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Sialic Acid-Mediated Gene Expression in *Streptococcus pneumoniae* and Role of NanR as a Transcriptional Activator of the *nan* Gene Cluster

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In this study, we investigated the transcriptomic response of *Streptococcus pneumoniae* D39 to sialic acid (*N*-acetylneuraminic acid [Neu5Ac]). Transcriptome comparison of wild-type D39 grown in M17 medium with and without sialic acid revealed the elevated expression of various genes and operons, including the *nan* gene cluster (*nan* operon I and *nanA* gene). Our microarray analysis and promoter-*lacZ* fusion studies showed that the transcriptional regulator NanR acts as a transcriptional activator of *nan* operon I and the *nanA* gene in the presence of sialic acid. The putative regulatory site of NanR in the promoter region of *nan* operon I is predicted and confirmed by promoter truncation experiments. Furthermore, the role of CcpA in the regulation of the *nan* gene cluster is demonstrated through microarray analysis and promoter-*lacZ* fusion studies, suggesting that in the presence of sialic acid and glucose, CcpA represses the expression of *nan* operon I while the expression of the *nanA* gene is CcpA independent.

The low-GC Gram-positive bacterium *Streptococcus pneumoniae* is a major human pathogen and the causal agent of many diseases, including pneumonia, sepsis, meningitis, otitis media, and conjunctivitis, which result in over a million deaths each year worldwide (1, 2). *S. pneumoniae* colonizes the human nasopharynx during the first few months of life (3). For survival in the host, bacteria rely not only on the different virulence factors they possess but also on the appropriate use of nutrients available in their habitat (4, 5). The ability of *S. pneumoniae* to utilize a variety of carbohydrate sources is one of the crucial factors in successful colonization and in causing pneumococcal infections (6). Metabolic selection enables a bacterium to choose a preferred source of carbon over a nonpreferred one through a mechanism called carbon catabolite repression (CCR) (5). CcpA (carbon catabolite protein A) is a transcription factor that mediates CCR in the presence of a preferred sugar source, e.g., glucose, by binding to catabolite repression elements (*cre* boxes) found in the promoter regions of CcpA-targeted genes (5, 7–9). Despite the importance of carbohydrates in the pathogenesis of *S. pneumoniae*, research concerning metabolic pathways of *S. pneumoniae* still demands more attention.

Sialic acid is one of the most important carbohydrates for *S. pneumoniae*, since it plays a vital role as a carbon/energy source, a receptor for adhesion and invasion, and a molecular signal for the promotion of biofilm formation, nasopharyngeal carriage, and invasion of the lungs (10). It has been shown that *S. pneumoniae* can utilize sialic acid as a carbon source, which results in improved pneumococcal biofilm formation *in vitro*, at concentrations comparable to those of free sialic acid in human saliva (11, 12). Of the 43 known naturally occurring derivatives of the nine-carbon sugar neuraminic acid, *N*-acetylneuraminic acid (Neu5Ac) is the only one found in humans (13). Another form of sialic acid is *N*-glycolylneuraminic acid (Neu5Gc), which is not synthesized by humans. Some factors, including a number of extracellular glycosidases and carbohydrate transporters, affect the interaction of the bacterium with the host (10). At least 10 surface-associated glycosidases (NanA, NanB, NanC, BgaA, EndoD, Eng, Hyl, SpuA, BgaC, and StrH) are expressed in *S. pneumoniae*, and these glycosidases scavenge and modify the host glycans and sialic acid residues (14) to use them as a carbon source for growth, making host clearance multifunctional molecules, helping the pneumococcus to fight against other bacteria for a niche, assisting it in driving through the mucin film, and stimulating its attachment to epithelial cells (15–20). Three neuraminidases are encoded by the genome of *S. pneumoniae* (NanA, NanB, and NanC), and their importance in pathogenicity has made pneumococcal neuraminidases the most-studied surface-located glycosyl-hydrolases (10). The regulatory mechanisms of neuraminidase expression are important, since release of sialic acid from O-glycans has been linked to pathogenesis. Moreover, sialic acid has been proposed as a molecular signal in stimulating *in vitro* biofilm production and *in vivo* nasopharyngeal carriage and lung invasion by *S. pneumoniae* (12, 21). NanA is the best-understood neuraminidase and aids the progression of otitis media in a chinchilla animal model (22) and of respiratory tract infection and sepsis in mice (23). Less is known about NanB, but NanB has also been suggested to play a significant role during pneumococcal infection of the respiratory tract and

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
S. pneumoniae strains		
D39	Serotype 2 strain, *cps-2*	Laboratory of P. Hermans
Δ*ccpA* strain	D39 Δ*ccpA* Spec^r	8
MA200	D39 Δ*nanR* Spec^r	This study
MA201	D39 Δ*bgaA*::P*nanA-lacZ* Tet^r	This study
MA202	D39 Δ*bgaA*::P*spd_1503-lacZ* Tet^r	This study
MA203	D39 Δ*bgaA*::P*nanE-lacZ* Tet^r	This study
MA204	MA200 Δ*bgaA*::P*nanA-lacZ* Tet^r	This study
MA205	MA200 Δ*bgaA*::P*spd_1503-lacZ* Tet^r	This study
MA206	MA200 Δ*bgaA*::P*nanE-lacZ* Tet^r	This study
MA207	Δ*ccpA* Δ*bgaA*::P*nanA-lacZ* Tet^r	This study
MA208	Δ*ccpA* Δ*bgaA*::P*spd_1503-lacZ* Tet^r	This study
MA209	Δ*ccpA* Δ*bgaA*::P*nanE-lacZ* Tet^r	This study
MA210	D39 Δ*bgaA*::P*nanE-F-lacZ* Tet^r	This study
MA211	D39 Δ*bgaA*::P*nanE-H-lacZ* Tet^r	This study
MA212	D39 Δ*bgaA*::P*nanE-N-lacZ* Tet^r	This study
E. coli EC1000	Km^r; MC1000 derivative carrying a single copy of the pWV1 *repA* gene in *glgB*	Laboratory collection
Plasmids		
pPP2	Amp^r Tet^r; promoterless *lacZ*; for replacement of *bgaA* with promoter *lacZ* fusion; derivative of pPP1	30
pORI38	Spec^r *ori*$^+$; *repA* deletion; derivative of pWV01	47
pMA201	pPP2 P*nanA-lacZ*	This study
pMA202	pPP2 P*spd_1503-lacZ*	This study
pMA203	pPP2 P*nanE-lacZ*	This study
pMA204	pPP2 P*nanE-F-lacZ*	This study
pMA205	pPP2 P*nanE-H-lacZ*	This study
pMA206	pPP2 P*nanE-N-lacZ*	This study

sepsis (23). NanA and NanB stimulate the pneumococcal colonization of the upper respiratory tract by cleaving sialic acid from cell surface glycans and mucin and by presenting host cell surface receptors for pneumococcal adherence (23, 24). Mouse model studies have also revealed that NanA and NanB are essential for *S. pneumoniae* infection (23).

