, BgaA, and StrH are predicted to be located in single-gene transcriptional units or present as the last gene in an operon (14, 35). Therefore, insertion-deletion mutants were constructed as previously described and confirmed by PCR and sequencing of *nanA* (primers A.1 and A.2 and primers A.3 and A.4), *bgaA* (primers G.1 and G.2), and *strH* (primers S.1 and S.2) (18, 19). All genomic DNA was prepared as previously described (41).

The gene encoding NanB, a second pneumococcal neuraminidase, is located in an operon. For this reason, we created an unmarked, nonpolar deletion of *nanB* by using a Janus cassette selection system (34). The construction of a mutant strain using this method required two rounds of transformation. The first round introduced a Janus cassette, which contains kanamycin resistance and *rpsL*$^+$ genes, into the genome of a streptomycin-resistant (Sm^r) *S. pneumoniae* strain in the place of a gene of interest. The Janus construct used to transform our *S. pneumoniae* strains was engineered using a variation on the process of splicing by overlap extension (SOE) by PCR, first described by Horton et al. (11). In order to minimize PCR-generated errors, all PCRs were conducted using a high-fidelity proofreading polymerase (*Pfx-50*; Invitrogen); furthermore, all PCR products were purified and concentrated using a PCR purification kit (QIAGEN). DNA fragments flanking *nanB* were amplified using primers B.1 and B.2 (upstream fragment) and B.4 and B.5 (downstream fragment). Each primer pair contained one hybrid primer such that sequences complementary to the Janus cassette primers were introduced at the 3′ end of the upstream DNA fragment and the 5′ end of the downstream DNA fragment (Table 2. These PCR products were sequentially joined to the Janus cassette PCR product (primers J.1 and J.2) by using SOE. Transformants were selected on kanamycin-treated TS plates, and their antibiotic susceptibility phenotype was subsequently confirmed to be kanamycin resistant (Km^r) and Sm^s.

The second round of transformation replaced the Janus cassette with an engineered segment of DNA consisting of the two DNA fragments flanking *nanB* spliced together via SOE. Fragments flanking *nanB* were amplified using primers B.1 and B.3 (upstream fragment) and B.5 and B.6 (downstream fragment). Primers were designed such that the 3′ end of the upstream fragment contained a sequence complementary to the 5′ end of the downstream fragment. Unmarked *nanB* mutants were selected on streptomycin-treated TS plates, and their antibiotic susceptibility phenotype was subsequently confirmed to be Km^s Sm^r. The generation of an unmarked *nanB* mutant was confirmed by PCR with primers flanking the construct (B.7 and B.8) and sequencing.

***nanC* region PCR.** To determine whether *nanC* was present in *S. pneumoniae* strains, PCR analysis across the boundaries of the genetic region containing *nanC* (primers C.1 and C.3 and primers C.2 and C.5) or the alternative region found in strains lacking *nanC* (primers C.1 and C.4 and primers C.2 and C.6) was conducted (25, 33, 35). Chromosomal DNA from sequenced strains R6 and TIGR4 was used as negative and positive controls for the presence of the *nanC* region, respectively.

Dialysis of human AGP and HSA. Dialysis was conducted in order to remove any free sugar from our human proteins by using a Slide-A-Lyzer dialysis cassette (molecular weight cutoff, 10,000) according to the instructions of the manufacturer (Pierce). Human AGP and fatty acid-free human serum albumin (HSA) were reconstituted at 10 mg/ml in distilled water (dH_2O) and dialyzed against 2 liters of dH_2O at 4°C for 2 h and then overnight in fresh, prechilled dH_2O (2 liters). The samples were concentrated using Slide-A-Lyzer concentrating solution (Pierce) according to the instructions provided, and the proteins were then extracted, diluted to the original volume, filter sterilized, and stored at 4°C.

Thirty-hour growth assays. C+Y semidefined medium, pH 6.8, was supplemented with either lactose (10 mM) and sucrose (10 mM), AGP (5 mg/ml), HSA (5 mg/ml), or no sugar. *S. pneumoniae* strains were grown in Todd-Hewitt broth supplemented with 0.2% (wt/vol) yeast extract to an optical density at 600 nm (OD_{600}) of 0.3 ± 0.05 (early exponential growth phase), washed, resuspended, and diluted 10-fold in phosphate-buffered saline (PBS). Twenty-microliter aliquots of either PBS alone (PBS control) or diluted bacterial suspensions were transferred into wells containing 180 μl of C+Y medium: the final bacterial dilution was 1:100. Medium supplemented with lactose and sucrose served as a positive control, indicating that the bacteria were viable and that mutant strains showed no defect in growth relative to their parent strain. Medium with no added sugar served as a negative control for pneumococcal growth within the semidefined media. Medium supplemented with 5 mg/ml of HSA served as a protein-

