Sialic Acid Catabolism in \textit{Staphylococcus aureus}

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\textit{Staphylococcus aureus} is a ubiquitous bacterial pathogen that is the causative agent of numerous acute and chronic infections. \textit{S. aureus} colonizes the anterior nares of a significant portion of the healthy adult population, but the mechanisms of colonization remain incompletely defined. Sialic acid (N-acetylneuraminic acid [Neu5Ac]) is a bioavailable carbon and nitrogen source that is abundant on mucosal surfaces and in secretions in the commensal environment. Our findings demonstrate that Neu5Ac can serve as an \textit{S. aureus} carbon source, and we have identified a previously uncharacterized chromosomal locus (\textit{nan}) that is required for Neu5Ac utilization. Molecular characterization of the \textit{nan} locus indicates that it contains five genes, organized into four transcripts, and the genes were renamed \textit{nanE, nanR, nanK, nanA}, and \textit{nanT}. Initial studies with gene deletions indicate that \textit{nanT}, predicted to encode the Neu5Ac transporter, and \textit{nanA} and \textit{nanE}, predicted to encode catabolic enzymes, are essential for growth on Neu5Ac. Furthermore, a \textit{nanE} deletion mutant exhibits a growth inhibition phenotype in the presence of Neu5Ac. Transcriptional fusions and Northern blot analyses indicate that NanR represses the expression of both the \textit{nanAT} and \textit{nanE} transcripts, which can be relieved with Neu5Ac. Electrophoretic mobility studies demonstrate that NanR binds to the \textit{nanAT} and \textit{nanE} promoter regions, and the Neu5Ac catabolic intermediate N-acetylmannosamine-6-phosphate (ManNAc-6P) relieves NanR promoter binding. Taken together, these data indicate that the \textit{nan} gene cluster is essential for Neu5Ac utilization and may perform an important function for \textit{S. aureus} survival in the host.

\textit{Staphylococcus aureus} is a Gram-positive bacterium that produces an impressive arsenal of tissue-degrading enzymes and pore-forming toxins (1). This opportunistic pathogen causes a diverse spectrum of acute and chronic infections, and recent community-associated methicillin-resistant \textit{S. aureus} (CA-MRSA) isolates are notorious for invasive disease associated with severe morbidity and mortality, even in otherwise healthy subjects (2, 3). \textit{S. aureus} colonizes the nasal and throat regions of the upper respiratory tract of approximately 30% of the healthy adult population (4). Epidemiology studies have revealed that some strain groups prevail in asymptomatic colonization, whereas others dominate invasive infections (5, 6). Multiple studies have demonstrated that the majority of \textit{S. aureus} infections are caused by the strain the infected individual carries (7–11).

While the pathogenic roles of many \textit{S. aureus} secreted factors and surface proteins have been extensively studied, the physiology of this commensal in the colonization state and other host environments has received only limited attention (12, 13). Sialic acid (N-acetylneuraminic acid [Neu5Ac]) is one example of a bioavailable energy source present in many host niches (14). Neu5Ac is the terminal moiety of glycan molecules on the surface of eukaryotic cells and is involved in diverse cellular functions ranging from adhesion to cell signaling (15). Additionally, Neu5Ac serves as an attachment and recognition point for a variety of pathogens (14, 16). Given that this molecule is at the interface of commensal colonization, we hypothesized that \textit{S. aureus} may sense and utilize Neu5Ac during growth in the host.

Although the total Neu5Ac concentration in humans is quite high (2 mM), much of the molecule is incorporated into glycoproteins or lipid acceptors, making it inaccessible (17). There is speculation that sialidases (also called neuraminidases) secreted by members of the normal flora, such as \textit{Streptococcus pneumoniae}, can release sialic acid from glycans, making it bioavailable to other commensals (18). Breakdown of Neu5Ac involves transport into the cell and degradation through a series of reactions to the end product fructose-6-phosphate (fructose-6P), which can then enter central metabolism (Fig. 1). Many species that utilize Neu5Ac lack the anabolic pathways and rely on the human host to generate the compound (14, 19). Acquisition and use of Neu5Ac have been shown to aid \textit{Escherichia coli} colonization of the mouse colon (20), and liberation of Neu5Ac gives a competitive growth advantage to \textit{Vibrio cholerae} and \textit{S. pneumoniae} (21–23). In the case of \textit{Haemophilus influenzae}, Neu5Ac is obtained either through polymicrobial action of other sialidases, either due to commensals or pathogens residing in the same niche or from host-derived sialidases, which are induced during the inflammation process (19, 24).

In this study, we investigated the ability of \textit{S. aureus} to catabolize Neu5Ac. All \textit{S. aureus} strains tested, and three other species of staphylococci, were shown to utilize Neu5Ac as a carbon source, and we identified a five-gene locus, called \textit{nan}, that is responsible for \textit{S. aureus} catabolism. The molecular architecture of the \textit{nan} locus was examined, and the function of a transcriptional regulator encoded in the locus, NanR, was investigated.