The present study was aimed at elucidating the effect of sialic acid (*N*-acetylneuraminic acid [Neu5Ac]) on the global gene expression of *S. pneumoniae*. We characterized the *nan* regulon (*nan* operon I and the *nanA* gene) in *S. pneumoniae* D39 and demonstrated that the transcriptional regulator NanR acts as a transcriptional activator for *nan* operon I and the *nanA* gene in the presence of sialic acid. We also demonstrated that regulation of *nan* operon I is CcpA dependent, whereas the regulation of the *nanA* gene is CcpA independent in the presence of glucose and sialic acid. The putative regulatory site (5′-TCTGAAASTACTTTCARA-3′) of NanR in the promoter region of *nanE* is also predicted and confirmed by promoter truncation experiments and was found to be highly conserved in other pneumococcal strains and streptococci.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and DNA isolation and modification. Bacterial strains and plasmids used in this study are listed in Table 1. The *S. pneumoniae* strain D39 was grown as described previously (25). For β-galactosidase assays, derivatives of the *S. pneumoniae* D39 strain were grown in M17 medium supplemented with different sugars (arabinose, cellobiose, dextrose, fructose, fucose, glucose, galactose, lactose, maltose, mannitol, mannose, melibiose, sorbitol, trehalose, and xylose) at concentrations given in Results. For selection on antibiotics, the medium was supplemented with 150 μg/ml spectinomycin and 2.5 μg/ml tetracycline for *S. pneumoniae* and with 100 μg/ml ampicillin for *Escherichia coli*. All bacterial strains used in this study were stored in 10% (vol/vol) glycerol at −80°C. All DNA manipulations in this study were performed as described before (26). For PCR amplification, chromosomal DNA of *S. pneumoniae* D39 (27) was used as a template. Primers used in this study are based on the sequence of the D39 genome and are listed in Table 2.

Construction of a *nanR* mutant. The *nanR* deletion mutant was made by allelic replacement with a spectinomycin resistance marker as described before (28). Briefly, primers nanR-1/nanR-2 and nanR-3/nanR-4 were used to generate PCR fragments of the left and right flanking regions of *nanR*. PCR products of the left and right flanking regions of *nanR* contain AscI and NotI restriction enzyme sites, respectively. The spectinomycin resistance marker was amplified with primers Spec-F/Spec-R from plasmid pORI38 (29). The spectinomycin resistance marker also contains AscI and NotI restriction enzyme sites. Then, by restriction and ligation, the left and right flanking regions of *nanR* were fused to the spectinomycin resistance cassette. The resulting ligation product was transformed into wild-type *S. pneumoniae* D39. For transformation, cells were grown at 37°C without shaking until an optical density at 600 nm (OD_{600}) of ~0.1 was reached. Bovine serum albumin (BSA; 0.2%) and 1 mM $CaCl_2$ were added to the grown cells. A 1-ml portion of the grown culture was transferred to a 1.5-ml Eppendorf tube, and 100 ng/μl of competence-stimulating peptide 1 (CSP1) was added to the culture. Cells were incubated at 37°C for 10 to 12 min. Then the ligation mixture was added to the incubated cells, and the cells were allowed to grow for 90 to 120 min at 37°C. After growth, the culture was spun for 1 min at 7,000 rpm, and most of the supernatant was discarded. The cell pellet was dissolved in the remaining medium (50 to 100 μl) and plated on GM17 (0.5% glucose plus M17) plus 1% sheep blood agar. Selection of the *nanR* mutant strain was done on the appropriate concentration of spectino-

TABLE 2 Primers used in this study

Primer	Nucleotide sequence (5′→3′)	Restriction site
nanA-R	CATGGGATCCATATTATTCCCCTTTTCTAAGC	BamHI
nanA-F	CATGGAATTCGCTGACTTCGTCAGTTCTATCC	EcoRI
spd-1503-R	CATGGGATCCCATCTCTTCTAACCTCCTTTCTC	BamHI
spd-1503-F	CATGGAATTCGTCCACTCGAAACAAGTATTGTAAG	EcoRI
nanE-Rv	CATGGGATCCCTAATCTGTGGCATCCTCTTTCC	BamHI
nanE-Fr	CATGGAATTCCAGAGATGGATGGAGCAATTGC	EcoRI
nanE-N	CATGGAATTCGCTGTTTGACTTGGCTAGTTTTTG	EcoRI
nanE-H	CATGGAATTCACTTTTAGAGGAGCTGTTTGACTTGG	EcoRI
nanE-F	CATGGAATTCCGACTTGCTCCTCTGAAAGTAC	EcoRI
nanR-1	GCCGTCATTTTATTGCTACGCG	
nanR-2	GCATAGGCGCGCCCGTTTGGTCAGTGGACTGTGC	AscI
nanR-3	CGATTGCGGCCGCCCTATACAGAGACTGTTCTTGTAGC	NotI
nanR-4	GGGTATATGCATATGCAGGATGG	
Spec-R	GCTAAGCGGCCGCACTAAACGAAATAAACGC	NotI
Spec-F	GCTATGGCGCGCCCTAATCAAAATAGTGAGGAGG	AscI
RT-PCR primers		
IR-I-F	GGATTGAGCAGGAAGTATGG	
IR-I-R	GGAGACGTTCCAAATACCACTGCTCC	
IR-II-F	GTCCACTCGAAACAAGTATTGTAAG	
IR-II-R	CATCTCTTCTAACCTCCTTTCTC	
IR-III-F	GGTTCATTTGAGAGCTGGTG	
IR-III-R	GCCGCTAGCGCAATTCCAGC	
IR-IV-F	GAGGCTGTTCAGTAGATGTA	
IR-IV-R	CCTACGATAATCATAGCTGGC	
IR-V-F	GCTATCATCAGTAAGTGGG	
IR-V-R	ACTGCAACGATGGTCGCACC	
IR-VI-F	GCCAAAGAGTCTATTAGTGCTAG	
IR-VI-R	GGCTAATTTTTGTAATAATCATC	
IR-VII-F	CGTGGTCGAGAAATGAATTGC	
IR-VII-R	GCGAGCCAATTCAGCTCC	
IR-VIII-F	CAGAGATGGATGGAGCAATTGC	
IR-VIII-R	CTAATCTGTGGCATCCTCTTTCC	
IR-IX-F	GGTGGCGCCATTACTAGACC	
IR-IX-R	GCAGGAAGTATAGCTATAGG	
IR-X-F	GGTGTAGATGATTAAGTACTTACTG	
IR-X-R	CCAGAATTGCCACAAGCAGC	
IR-XI-F	GCTATGAAACAATAGTCCTTAGTTATTC	
IR-XI-R	TGAAGCCCATCACCATCGGAGC	
IR-XII-F	GCAGTCATGATTGCTATCG	
IR-XII-R	GGAAAGATGAACAGCACAGTC	
IR-XIII-F	GGGTATTACTATGGGAGCGG	
IR-XIII-R	GAGGTAGTCGATAGCCTTGTC	
IR-XIV-F	CCCAGGTGAGCCACATCAG	
IR-XIV-R	CTAGCTCTTGCAGCATAC	
IR-XV-F	GCTTTACTGACAAAAAAGAAAACAGTTTACAAA	
IR-XV-R	CTCAAACATGGTCTCGTAGAGGC	
IR-XVI-F	GCAATTATTTCTGAAACTACTTTCAAAGGC	
IR-XVI-R	CCTTTGGCTACCGCCATGACTTC	
IR-XVII-F	CTGTTCGTTCACCATTGACACC	
IR-XVII-R	TGCAACGTAGTATGTCATAAATAC	

mycin. The *nanR* mutant was further confirmed by PCR and DNA sequencing.