TABLE 2. Primers used in this study

Group	No.	Primer sequence (5′→3′)[a]	Location[b] (accession no.)
Janus	J.1	CCGTTTGATTTTTAATGGATAATG	7–30 (AY334019)
	J.2	GGGCCCCTTTCCTTATGCTT	247511–247527 (AE005672)
nanA	A.1	TATCGAGTAGGGTAGTTCTT	149–169 (X72967)
	A.2	ACGGGGCAGGTTAGTGACAT	1953–1972 (J01754)
	A.3	TAGTTCAACAAAGGAAAATTGGATAA	1101–1126 (J01754)
	A.4	ATTCTGCAGTTCTAGCACTAATAGACTCTT	1008–1037 (U43526)
nanB	B.1	CATATGCAGTTTGGCTCTTAGTT	4708–4730 (U43526)
	B.2	CATTATCCATTAAAAATCAAACGGTTTTCCTTCTTTCGTCTACTTCAC[1]	5076–5099 (U43526)
	B.3	ACCTATGTGATGATTTGGCAATTTTCCTTCTTTCGTCTACTTCAC[2]	5076–5099 (U43526)
	B.4	GGAAAGGGGCCCAGGTGTTGCCAAATCATCACATAGGT[3]	7076–7096 (U43526)
	B.5	TCATCGCTAGAAACCAACTCATC	7363–7385 (U43526)
	B.6	TTGCCAAATCATCACATAGGT	7076–7096 (U43526)
	B.7	GGAATTAACTCAGTATCCAACA	4370–7391 (U43526)
	B.8	TTACACGCATCTACCATCTC	7510–7529 (U43526)
nanC	C.1	GCAAACACTATTCCAATCATCACC	1234554–1234577 (AE005672)
	C.2	TAGTGGTGTTTTTGGGGCTG	1261423–1261442 (AE005672)
	C.3	GACAACAGCTGAACGTGGACATAA	1239066–1239089 (AE005672)
	C.4	TAGTCCCATATTCGTTCTCAAGTC	9376–9399 (AE008490)
	C.5	GTGTGGTAAGTATTGTGGTGAACG	1259130–1259130 (AE005672)
	C.6	CAATCGTTATTCCTTTATCTGGAG	8344–8367 (AE008491)
bgaA	G.1	AACTAGGTTGTCATACCATG	149–168 (AE007374)
	G.2	GTAACTACTAATCCTGCACT	6888–6907 (AE007374)
strH	S.1	CAGGAGGTTTTGCAATGAAAC	719–739 (L36923)
	S.2	GTGTGGACATGAGTCCTG	4613–4630 (L36923)

[a] Underlining indicates the reverse complement sequence of primers J.1 ([1]), B.6 ([2]), and J.2 ([3]).
[b] Locations are given as nucleotide positions in the indicated accession numbers.

only control to confirm that glycans were required for *S. pneumoniae* growth on glycoconjugates.

Growth plates were sealed with Breath-Easy gas-permeable sealing membranes (USA Scientific) and incubated at 37°C for 30 h in a Bio-Tek Synergy HT plate reader. Prior to each OD reading, plates were shaken gently for 2 s; the OD_{600} was measured every 20 min. All data are adjusted to a path length of 1 cm. All growth assays were run in triplicate on three independent occasions.

Dilutions of media. To determine whether pneumococcal growth was limited by the concentration of AGP in the medium, C+Y media supplemented with 4, 3, 2, and 1 mg of AGP/ml were also prepared. To test the growth of *S. pneumoniae* on an equivalent amount of free sugar, we calculated the number of sugar residues made available by the deglycosylation of 5 mg of AGP/ml. Assuming the average of 16 glycan branches per molecule of AGP and complete glycosylation of each branch, the level of sugar residues in 5 mg of AGP/ml is approximately equivalent to that in a 3 mM solution of disaccharides. C+Y media were prepared with 3, 2.4, 1.8, 1.2, and 0.6 mM concentrations of total disaccharide, with lactose and sucrose each contributing 50% of the disaccharide concentration.

Protein and glycan analyses. Lectin and Western blotting analyses were conducted to investigate the deglycosylation of AGP following incubation with different *S. pneumoniae* strains. Samples were harvested from growth plates after 30 h and stored at −20°C until use (for no more than 48 h). Protein samples (0.2 μg of AGP) were electrophoresed on a 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto Immobilon-P membranes (Millipore). Analysis with *Sambucus nigra* (SNA), *Maackia amurensis* (MAA), and *Galanthus nivalis* (GNA) lectins to detect terminal α2,6- and α2,3-linked sialic acid and terminal mannose, respectively, was conducted as previously described (19). Immunoblotting was conducted using mouse anti-human AGP (1:10,000; primary antibody) and alkaline phosphatase (AP)-conjugated donkey anti-mouse immunoglobulin G (IgG [1:10,000; secondary antibody; Jackson ImmunoResearch]). Contrast on digitized images was manipulated using Microsoft Office Picture Manager or Adobe Photoshop Elements 2.0. To investigate whether *S. pneumoniae* degraded the protein component of AGP during growth assays, we electrophoresed 1- and 5-μg samples of the control protein HSA, which has no carbohydrate component, on a 12.5% SDS-PAGE gel following 30 h of incubation with strain 6A-T. Coomassie staining was conducted to visualize HSA.

Statistical analysis. Growth assays were run in triplicate on three independent occasions, and all data shown on the same graph were collected from the same three microtiter plates. After data were corrected for path length, the average from triplicate medium blanks was subtracted from values for appropriate experimental wells. Results for triplicate wells were then averaged, and the average was considered as one datum point for further analysis. Data from three independent experiments were averaged, and the 95% confidence interval was calculated for each time point.

RESULTS

***S. pneumoniae* can utilize human AGP as a sole carbon source for growth.** Our previous studies have demonstrated that *S. pneumoniae* can deglycosylate human glycoconjugates through the sequential activity of three pneumococcal exoglycosidases, NanA, BgaA, and StrH (19). To determine whether *S. pneumoniae* can utilize monosaccharides liberated from glycoconjugates via exoglycosidase activity, we selected the model glycoprotein, purified human AGP, as a substrate for pneumococcal growth. Human AGP is a well-characterized serum glycoprotein with an average of 16 glycan branches per molecule (38). Although not present in the airway, AGP exhibits the same complex N-linked glycan structure as host defense molecules, which have been previously demonstrated to coat the pneumococcal surface in vivo. Sequential activity of pneumococcal exoglycosidases is predicted to result in the deglycosylation of this glycan structure to expose mannose (Fig. 1A) (12, 13).