Construction of promoter-*lacZ* fusions and β-galactosidase assays. Chromosomal transcriptional *lacZ* fusions to the *nanA*, *spd_1503*, and *nanE* promoters were constructed in the integration plasmid pPP2 (30) via double crossover in the *bgaA* locus with primer pairs listed in Table 2, resulting in pMA201 to pMA203, respectively. These constructs were further introduced into wild-type D39, resulting in strains MA201 to MA203, respectively. pMA201, pMA202, and pMA203 were also transformed into the Δ*nanR* strain, resulting in strains MA204, MA205, and MA206, respectively. Similarly, pMA201, pMA202, and pMA203 were transformed into a Δ*ccpA* strain (8), resulting in strains MA207, MA208, and MA209. The following subclones of P*nanE* were made in pPP2 (30) using the primer pairs mentioned in Table 2: P*nanE-F* (truncated just a few bases upstream of the NanR regulatory site), P*nanE-H* (deletion of half of the NanR regulatory site), and P*nanE-N* (deletion of the full NanR regulatory site), resulting in plasmids pMA204 to pMA206. These constructs were introduced into the wild type, resulting in strains MA210, MA211, and

MA212. All plasmid constructs were further checked for the presence of insert by PCR and DNA sequencing.

β-Galactosidase assays were performed as described before (30) using cells that were harvested in the mid-exponential growth phase and grown in M17 medium with appropriate sugars, as described in Results.

Microarray analysis. For DNA microarray analysis of *S. pneumoniae* in the presence of sialic acid, the transcriptome of wild-type *S. pneumoniae* D39, grown in replicates in SM17 (0.5% sialic acid plus M17) medium, was compared to that grown in M17 medium (no added sugar). To analyze the effect of *nanR* deletion on the transcriptome of *S. pneumoniae* in the presence of sialic acid, wild-type D39 and its isogenic mutant *nanR*, were grown in replicates in SM17 medium and harvested at the mid-exponential growth phase. For the identification of differentially expressed genes, a Bayesian *P* value of <0.001 and a cutoff of a >2-fold change were applied. *nanR* is downregulated less than 2-fold but is listed in the table to show the deletion of *nanR*. Similarly, to analyze the effect of a *ccpA* deletion on the transcriptome of *S. pneumoniae* in the presence of sialic acid and glucose, wild-type D39 and its isogenic Δ*ccpA* mutant were grown in replicates in SM17 and GM17 medium and harvested at the mid-exponential growth phase.

For RNA isolation, the following procedure was performed. The pellet of the harvested cells was resuspended in 400 μl Tris-EDTA (TE) containing diethyl pyrocarbonate (DEPC), and the resuspended cells were added to RNA-free screw-cap tubes containing 0.5 g glass beads, 50 μl 10% SDS, 500 μl phenol-chloroform–isoamyl alcohol (IAA), and a Macaloïd layer (Bentone MA; Rheox Inc.) (150 to 175 μl; the value is not exact, as the layer is very viscous). To break the cells, the screw-cap tubes were placed in a bead beater and two 60-s pulses were applied with a 60-s interval on ice. The samples were centrifuged for 10 min at 10,000 rpm (4°C). Chloroform-IAA (500 μl; 24:1) was added to tubes containing the upper phase from the centrifuged tubes, and the samples were again centrifuged for 5 min at 10,000 rpm (4°C). A 500-μl portion of the upper phase was transferred to the fresh tubes, and 2 volumes (1 ml) of lysis/binding buffer was added and mixed by pipetting up and down. Total RNA was isolated using the RNA isolation kit, and to remove contaminating DNA from total RNA, 100 μl DNase I mix (90 μl DNase buffer and 10 μl DNase I) was added and the samples were incubated for 20 to 30 min at 15 to 25°C. RNA was wash-cleaned using the RNase kit, and 50 μl of eluted volume was obtained. To check the quality of the RNA isolated, the RNA sample was diluted with DEPC water to get a concentration of 20 to 200 ng/μl. A 1-μl portion of the diluted RNA sample was used to check quality on the Bioanalyzer according to the manufacturer's instructions, and a ratio of 23S to 16S of around 2.0 was found, which is considered good. Microarray analysis was performed as described before (26, 31, 32). Homemade slides were used for sample hybridization, and in-house-made software packages were used for data analysis (33, 34).

All other procedures regarding the DNA microarray experiment and data analysis were performed as previously described (35–37).

Reverse transcription-PCR. To confirm that *nan* operons I and II are transcribed into single transcriptional units, wild-type D39 was grown in SM17, and total RNA was isolated as described above. Reverse transcription-PCR (RT-PCR) was performed as described before (25) on all possible intergenic regions of *nan* operons I and II with primer pairs mentioned in Table 2. For a fair comparison of the PCR products, 100 ng of RNA and 20 ng of DNA were used.

Microarray data accession number. Microarray data have been submitted to the Gene Expression Omnibus (GEO) under accession number GSE66561.

RESULTS

Genetic organization of the *nan* gene cluster in *S. pneumoniae* D39. Based on the annotation of the D39 genome, the *nan* gene cluster consists of *nan* operon I (*spd_1497* to *spd_1488*), the *nanA* gene (*spd_1504*), and *nan* operon II (*spd_1503* to *spd_1498*) (Fig. 1A). *nanA* encodes a sialidase that has been shown to cleave sialic acid and plays a role in pneumococcal pathogenesis (21). *nan* operon I consists of 10 genes (*spd_1488* to *spd_1497*), which code for an *N*-acetylmannosamine-6-phosphate 2-epimerase (NanE) (*spd_1497*), a phosphotranferase system (PTS) transporter (NanP) (*spd_1496*), three ABC transporters (NanUVW) (*spd_1493* to *spd_1495*), three hypothetical proteins (*spd_1490* to *spd_1492*), an *N*-acetylneuraminate lyase (*spd_1489*), and a ROK family protein (NanK) (*spd_1488*). The putative *nan* operon II (*spd_1498* to *spd_1503*) is composed of six genes, which code for a hypothetical protein (*spd_1503*), three ABC transporters (*spd_1500* to *spd_1502*), a neuraminidase *nanB* (*spd_1499*), and an oxidoreductase (*spd_1498*). The gene encoding a phosphosugar-binding transcriptional regulator, NanR, belonging to the RpiR family is present downstream of the *nan* gene cluster (Fig. 1A). The presence of *nanR* next to the *nan* gene cluster may indicate its putative function in the regulation of the *nan* gene cluster.

To confirm whether *nan* operons I and II are transcribed into single transcriptional units and whether *nanA* has its own promoter, we performed RT-PCR on all possible intergenic regions present in *nan* operon I, in *nan* operon II, and on the upstream and downstream regions of *nanA* with the primer pairs listed in Table 2. RT-PCR data revealed that *nan* operons I and II are transcribed separately as single transcriptional units and that *nanA* has its own promoter (Fig. 1B).

Sialic acid-dependent gene expression in *S. pneumoniae*. To elucidate the transcriptomic response of *S. pneumoniae* to sialic acid, transcriptome comparison of wild-type D39 grown in SM17 medium (0.5% sialic acid plus M17) to that grown in M17 (no added sugar) medium was performed. Table 3 summarizes the transcriptome changes induced in *S. pneumoniae* in the presence of sialic acid. After the criteria of a >2.0-fold difference and a *P* value of <0.001 were applied, 34 genes were upregulated in the presence of sialic acid, whereas 13 genes were downregulated under the tested conditions, including *nan* operon I and the *nanA* gene. Upregulation of *nan* operon I and the *nanA* gene in the presence of sialic acid indicates that the *nan* gene cluster is functional in *S. pneumoniae* D39 and responds to sialic acid. No change in the expression of *nan* operon II was observed. This indicates that *nan* operon II does not respond to sialic acid under our tested conditions. Some other genes and operons were found to be up- and downregulated in our transcriptome experiment (Table 3). The glutamine regulon, consisting of genes involved in nitrogen metabolism and known to contribute to the colonization of the nasopharynx by *S. pneumoniae* (26), was downregulated in the presence of sialic acid. The expression of this regulon is repressed in the presence of a nitrogen source (which in this case is sialic acid). There were other amino acid utilization and transport genes (*spd_0334* and *spd_0901*) that were also downregulated in our microarray analysis (Table 3). Two bacteriocin secretion accessory proteins (encoded by *spd_0115* and *spd_0116*) and some hypothetical proteins (encoded by *spd_0091*, *spd_0373*, *spd_1159*, *spd_1265*, *spd_1505*, *spd_1515*, *spd_1516*, and *spd_1800*) were also upregulated in the presence of sialic acid in addition to some ABC transporters (encoded by *spd_1263*, *spd_1264*, *spd_1267*, *spd_1330*, and *spd_1514*) (Table 3). *spd_1265* codes for an integral membrane protein, whereas *spd_1800* codes for an ABC-type multidrug transport system permease. An alpha-glycerophosphate oxidase gene and a glycerol kinase gene were also among the genes upregulated in our microarray experiment.

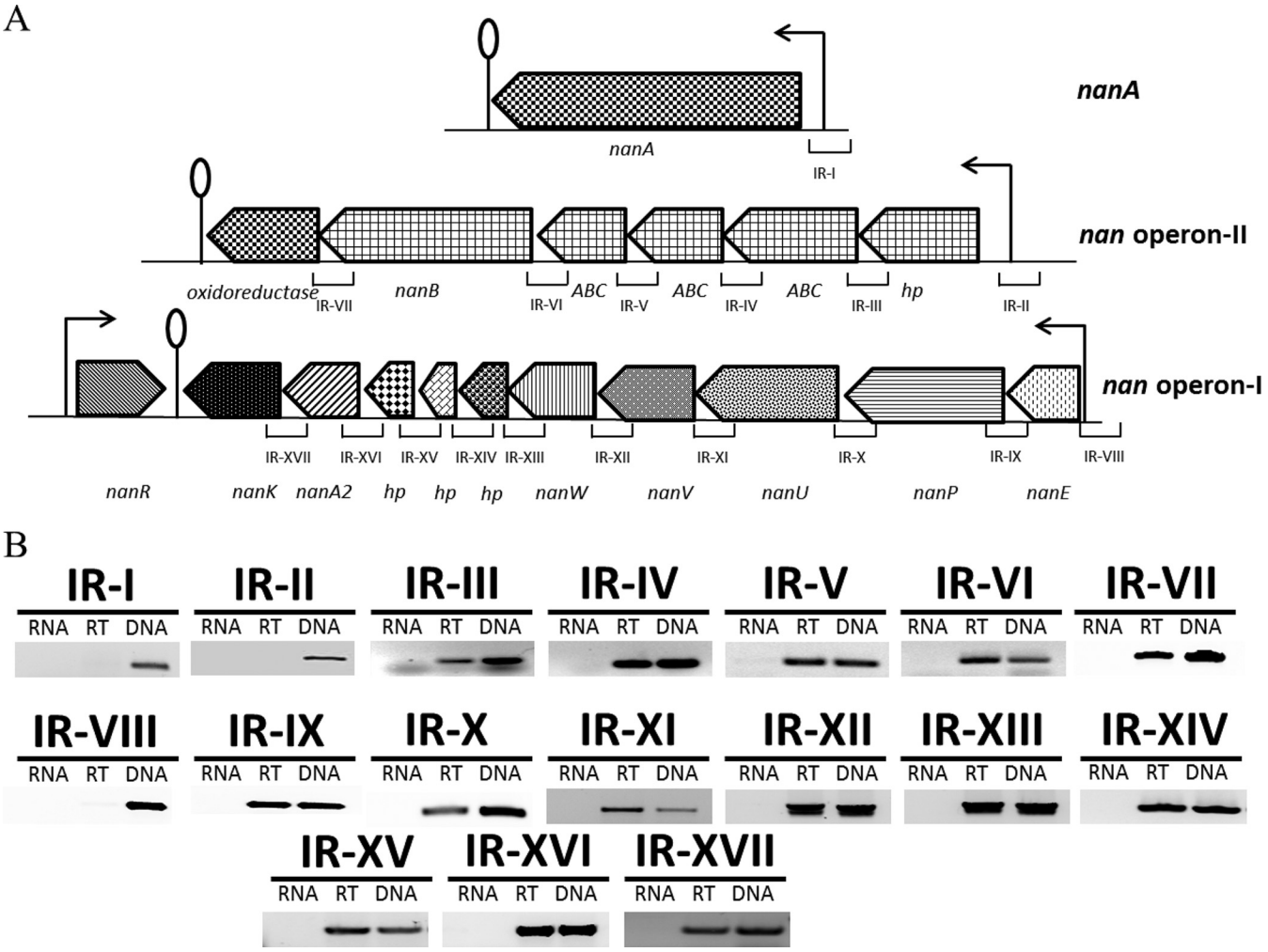

FIG 1 (A) Organization of the *nan* gene cluster in *S. pneumoniae* D39. A lollipop structure represents a putative transcriptional terminator, while black arrows indicate the promoter regions. See the text for further details. (B) RT-PCR analysis to confirm the polycistronic nature of *S. pneumoniae nan* operons I and II. RT-PCR was performed on total RNA isolated from wild-type D39 grown in SM17 (0.5% sialic acid plus M17) medium with (RT) and without (RNA) reverse transcriptase treatment using the intergenic region primer pairs. DNA was used as a positive control. The sizes of RT-PCR products range from 100 to 300 bp.