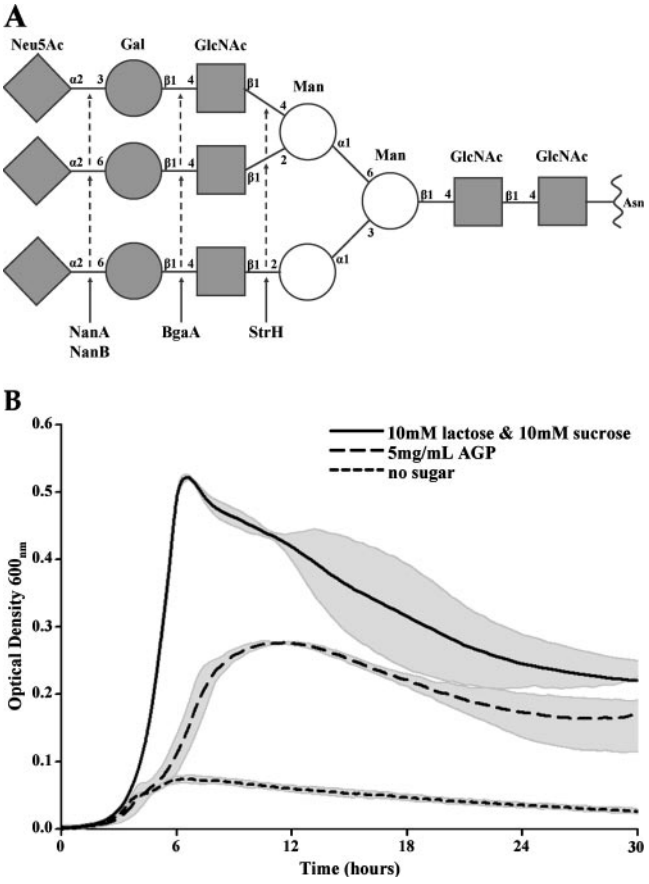

FIG. 1. Demonstration that *S. pneumoniae* can utilize human AGP to sustain growth, an ability that is hypothesized to be dependent upon the cleavage and utilization of the complex N-linked glycan. (A) A schematic representation of the triantennary N-linked glycan structure of human AGP is shown: this antennary structure comprises approximately 38% of all glycans bound to AGP, with bi- and tetra-antennary chains accounting for 62% (38). Sugar residues are labeled above their corresponding symbols, and arrows indicate cleavage sites for pneumococcal neuraminidase (NanA), β-galactosidase (BgaA), and *N*-acetylglucosaminidase (StrH). Lines indicate linkages between sugar residues, and linkages are specified by characters found either above or below a given line. This N-linked glycan structure is commonly found in the human airway and has been identified on host defense molecules, which coat the pneumococcal surface in vivo (19). (B) Growth of *S. pneumoniae* strain 6A-T on semidefined C+Y medium supplemented with either 10 mM lactose and 10 mM sucrose, 5 mg of human AGP/ml, or no sugar as described in Materials and Methods. OD_{600} values are the means from three independent experiments each run in triplicate; 95% confidence intervals are represented by gray shading around each growth curve, indicating statistically significant differences in pneumococcal growth exhibited in each of the three media.

In order to demonstrate that *S. pneumoniae* can grow on human glycoconjugates, a semidefined C+Y medium was supplemented with either free lactose and sucrose, AGP, or no sugar and inoculated with clinical isolate 6A-T. 6A-T exhibited statistically significant growth on lactose and sucrose ($OD_{600} > 0.5$), indicating that the bacteria were viable when inoculated into the medium. The limited growth of 6A-T on the no-sugar medium may be attributed to the presence of carbon from the yeast extract or Casamino Acids in the semidefined medium. Pneumococcal strain 6A-T exhibited statistically significant growth ($OD_{600} \approx 0.3$) on AGP beyond the baseline levels exhibited on no-sugar medium (Fig. 1B). These data indicate that *S. pneumoniae* is able to utilize human AGP as a sole source of carbon to sustain growth. To confirm that pneumococcal growth was dependent upon the presence of glycans attached to AGP rather than the degradation and utilization of the protein component, growth assays in which 6A-T was inoculated into C+Y medium supplemented with fatty acid-free HSA were conducted. HSA, a human serum protein with no carbohydrate component, did not support pneumococcal growth that was statistically significantly different from growth on no-sugar medium (data not shown). Furthermore, SDS-PAGE and Coomassie staining revealed no change in the molecular weight of HSA incubated with 6A-T versus that incubated with medium alone, indicating that the protein was not degraded by the pneumococci (data not shown). These data indicate that pneumococcal growth on AGP is dependent on the presence of glycans.

The growth exhibited by 6A-T on lactose and sucrose medium was significantly greater than that on AGP. The serial dilution of AGP revealed that the growth of 6A-T was limited by the concentration of AGP in the medium (data not shown), which was in turn limited by the solubility of AGP. In order to determine whether the concentration of available sugar, the efficiency of sugar metabolism, or both contributed to this difference in growth, pneumococcal growth rates on dilutions of each medium preparation were compared. The maximum OD_{600} of 6A-T on 5 mg/ml of AGP (0.33 ± 0.02) was statistically significantly less than that on a lactose and sucrose medium (3 mM total disaccharide) containing an equivalent number of available sugar residues ($OD_{600} = 0.45 \pm 0.03$) ($P < 0.01$). The concentration of free sugar in lactose and sucrose medium became limiting below 1.2 mM total disaccharide, and pneumococcal growth on 0.6 mM total disaccharide was approximately equivalent to growth on 5 mg/ml of AGP (data not shown). These data indicate that while growth on AGP was limited by the concentration of the glycoconjugate in the medium, it was also less efficient than growth on an equivalent number of free sugar residues.

While growth on glycoconjugates may be less efficient than growth on free sugar, it is likely that pneumococci utilize glycoconjugate-derived monosaccharides in vivo where free-sugar concentrations are low. To expand our observations, we demonstrated that six minimally passaged recent clinical isolates representing six different multilocus sequence types and six distinct serotypes grew at least as efficiently on AGP as 6A-T (data not shown).

Exoglycosidases NanA, BgaA, and StrH are required for efficient growth on human AGP. To determine whether three pneumococcal exoglycosidases previously implicated in the deglycosylation of host glycoprotein—NanA, BgaA, and StrH—contributed to the growth of pneumococci on AGP, we conducted growth assays with each of the three insertion-deletion exoglycosidase mutants on AGP (Fig. 2A). Because the enzymes must act consecutively to deglycosylate glycoconjugates, the inability to cleave a terminal sugar residue results in the inaccessibility of all internal sugars. Therefore, we expected to see a stepwise reduction in maximal growth, as follows: growth of

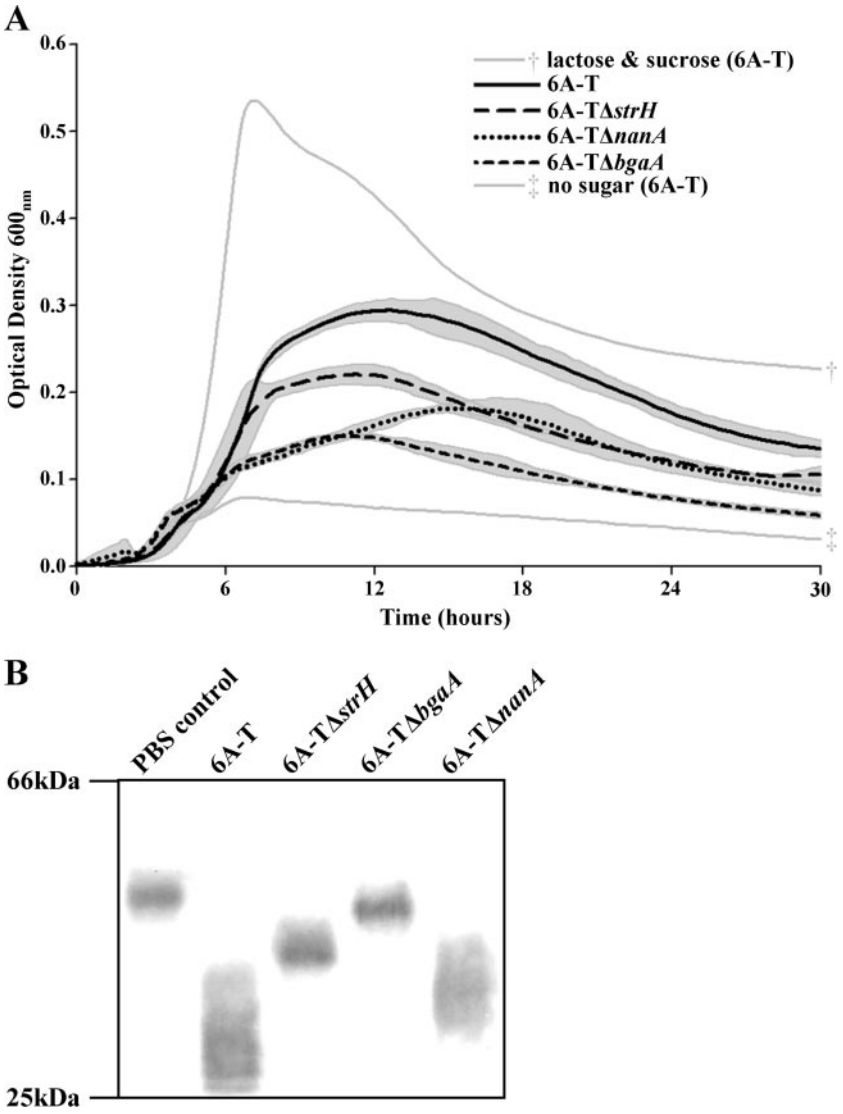

FIG. 2. Demonstration that the growth of *S. pneumoniae* on human AGP is dependent upon exoglycosidase activity and that the extent of growth correlates to the ability of the strain to deglycosylate AGP. (A) Growth of wild-type and exoglycosidase mutants of *S. pneumoniae* strain 6A-T on semidefined C+Y medium supplemented with 5 mg of human AGP/ml. OD_{600} data for 6A-T on medium supplemented with either 10 mM lactose and 10 mM sucrose or no sugar are provided for reference. Growth was analyzed as described in the legend to Fig. 1. (B) Western blotting for human AGP. Samples were harvested from 96-well plates after a 30-h growth assay, electrophoresed on a 12.5% SDS-PAGE gel, and transferred onto an Immobilon-P membrane, and AGP was detected with monoclonal mouse anti-human AGP (primary antibody) and AP-conjugated donkey anti-mouse IgG (secondary antibody).

wild-type 6A-T > growth of 6A-TΔ*strH* > growth of 6A-TΔ*bgaA* > growth of 6A-TΔ*nanA*.

Equivalent growth of all strains on lactose and sucrose medium demonstrated that mutants exhibited no statistically significant reduction in growth rate or maximum OD_{600} relative to the parent strain (data not shown). Furthermore, there was no statistically significant difference in the growth of mutant and parent strains on no-sugar medium (data not shown). For clarity, only the growth of parent strains on lactose and sucrose and no-sugar media is shown; as these data are provided for reference only, 95% confidence intervals have been omitted.

Both the StrH and BgaA mutants exhibited stepwise reductions in growth relative to the wild type, as predicted. However, the growth of the NanA mutant was not reduced to the extent predicted, and this mutant actually exhibited growth to a maximum OD_{600} beyond that of 6A-TΔ*bgaA*. This suggested either the presence of unsialylated glycans in our AGP sample or NanA-independent desialylation of the glycoconjugate.