Sialic acid induces, while glucose represses, the expression of *nan* operon I and the *nanA* gene. To further confirm sialic acid-dependent transcriptome results, we made transcriptional *lacZ* fusions of P*nanA*, P*spd_1503*, and P*nanE* and transformed these *lacZ* fusions into the D39 wild type. β-Galactosidase assays were performed with the strains containing these transcriptional *lacZ* fusions grown in M17 (no added sugar), GM17 (0.5% glucose plus M17), and SM17 medium. β-Galactosidase assays showed that the expression of P*nanA-lacZ* and P*nanE-lacZ* was strikingly higher in the presence of sialic acid in the medium than in the presence of glucose (Fig. 2A). However, no significant change in the expression of P*spd_1503-lacZ* was observed in the presence of sialic acid compared to glucose. This may suggest that *nan* operon II does not respond to sialic acid under our tested conditions. These results further suggest that the expression of *nan* operon I and the *nanA* gene is activated in the presence of sialic acid. Moreover, these results are in accordance with our transcriptome data mentioned above.

To investigate the impact of other sugars (arabinose, cellobiose, dextrose, fructose, fucose, glucose, galactose, lactose, maltose, mannitol, mannose, melibiose, sorbitol, trehalose, and xylose) on the expression of the *nan* gene cluster, we checked the activity of P*nanE-lacZ* in the presence of various sugars (Table 4). β-Galactosidase assay data revealed that the activity of P*nanE-lacZ* was highest in the presence of sialic acid and lowest in the presence of glucose. These data suggest that the *nan* gene cluster specifically responds to sialic acid.

NanR acts as a transcriptional activator of *nan* operon I and the *nanA* gene. NanR, a phosphosugar-binding transcriptional regulator belonging to the RpiR family of transcriptional regulators, is present downstream of the *nan* gene cluster. To investigate the role of NanR in the regulation of the *nan* gene cluster, we constructed a *nanR* deletion mutant in *S. pneumoniae* D39 by replacing *nanR* with a spectinomycin resistance marker and transformed P*nanA-lacZ*, P*spd_1503-lacZ*, and P*nanE-lacZ* transcriptional fusions into the Δ*nanR* strain. β-Galactosidase assays were performed with the strains containing these transcriptional *lacZ* fusions grown in M17 (no added sugar), GM17, and SM17 medium. The assays showed that the deletion of *nanR* led to

TABLE 3 Summary of transcriptome comparison of wild-type *S. pneumoniae* D39 grown in SM17 (0.5% sialic acid plus M17) to that grown in M17

Category and D39 locus tag	Function[a]	Ratio[b]
Upregulated genes		
spd_0091	Hypothetical protein	2.4
spd_0115	Hypothetical protein	2.5
spd_0116	Hypothetical protein	3.0
spd_0373	Hypothetical protein	2.2
spd_1263	ABC transporter, ATP-binding/permease protein	2.2
spd_1264	ABC transporter, ATP-binding protein	2.7
spd_1265	Hypothetical protein	2.9
spd_1267	ABC transporter, ATP-binding protein	3.1
spd_1330	Amino acid ABC transporter, permease protein	5.6
spd_1488	ROK family protein, NanK	4.8
spd_1489	*N*-Acetylneuraminate lyase, putative	3.8
spd_1490	Hypothetical protein	2.6
spd_1491	Hypothetical protein	2.8
spd_1492	Hypothetical protein	2.6
spd_1493	Sugar ABC transporter, permease protein, NanW	2.7
spd_1494	Sugar ABC transporter, permease protein, NanV	2.9
spd_1495	Sugar ABC transporter, sugar-binding protein, NanU	4.4
spd_1496	PTS, IIBC components, NanP	4.2
spd_1497	*N*-Acetylmannosamine-6-phosphate 2-epimerase 2, NanE	2.8
spd_1504	Sialidase A precursor, NanA	6.8
spd_1505	Hypothetical protein	2.0
spd_1800	Hypothetical protein	2.1
spd_2012	Alpha-glycerophosphate oxidase	3.2
spd_2013	Glycerol kinase	3.3
Downregulated genes		
spd_0334	Oligopeptide ABC transporter, oligopeptide-binding protein AliA	−2.4
spd_0447	Transcriptional regulator, GlnR	−5.5
spd_0448	Glutamine synthetase, GlnA	−6.0
spd_0901	Dihydrodipicolinate synthase	−2.0
spd_1098	Amino acid ABC transporter, GlnP	−4.0
spd_1099	Amino acid ABC transporter, GlnQ	−4.0
spd_1158	NADP-specific glutamate dehydrogenase	−3.5
spd_1514	ABC transporter, ATP-binding protein	−3.3
spd_1515	Hypothetical protein	−3.9
spd_1516	Hypothetical protein	−3.6
spd_1956	Dihydroxy-acid dehydratase	−2.0

[a] D39 annotation or TIGR4 annotation (27). PTS, phosphotransferase system.
[b] Fold increase or decrease in the expression of genes in SM17 compared to M17. Errors in the ratios never exceeded 10% of the given values.

inactivation of P*nanA-lacZ* and P*nanE-lacZ* even in the presence of sialic acid, suggesting the role of *nanR* as a transcriptional activator of *nan* operon I and the *nanA* gene in the presence of sialic acid (Fig. 2B). However, no effect of the *nanR* deletion was observed on the activity of P*spd_1503*, suggesting that NanR plays no role in the regulation of *nan* operon II under our tested conditions.

DNA microarray analysis with the Δ*nanR* strain. To elucidate the effect of the *nanR* deletion on the transcriptome of *S. pneumoniae*, DNA microarray analyses were performed with wild-type D39 against its isogenic Δ*nanR* mutant grown in SM17 medium. SM17 medium was used, as our β-galactosidase assays showed that NanR activates its targets in the presence of sialic acid (data shown above). Table 5 lists the results of transcriptome changes induced in *S. pneumoniae* due to the deletion of *nanR*. *nan* operon I and the *nanA* gene were downregulated significantly in the Δ*nanR* strain, confirming the role of NanR as a transcriptional activator of *nan* operon I and the *nanA* gene in the presence of sialic acid. We did not observe any significant differences in the expression of *nan* operon II in our microarray study, confirming that NanR has no role in the regulation of *nan* operon II. These data are also in accordance with the β-galactosidase assay data mentioned above.

Role of CcpA in the regulation of the *nan* gene cluster. CcpA is the global transcriptional regulator in *S. pneumoniae* that represses the expression of genes involved in the utilization of nonpreferred sugars in the presence of a preferred one (8). To study the role of CcpA in the regulation of the *nan* gene cluster, we transformed P*nanA-lacZ*, P*spd_1503-lacZ*, and P*nanE-lacZ* into the Δ*ccpA* strain and performed β-galactosidase assays. The β-galactosidase assay data showed that *ccpA* deletion led to the increased expression of P*nanE-lacZ* in the presence of sialic acid and glucose (Fig. 2C), whereas no change in the expression of P*nanA-lacZ* and P*spd_1503-lacZ* was observed in the presence of sialic acid and glucose (Fig. 2C). These results suggest that CcpA represses the expression of *nan* operon I in the presence of glucose and sialic acid but that regulation of the *nanA* gene and *nan* operon II is CcpA independent. Furthermore, we analyzed the promoter regions of *nanA*, *spd_1503*, and *nanE* to check for the presence of a putative *cre* box. Interestingly, a *cre* box (5′-TTGAA AGCGTTTTAAT-3′) is present in the *nanE* promoter region, supporting the putative role of CcpA in the regulation of *nan* operon I. However, no *cre* box was found in P*nanA* and P*spd_1503*, which suggests the CcpA-independent regulation of *nanA* by the transcriptional activator NanR and *nan* operon II by another, unknown transcriptional regulator.

DNA microarray analysis of the D39 Δ*ccpA* strain. To further verify our β-galactosidase results and to find more targets of CcpA in the presence of sialic acid, we performed microarray analysis with Δ*ccpA* against the wild-type D39 strain in the presence of sialic acid and glucose. Table 6 summarizes the transcriptome changes induced in *S. pneumoniae* due to the deletion of *ccpA* in the presence of sialic acid and glucose. *nan* operon I was significantly upregulated in Δ*ccpA* in the presence of sialic acid and glucose (Table 6), which also confirms our β-galactosidase assay results mentioned above. These results confirm the role of CcpA in the regulation of *nan* operon I. We could not observe any changes in the expression of the *nanA* gene and *nan* operon II in our microarray analysis, which also confirms our β-galactosidase assay results mentioned above. These findings are also consistent with the findings of Carvalho et al. (8), who performed transcriptome analysis with a *ccpA* mutant in the presence of glucose and galactose (in minimal medium). Various other genes and operons were also regulated in our microarray experiment (see Table S1 in the supplemental material). These genes are grouped in COG functional categories according to their putative function (see Table S1 in the supplemental material). Most of these genes have a *cre*

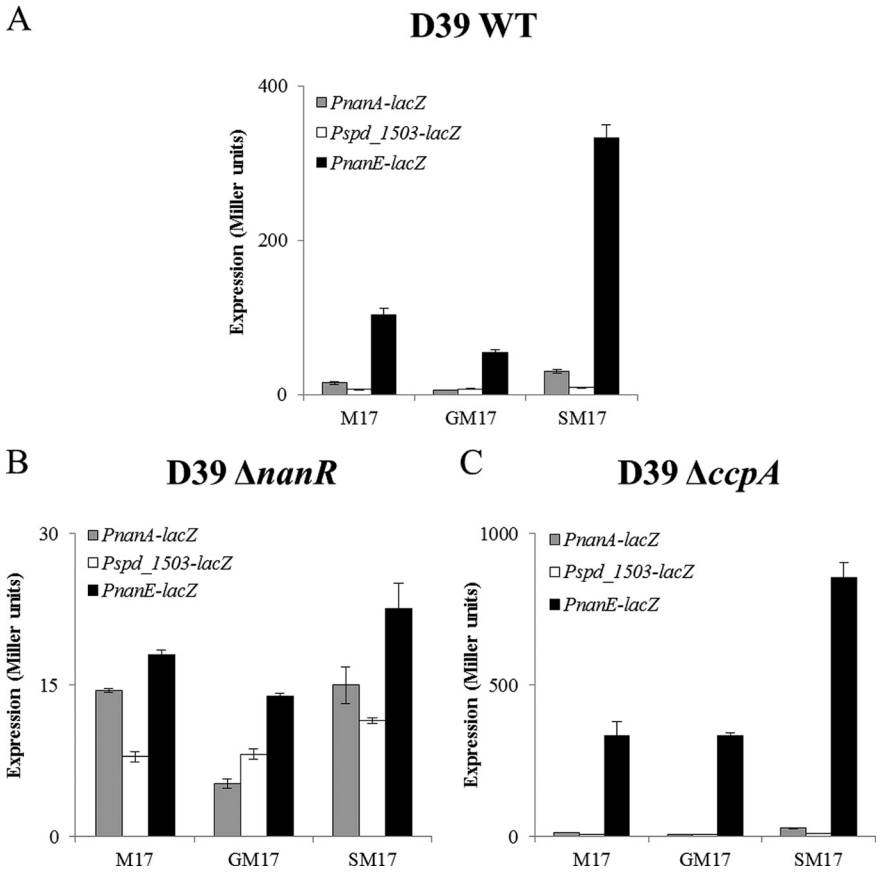

FIG 2 Expression levels (in Miller units) of P*nanA-lacZ*, P*spd_1503-lacZ*, and P*nanE-lacZ* in wild-type D39 (A), D39 Δ*nanR* (B), and D39 Δ*ccpA* (C) grown in M17 (without sugar), GM17 (0.5% glucose plus M17), and SM17 (0.5% sialic acid plus M17). Standard deviations from three independent experiments are indicated by error bars.

box in their promoter regions, suggesting that CcpA regulates these genes in the presence of sialic acid. Most of the genes affected in the microarray experiment were carbohydrate transport and metabolism genes. Amino acid transport and utilization genes were also among the ones that were affected significantly in the transcriptome analysis.

TABLE 4 Expression levels of the P*nanE-lacZ* transcriptional fusion in wild-type *S. pneumoniae* D39 grown in the presence of various sugars in M17 medium

Sugar	β-Galactosidase activity (Miller units)[a]
None	98 (8)
Glucose	51 (1)
Melibiose	94 (1)
Dextrose	46 (4)
Fucose	66 (3)
Sorbitol	114 (13)
Trehalose	89 (2)
Galactose	76 (1)
Maltose	114 (1)
Lactose	60 (2)
Arabinose	97 (2)
Sialic acid	332 (16)
Mannose	151 (2)
Fructose	70 (1)
Xylose	101 (9)
Cellobiose	103 (7)

[a] Standard deviations of three independent experiments are in parentheses.

Prediction and verification of a NanR regulatory site in P*nanE*. By using Genome2D software (34) and a MEME motif sampler search (38), an 18-bp palindromic sequence was found in the promoter regions of *nanA* and *nanE* in *S. pneumoniae* D39 (Fig. 3A). A weight matrix of these putative NanR regulatory sites (5′-TCTGAAASTACTTTCARA-3′) was constructed using these DNA regions. This DNA sequence may serve as the NanR regulatory site in *S. pneumoniae*. We also analyzed the *spd_1503* promoter region for the presence of a putative NanR regulatory site. We could not find any DNA stretch matching the NanR regulatory site in the promoter region of *spd_1503*, which further confirms the NanR-independent regulation of *nan* operon II. P*nanA* and P*nanE* of other streptococcal species were studied to check if the NanR regulatory site is also conserved in those streptococci. From this study, we conclude that the NanR regulatory sequence is highly conserved in these streptococci as well (Fig. 3B).