Western blot analysis of AGP samples harvested after 30-h growth assays revealed that the extent to which AGP was deglycosylated by a given strain (indicated by the mobility shift) corresponded to the growth observed for that strain (Fig. 2B). Stepwise shifts in mobility were observed for samples incubated with 6A-TΔ*bgaA*, 6A-TΔ*strH*, and wild-type 6A-T relative to the PBS control, demonstrating that the extent of deglycosylation by wild-type 6A-T was greater than that by 6A-TΔ*strH*, which was greater than that by 6A-TΔ*bgaA*. The mobility shift observed for the AGP sample incubated with

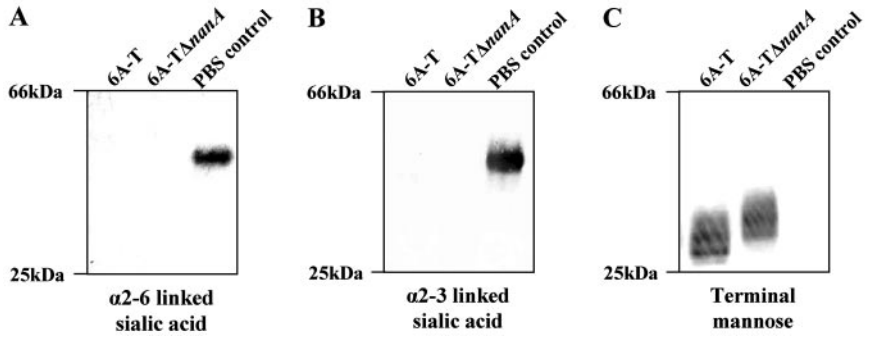

FIG. 3. Demonstration that 6A-TΔ*nanA* desialylates human AGP in a NanA-independent manner. Following the growth of bacteria on AGP for 30 h, 0.2-μg samples of AGP were electrophoresed on 12.5% SDS-PAGE gel and transferred onto an Immobilon-P membrane. SNA (A), MAA (B), and GNA (C) lectins were used to detect α2,6-linked sialic acid, α2,3-linked sialic acid, and terminal mannose, respectively.

6A-TΔ*nanA* indicated that it was further deglycosylated than samples incubated with either BgaA or StrH mutants but not deglycosylated to the extent of the sample incubated with wild-type 6A-T.

***S. pneumoniae* desialylates human AGP in a NanA-independent manner.** To determine whether AGP was desialylated in a NanA-independent manner, we performed lectin blotting with SNA (Fig. 3A), MAA (Fig. 3B), and GNA (Fig. 3C) lectins to detect α2,6- and α2,3-linked Neu5Ac and terminal mannose, respectively. 6A-TΔ*nanA* cleaved both linkages of terminal Neu5Ac and exposed mannose via further exoglycosidase activity; however, AGP incubated with 6A-TΔ*nanA* exhibited a reduced shift in mobility relative to AGP incubated with wild-type 6A-T. These data indicate that Neu5Ac can be cleaved in a NanA-independent manner but that the NanA mutant is less efficient than the wild type at deglycosylating AGP.

To account for this NanA-independent mechanism of desialylation, we investigated the potential role of other neuraminidases in the growth of 6A-T on human glycoconjugates; two pneumococcal enzymes have been demonstrated to have neuraminidase activity, and one additional putative neuraminidase has been identified (2, 5, 35). The gene encoding NanA is part of the core pneumococcal genome: it is both present in and expressed by 100% of tested strains (15, 18, 20, 26). To date, all known functions for pneumococcal neuraminidase have been attributed to NanA. The gene encoding NanB is present in 96% of strains tested; NanB has previously been demonstrated to exhibit neuraminidase activity against a fluorogenic substrate, albeit 100-fold less than that of NanA (2, 17, 28). Additionally, NanB has recently been reported to be important in mouse models of infection (23). A putative third neuraminidase is encoded by *nanC*, which is present in fewer than 51% of strains examined; *nanA* and *nanB* but not *nanC* have been detected in strain 6A-T by PCR (data not shown) (17, 25, 28, 33, 35).

NanB contributes to pneumococcal growth on AGP in both the presence and the absence of *nanA*. Growth of 6A-*T*Δ*nanB* on AGP exhibited a small yet statistically significant 6.4% reduction in maximum OD_{600} relative to wild-type 6A-T, indicating that NanB contributes to pneumococcal growth on AGP even in the presence of NanA (Fig. 4A). 6A-TΔ*nanA* was reduced in growth as previously observed. The growth of the double neuraminidase mutant (6A-TΔ*nanA*Δ*nanB*) was reduced almost to the baseline levels of growth on no-sugar medium, indicating that the activity of NanB is primarily responsible for the growth of 6A-TΔ*nanA* on AGP and demonstrating that in the absence of NanA, NanB can partially support the growth of pneumococci on glycoconjugates.

Lectin blotting for both α2,6-linked Neu5Ac (Fig. 4D*i*) and terminal mannose (Fig. 4D*ii*), as well as Western blotting for AGP (Fig. 4C), indicated that 6A-TΔ*nanB* cleaved terminal sialic acid and deglycosylated AGP to expose mannose comparably to wild-type 6A-T. The double neuraminidase mutant was unable to expose mannose, and α2,6-linked Neu5Ac was still detected. These data indicate that 6A-TΔ*nanA*Δ*nanB* was unable to desialylate AGP and that NanB was responsible for the NanA-independent desialylation observed for 6A-TΔ*nanA*.

To determine whether the limited growth of the double neuraminidase mutant was due to the activity of BgaA and StrH on unsialylated glycans, which are found naturally in glycoconjugate samples, we constructed and characterized a quadruple mutant lacking both neuraminidases, BgaA, and StrH. Growth assays revealed that the growth of the quadruple mutant on AGP was not statistically significantly different from the growth exhibited on no sugar (Fig. 4B). Lectin and Western blots demonstrated that the quadruple mutant was unable to cleave terminal sialic acid or otherwise deglycosylate AGP to expose mannose (Fig. 4C and D).

NanC plays no detectable role in the growth of *S. pneumoniae* on human AGP. In order to determine whether the contribution of NanB to growth on human glycoconjugates is strain dependent, as well as to investigate a potential role for NanC in our system, we conducted further studies with the sequenced *S. pneumoniae* strain TIGR4, which is known to contain not only *nanA* and *nanB* but also *nanC* and is of a different genetic background than strain 6A-T (31).

Both TIGR4Δ*nanA* and TIGR4Δ*nanB* exhibited statistically significant reductions in growth on AGP, with the maximum OD_{600} of TIGR4Δ*nanB* being reduced by 46.0 to 51.3% relative to that of wild-type TIRG4 in three independent experiments (Fig. 5A). These data indicate that NanB contributes to the growth of TIGR4 on glycoconjugates to a greater extent than it contributes to the growth of 6A-T on the same substrate. The growth of double mutant TIGR4Δ*nanA*Δ*nanB* on AGP was reduced almost to the baseline levels on no-sugar

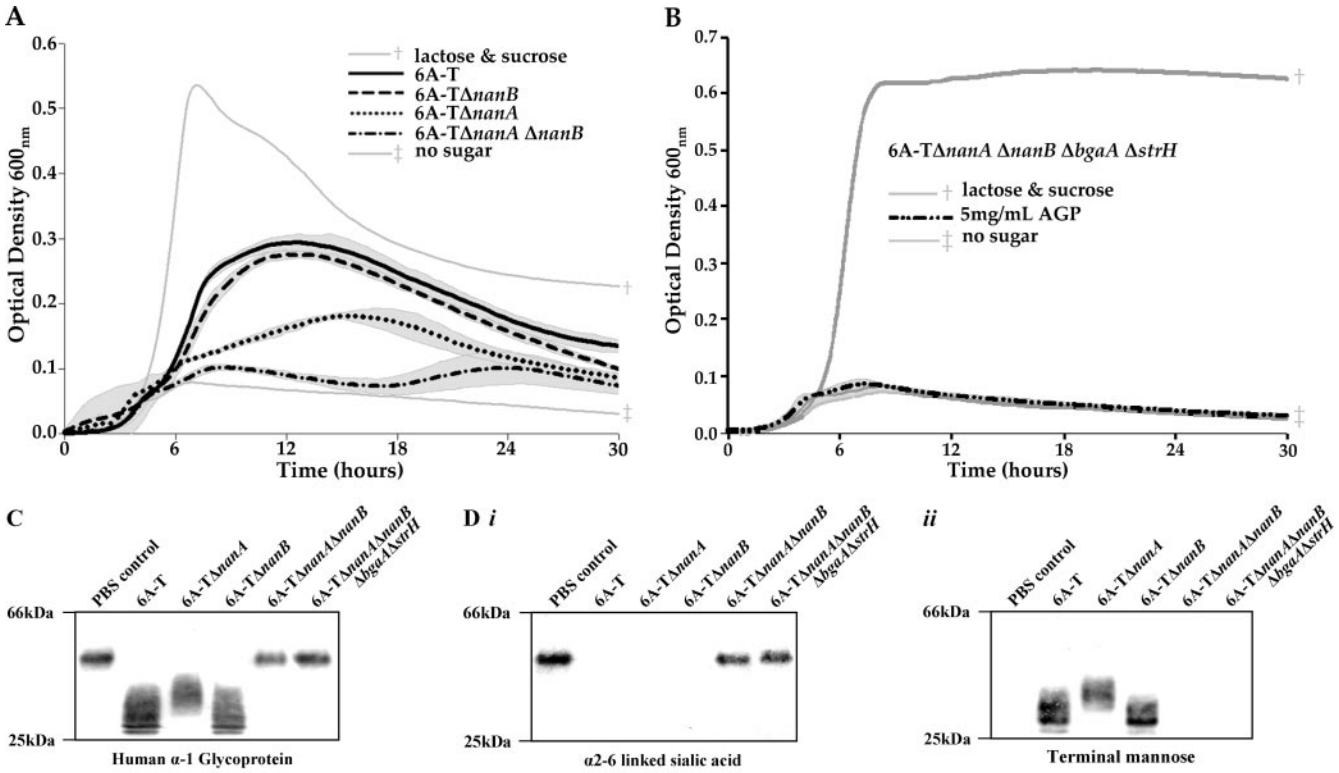

FIG. 4. Both NanA and NanB contribute to the growth of *S. pneumoniae* on human glycoconjugates. (A) Growth of wild-type 6A-T, 6A-TΔ*nanA*, 6A-TΔ*nanB*, and 6A-TΔ*nanA*Δ*nanB* on semidefined C+Y medium supplemented with 5 mg of AGP/ml. Growth curves of 6A-T on medium supplemented with either 10 mM lactose and 10 mM sucrose or no sugar are provided for reference. Growth was analyzed as described in the legend to Fig. 1. (B) Growth of 6A-TΔ*nanA*Δ*nanB*Δ*bgaA*Δ*strH* on lactose and sucrose, AGP, and no-sugar media. OD_{600} data are the means from three independent experiments each run in triplicate. For clarity, 95% confidence intervals have been omitted from the data demonstrating bacterial growth on lactose and sucrose as well as on no sugar. Following the growth of bacteria on AGP for 30 h at 37°C, 0.2-μg samples of AGP were electrophoresed on 12.5% SDS-PAGE gel and transferred onto Immobilon-P membranes. (C) Western blotting for human AGP using monoclonal mouse anti-human AGP (primary antibody) and AP-conjugated donkey anti-mouse IgG (secondary antibody) for detection. (D) 6A-TΔ*nanA*Δ*nanB* does not desialylate AGP and is unable to expose detectable amounts of mannose. SNA (*i*) and GNA (*ii*) lectins were used to detect α2,6-linked sialic acid and terminal mannose, respectively.