We further conducted a genome-wide search with the putative NanR regulatory site. The NanR regulatory site was also found in the upstream region of the *nanA2* gene. We had already shown by RT-PCR that *nanA2* is part of *nan* operon I (Fig. 1B). To confirm this further, we constructed a transcriptional *lacZ* fusion with the upstream region of the *nanA2* and performed a β-galactosidase

TABLE 5 Summary of transcriptome comparison of *S. pneumoniae* D39 Δ*nanR* and wild-type D39 grown in SM17 (0.5% sialic acid plus M17)

D39 locus tag	Function[a]	Ratio[b]
spd_1487	Phosphosugar-binding transcriptional regulator, NanR	−1.4
spd_1488	ROK family protein, NanK	−8.0
spd_1489	*N*-Acetylneuraminate lyase, NanA2	−6.1
spd_1490	Hypothetical protein	−3.4
spd_1491	Hypothetical protein	−2.1
spd_1492	Hypothetical protein	−2.2
spd_1493	Sugar ABC transporter, permease protein, NanW	−4.1
spd_1494	Sugar ABC transporter, permease protein, NanV	−5.2
spd_1495	Sugar ABC transporter, sugar-binding protein, NanU	−4.1
spd_1496	PTS, IIBC components, NanP	−5.0
spd_1497	*N*-Acetylmannosamine-6-phosphate 2-epimerase 2, NanE	−4.6
spd_1504	Sialidase A, NanA	−5.9

[a] D39 annotation or TIGR4 annotation (27). PTS, phosphotransferase system.
[b] Fold decrease in the expression of genes in the Δ*nanR* mutant compared to the wild type. *nanR* is downregulated less than 2-fold but is listed in the table to confirm the deletion of *nanR*. Errors in the ratio numbers never exceeded 10% of the given values.

TABLE 6 Summary of transcriptome comparison of *S. pneumoniae* D39 Δ*ccpA* and wild-type D39 grown in SM17 (0.5% sialic acid plus M17) and GM17 (0.5% glucose plus M17)

D39 locus tag	Function[a]	Ratio[b] in:	
		SM17	GM17
spd_1488	ROK family protein, NanK	1.6	4.2
spd_1489	*N*-Acetylneuraminate lyase, NanA2		4.5
spd_1490	Hypothetical protein	1.6	3.7
spd_1491	Hypothetical protein	2.0	4.7
spd_1492	Hypothetical protein	2.0	4.4
spd_1493	Sugar ABC transporter, permease protein, NanW	2.5	6.2
spd_1494	Sugar ABC transporter, permease protein, NanV	2.2	6.4
spd_1495	Sugar ABC transporter, sugar-binding protein, NanU	5.4	
spd_1496	PTS, IIBC components, NanP	4.7	8.8
spd_1497	*N*-Acetylmannosamine-6-phosphate 2-epimerase 2, NanE	1.6	4.6

[a] D39 annotation or TIGR4 annotation (27). PTS, phosphotransferase system.
[b] Fold increase in the expression of genes in the Δ*ccpA* mutant compared to the wild type. Errors in the ratio numbers never exceeded 10% of the given values.

assay with strain D39 containing P*nanA2-lacZ* in the presence of glucose and sialic acid. We could not see any changes in the expression of P*nanA2-lacZ*, even in the presence of sialic acid (data not shown). Our β-galactosidase assay with P*nanA2-lacZ* also suggests that the NanR regulatory site present upstream of *spd_1489* is most likely not functional.

To verify the NanR regulatory site present in P*nanE*, transcriptional *lacZ* fusions to 5′ truncations of P*nanE* were constructed (Fig. 3C). β-Galactosidase assays revealed that deletion of half or full of the predicted NanR regulatory site in P*nanE* abolished activity in M17, SM17, or GM17 medium. However, we observed expression similar to that of the full-length promoter when the promoter (P*nanE*) was truncated only a few bases upstream of the predicted regulatory site (Fig. 3C). These data confirm that the NanR regulatory site present in P*nanE* is functional and acts as a NanR regulatory site in *S. pneumoniae*.

DISCUSSION

In this study, we explored the regulatory mechanism of the *nan* gene cluster and found that the *nan* gene cluster consisting of *nan* operon I and the *nanA* gene is functional and responds to sialic acid in *S. pneumoniae* D39. The results presented in this study will increase our understanding regarding sialic acid-dependent regulation of the *nan* gene cluster in *S. pneumoniae* significantly. The *nan* gene cluster, putatively responsible for the utilization of sialic acid in *S. pneumoniae*, shows many variations among various pneumococcal strains. The provided annotation of *S. pneumoniae* TIGR4 genome suggests that there are two putative systems for sialic acid utilization, which are most likely regulated by two different transcriptional regulators (NanR1 and NanR2) (39). However, in *S. pneumoniae* D39, only one system for sialic acid utilization is present that is regulated by a transcriptional regulator NanR (this study). Based on the number of sialic acid systems, pneumococcal strains available in the RegPrecise database (http://regprecise.lbl.gov/RegPrecise/) can be divided into two groups. The first group consists of *S. pneumoniae* CDC0288-04, CGSP14, G54, Hungary19A-6, JJA, MLV-016, SP11-BS70, and SP9-BS68. Like the TIGR4 strain, these strains have two *nan* systems, whereas the second group possesses only one *nan* system, like D39, and includes R6, SP195, SP23-BS72, SP3-BS71, SP6-BS73, SP14-BS69, and P1031. These variations in the *nan* gene cluster among pneumococcal strains suggest the importance of the *nan* gene cluster in the physiology and lifestyle of *S. pneumoniae*.

Sialic acid may enter the cell through either the ABC transporters (NanUVW), a secondary transporter, or tripartite ATP-independent periplasmic (TRAP) transporters (40). Once internalized, NanK and NanE lead the metabolism of sialic acid. NanK converts ManNAc into ManNAc-6P by phosphorylating it at C-6. NanE then converts ManNAc-6P to GlcNAc-6P. NagA (*N*-acetylglucosamine-6-phosphate deacetylase) and NagB (glucosamine-6-phosphate deaminase), which perform the final two reactions of the sialic acid utilization pathway, are the two enzymes not encoded by the *nan* gene cluster (40). The *S. pneumoniae* D39 *nan* gene cluster codes for NanA, which may start the catabolism of sialic acid, and NanK and NanE, which may lead the further metabolism of ManNAc. Interestingly, the genome of *S. pneumoniae* D39 also encodes NagA and NagB. We could not see any change in expression of *nagA* and *nagB* in our transcriptome study in the presence of sialic acid. Further studies focusing on the regulation of *nagA* and *nagB* are required.

NanA and NanB are the pneumococcal neuraminidases that cleave sialic acid, although the localization and cleavage specificity of NanA differ from those of NanB, which suggests distinct roles of these two neuraminidases in *S. pneumoniae* (41). NanA is a hydrolytic enzyme that is attached to the cell surface and cleaves α2-3- and α2-6-linked sialic acid, whereas NanB is a secreted neuraminidase that shows strict specificity for α2-3-linked sialic acid (41, 42). NanA is important for pathogenesis in the host, as it helps in unmasking carbohydrate receptors for attachment, providing a carbon source for the bacteria, changing the surface of other bacteria in the same niche, and disturbing the function of host defense molecules (41). NanA was highly expressed under our tested conditions. Moreover, we show that NanR activates the