medium, while the growth of the quadruple mutant on AGP was not statistically significantly different from the growth exhibited on no sugar (Fig. 5B). These data indicate that the growth of TIGR4Δ*nanA*Δ*nanB* was due to the presence of unsialylated glycans in the AGP sample and therefore do not support a role for NanC in our system.

DISCUSSION

Little is known about how *S. pneumoniae* acquires energy during the colonization of the human upper airway, where the concentration of free sugar is generally low; however, the glycosidase-dependent release of monosaccharides from glycoconjugates in close proximity to the pneumococcal surface may provide *S. pneumoniae* with a source of carbon in vivo.

By using human AGP as a model substrate for pneumococcal growth, this study has demonstrated that *S. pneumoniae* can utilize monosaccharides liberated from complex N-linked human glycoconjugates to sustain growth. Although pneumococcal growth on AGP was not as efficient as that on an equivalent number of free sugar residues, this observation is consistent with the knowledge that sucrose and lactose are efficiently metabolized, preferred carbon sources (9). Furthermore, it is probable that growth on AGP comes at a greater metabolic cost than growth on free sugar, as it requires the production of many factors necessary to grow on glycoconjugates. This metabolic cost may manifest itself as a reduction in the maximum growth of *S. pneumoniae*.

All clinical isolates that were tested were able to grow efficiently on AGP, indicating that the sequential deglycosylation of glycans may be a mechanism used to acquire energy and grow in vivo. Other streptococcal species, including *S. oralis*, have also been demonstrated to utilize glycoconjugates as a sole source of carbon in vitro (4). Minimal growth of *S. pneumoniae* (mean maximum increase in absorbance at 620 nm, 0.05) on RNase B, a glycoconjugate posttranslationally modified by high-mannose glycoforms (Man_5 to Man_9), has been previously demonstrated. This growth was attributed to the cleavage and utilization of the Man_5 glycoform; however, the enzyme(s) and mechanisms for cleavage, transport, and utilization of the liberated six-sugar glycan have not yet been elucidated (10). These ex-

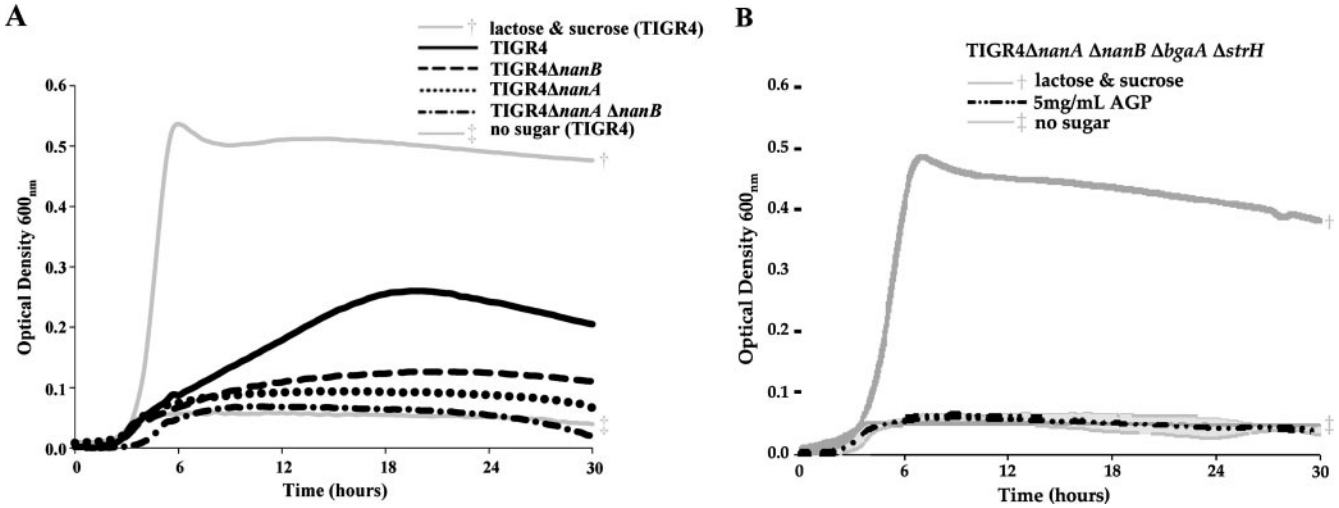

FIG. 5. Demonstration that both NanA and NanB but not NanC contribute significantly to the growth of TIGR4 on human AGP. (A) Growth of wild-type *S. pneumoniae* strain TIGR4, as well as single and double neuraminidase mutants, on semidefined C+Y medium supplemented with 5 mg of human AGP/ml. OD_{600} data for TIGR4 on medium supplemented with either 10 mM lactose and 10 mM sucrose or no sugar are provided for reference. OD_{600} data are representative of three independent experiments each run in triplicate. (B) Growth of TIGR4Δ*nanA*Δ*nanB*Δ*bgaA*Δ*strH* on lactose and sucrose, AGP, and no-sugar media. OD_{600} data are the means from three independent experiments each run in triplicate. For clarity, 95% confidence intervals have been omitted from the data demonstrating bacterial growth on lactose and sucrose as well as on no sugar.

periments were conducted with a type of N-linked glycan that does not contain the three terminal sugars—Neu5Ac, Gal, and GlcNAc—that contribute to the growth we observed on AGP.

The ability of *S. pneumoniae* to sequentially deglycosylate AGP was required for efficient growth: more extensive deglycosylation corresponded to increased growth, as was evidenced by stepwise reductions in maximum optical densities observed for exoglycosidase mutants. In this study, four pneumococcal exoglycosidases were implicated in the growth of two different *S. pneumoniae* strains of divergent genetic backgrounds on human glycoconjugates: NanA, NanB, BgaA, and StrH.

The desialylation of human glycoconjugates, along with all other functions of pneumococcal neuraminidase, has previously been attributed to NanA; however, our data demonstrate that NanB contributes to the deglycosylation of human glycoconjugates in both the presence and the absence of NanA (18, 19). This discrepancy may be accounted for, in part, by the differences in growth conditions employed by the relevant studies. Previous studies measured neuraminidase expression by *S. pneumoniae* grown in enriched medium to stationary phase; in this study, *S. pneumoniae* was provided with a complex substrate which required neuraminidase activity in order for pneumococci to grow (5, 18). It seems likely that both NanA and NanB are differentially regulated and may be induced by growth on a cleavable substrate such as human glycoconjugates and that NanB is not expressed during growth on enriched medium.

These data, indicating a role for NanB, are consistent with the observation that some sialic acid can be removed from the Eustachian tube epithelia of chinchillas infected with a NanA-deficient strain of *S. pneumoniae* (36). Furthermore, it has recently been demonstrated that NanB is important in a mouse model of pneumococcal infection and sepsis (23). This is the first described function for NanB in vitro that may contribute to the ability of *S. pneumoniae* to colonize and therefore cause disease in humans.

It is unclear whether pneumococcal strains lacking *nanB* would be reduced in their ability to grow on N-linked glycans or whether their growth in vivo would be affected (17, 28). However, our data demonstrate that the contribution of NanB varies between strains, with 6A-TΔ*nanB* exhibiting only a 6.4% reduction in growth on AGP relative to its parent strain whereas the growth of TIGR4Δ*nanB* was reduced more than 50% compared to that of its wild-type counterpart. It is possible, then, that strains lacking *nanB* would not show a reduction in growth. What determines the extent to which NanB contributes to the growth of a particular strain on glycoconjugates is unknown; however, it is possible that the contribution of NanB to the growth of TIGR4 on AGP is related to the secretion of NanA by TIGR4 (20, 27, 35).

In strain TIGR4, an 11-bp deletion within the 3′ region of *nanA* results in the termination of the gene product at amino acid 804 (20, 35). Consequently, the membrane-anchoring domain is not translated and NanA is secreted. Despite this change, TIGR4 was selected for further study because it is the only sequenced strain known to contain *nanC*. Wild-type TIGR4 exhibited linear growth on AGP to an OD_{600} comparable to that of 6A-T under the same conditions ($OD_{600} \approx 0.3$), suggesting that TIGR4's process for acquiring energy from glycoconjugates is less efficient than that of 6A-T (Fig. 5A). This result may reflect the reduced efficiency of sequential cleavage by enzymes not colocalized at the bacterial surface, as well as the reduced efficiency of transport due to sugars' being released away from the bacterial surface. To compensate, TIGR4 may have evolved to have increased expression of NanB, suggesting that NanB is able to compensate for the loss or dysfunction of NanA in vivo. This idea is supported by the increased contribution of NanB to the growth of strain 6A-T on glycoconjugates in the absence of NanA.

To determine whether other potential neuraminidases, like NanC, contributed to the residual growth of double neuraminidase mutants on AGP, we tested the growth of 6A-T and TIGR4 quadruple mutants on AGP. *nanC* is absent from the majority of *S. pneumoniae* strains and was confirmed to be absent from strain 6A-T by PCR (data not shown) (17, 28). Neither quadruple mutant exhibited growth above baseline levels on AGP, indicating that growth was due to the activity of BgaA and StrH against unsialylated glycans. Thus, our data do not support a role for NanC in the deglycosylation of glycoconjugates in our system. However, it is possible that the genetic region containing *nanC* confers another selective advantage for *S. pneumoniae*, that *nanC* is expressed under different conditions than are present in our system, or that NanC is not acting as a neuraminidase despite its high level of homology to NanB.

Previous studies have demonstrated that NanA is important in establishing colonization, which is essential to the development of disease (19, 26, 32, 37). Furthermore, both NanA and NanB have recently been demonstrated to be important in a mouse model of pneumococcal respiratory infection and sepsis (23). These data demonstrate a role for the previously identified pneumococcal virulence factors, NanA and NanB, in pneumococcal metabolism; in doing so, they suggest a link between metabolism and more direct mechanisms for colonization that may allow *S. pneumoniae* to subsequently cause disease. In this study, we have demonstrated that while exoglycosidase activity may contribute to pneumococcal pathogenesis, it may also provide pneumococci with a source of carbon in vivo. Therefore, exoglycosidase activity against glycoconjugates may contribute directly to the ability of *S. pneumoniae* to acquire energy to establish colonization and therefore persist in the airway.

Understanding such roles for previously identified virulence factors is critical: there is no proposed selective advantage for *S. pneumoniae* in causing invasive disease. Instead, it is more likely that factors that we classically consider to be virulence determinants were evolutionarily selected based upon their contribution to pneumococcal transmission and colonization and that invasion and disease are accidents, per se, of effective colonization tactics. These data stress the importance of understanding how previously identified virulence factors contribute to pneumococcal colonization and disease, as well as the necessity of investigating the role of metabolic enzymes in the colonization process and considering their possible contribution to pathogenesis.

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