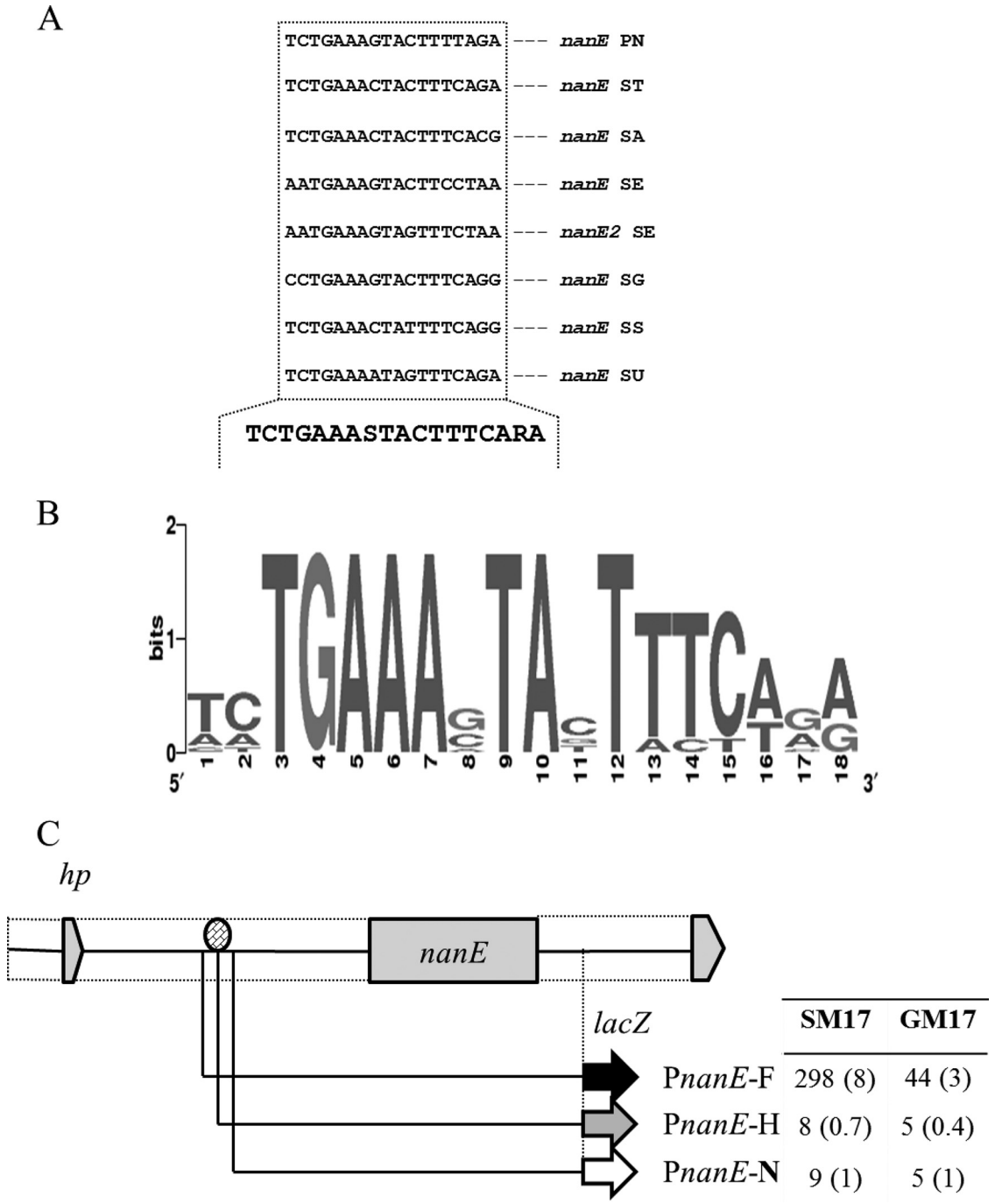

FIG 3 Identification of the NanR regulatory site in P*nanE*. (A) Position of the NanR regulatory site in the P*nanE* of different streptococci. PN, *S. pneumoniae*; ST, *S. mitis*; SA, *S. agalactiae*; SD, *S. dysgalactiae*; SE, *S. equi*; SM, *S. mutans*; SP, *S. pyogenes*; SG, *S. sanguinis*; SS, *S. suis*; SU, *S. uberis*. (B) Weight matrix of the identified NanR regulatory site in P*nanE* of different streptococci. (C) Verification of NanR regulatory site in the P*nanE*. A schematic illustration of P*nanE* truncations is shown. The oval indicates the putative NanR regulatory site. The table gives the β-galactosidase activity (Miller units) of the truncated promoters fused with *lacZ* in wild-type *S. pneumoniae* D39 grown in GM17 (0.5% glucose plus M17) and SM17 (0.5% sialic acid plus M17) medium. Standard deviations from three independent experiments are given in parentheses.

nanA gene and *nan* operon I in the presence of sialic acid. These observations suggest that NanA is a suitable target for drugs and vaccines against pneumococci. The gene coding for NanB is one of the six genes present in *nan* operon II. However, *nan* operon II is not regulated under our tested conditions, and NanR and CcpA also have no role in its regulation of *nan* operon II under our tested conditions. Future investigations focusing on the conditions and mechanisms under which *nan* operon II is regulated are required.

CcpA is the master transcriptional regulator of the sugar utili-

zation systems in *S. pneumoniae* and regulates multiple sugar systems in a pleiotropic manner (8, 9, 43). However, many systems dedicated to the utilization of nonpreferred sugars are regulated independently of CcpA in *S. pneumoniae*, like the *cel* and *lac* systems (25, 31). In this study, we show that CcpA plays an important role in the regulation of *nan* operon I in *S. pneumoniae* D39. Our results also show that the regulation of the *nanA* gene and *nan* operon II is CcpA independent. Similarly, recent transcriptome analysis of a *ccpA* mutant in the presence of glucose and galactose revealed that CcpA represses the expression of *nan* operon I in the presence of glucose and galactose (8). However, no effect of *ccpA* deletion on the expression of *nan* operon II and the *nanA* gene was observed in the presence of glucose and galactose (8). Therefore, data presented in our study are consistent with the observations presented in a previous study on CcpA (8).

In this study, we have shown that NanR (an RpiR-type transcriptional regulator), located downstream of the *nan* gene cluster, acts as a transcriptional activator of *nan* operon I and the *nanA* gene in the presence of sialic acid. NanR has a DNA-binding helix-turn-helix (HTH) domain and a sugar isomerase (SIS) domain and shares >90% sequence identity to its counterparts in *S. oralis*, *S. tigurinus*, *S. mitis*, and *S. pseudopneumoniae*. The SIS domain is a phosphosugar-binding domain found in many phosphosugar isomerases and phosphosugar binding proteins that regulate the expression of genes involved in the synthesis of phosphosugars possibly by binding to the end product of the pathway (44). RpiR-type transcriptional regulators have been shown to regulate various sugar systems in different bacteria. These regulators mostly control sugar and sugar phosphate catabolic pathways, including maltose, glucose, and ribose metabolism, the pentose phosphate pathway, inositol catabolism, and *N*-acetylmuramic acid catabolism (39). For example, HpxU is an RpiR-type transcriptional regulator in *Klebsiella pneumoniae* that has been shown to act as a transcriptional repressor of allantoate catabolism (45). In *E. coli*, NanR has also been shown to act as a transcriptional activator of the sialic acid gene cluster (46).

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