**MATERIALS AND METHODS**

**Strains and growth conditions.** Bacterial strains used in this study are listed in Table 1. \textit{E. coli} was grown on Luria-Bertani (LB) medium. \textit{S. aureus} cultures were grown in tryptic soy broth (TSB) without dextrose as the baseline rich medium, which was then supplemented with glucose or...
Neu5Ac. To assess carbon source utilization, a carbon-limiting medium (CLM) was generated which consisted of ammonium sulfate (7.5 mM), potassium phosphate (33 mM), dipotassium phosphate (60 mM) supplemented with NaCl (11 mM), KCl (2 mM), Casamino Acids (BD Biosciences) (0.5%), MgSO4 (0.1 mM), and the vitamins nicotinamide (500 μg/liter), thiamine (500 μg/liter), pantothenate (300 μg/liter), and biotin (0.3 μg/liter). Single-distilled water was used for all components, and they were filter sterilized and combined. This medium was further supplemented with 0.02% glucose, 0.01% Neu5Ac, or water when no additional carbon source was needed. CLM (or TSB in other experiments) was supplemented with less Neu5Ac than glucose in order to keep carbon equivalents at similar levels. All cultures were grown at 37°C with appropriate antibiotics at concentrations of 10 μg/ml for chloramphenicol, erythromycin, and tetracycline and 50 μg/ml for kanamycin, as needed. Chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

**Neu5Ac utilization, complementation, and sGFP reporter fusion testing.** Growth of various staphylococcal species and nan locus deletion mutants was performed in 5 ml of CLM in 18- by 150-mm tubes, and strains were incubated at 37°C with shaking at 200 rpm. For complementation, strains with plasmids were grown in CLM plus antibiotic. Growth was assessed by taking readings of the optical density at 600 nm (OD600) over time. For superfolder green fluorescent protein (sGFP) reporter plasmids, strains were grown in 5 ml of TSB in the same manner as described above. Either unsupplemented TSB or TSB supplemented with 0.2% glucose or 0.1% Neu5Ac was used as indicated below for the reporter experiments. Measurements of sGFP fluorescence were recorded on a Tecan Infinite M200 at 4, 7, 24, and 30 h. The values were reported as relative fluorescence after normalization for OD600 readings. For each approach outlined here, at least three biological replicates were performed.

**Recombinant DNA and genetic techniques.** Restriction enzymes, Antarctic phosphatase, Phusion DNA polymerase, and T4 DNA polymerase were obtained from New England Biolabs (Beverly, MA). Oligonucleotide primers were purchased from Integrated DNA Technologies (Coralville, IA) and are listed in Table S1 in the supplemental material. Competent S. aureus cells were prepared as previously described (25).
Plasmids were moved from RN4220 into various backgrounds by transduction using bacteriophage P11 or 80a as described previously (26).

Construction of *S. aureus* deletion mutants. ΔnanH, ΔnanE, ΔnanR, ΔnanT, and ΔnanK deletion strains were constructed by amplification of the ~500-bp flanking regions upstream and downstream of each target gene. All deletion mutants were generated using the same general scheme. Briefly, the flanking regions of DNA (such as for nanH) were amplified and joined by overlap PCR extension using genomic DNA from strain AH1263 as the template. The outermost primers were EcoRI and AvalI targeted, and the inner primers each have complementary Nhel/MulI overhangs to aid the overlap extension. For the ΔnanH deletion, oligonucleotides 11(EcoRI) and 17(AvalI) were used to generate the final cloning fragment from the PCR products 11-12(Nhel/MulI) and 16(Nhel/MulI)-17. For ΔnanE, oligonucleotides 48 and 51 generated the final products 48-49 and 50-51. For ΔnanK, oligonucleotides 33 and 36 generated the final products 33-34 and 35-36. For ΔnanT, oligonucleotides 28 and 31 generated the final products 28-29 and 30-31. Lastly, for ΔnanR, oligonucleotides 52 and 55 generated the final products 52-53 and 54-55. The purified PCR products were ligated with T4 DNA ligase (Invitrogen) into cleotides 52 and 55 generated the final products 52-53 and 54-55. The plasmids were constructed using the same 300 bp) upstream of the pre-}

End of the translational start site that was fused to sGFP. Purified PCR products were ligated with T4 DNA ligase (Invitrogen) into plasmid 53-54 and 55-56. The plasmids were constructed using the same general scheme. Purified PCR products were ligated with T4 DNA ligase (Invitrogen) into cleotides 52 and 55 generated the final products 52-53 and 54-55. The plasmids were constructed using the same general scheme.

**Constitution of complementation and reporter plasmids.** Complementation plasmids for each *nan* locus gene were constructed by PCR amplification of the promoter region (~300 bp) upstream of the predicted start site through the stop codon. The PCR product was purified and ligated into the BamHI and XmaI sites of pSKerm-MCS. GFP reporter plasmids were constructed using the same ~300-bp promoter region and ending at the translational start site that was fused to sGFP. Purified PCR fragments were cloned into the KpnI and HindIII sites of pCM11, allowing the promoter to drive expression of sGFP expression.

**RNA purification and Northern blots.** Bacteria were grown in TSB (prepared without glucose) supplemented with Neu5Ac or glucose. When the culture reached an OD₆₀₀ of 1.0, 5 ml of culture was harvested by centrifugation and stored in RNA Later (Qiagen) at −20°C. Cells were pelleted and suspended in buffer (50 mM Tris·HCl [pH 7.9], 0.15 M NaCl) and treated with 2.5 μg of lysostaphin (AMBI Products, Lawrence, NY) for 1 h at 37°C. RNA was purified using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Northern blot analysis of the promoter regions of *nanA* and *nanE* were amplified using primer sets MO122/MO123 and MO124/MO125, respectively. The EMSAs specific probe was amplified from the intergenic region between nanR and nanK using primer set MO126/MO127. Probes were end labeled (1 pmol) using 10 μCi of [γ⁻³²P]ATP (GE Healthcare) and 10 U of polynucleotide kinase (New England Biolabs) according to the manufacturer’s instructions. Unlabeled [γ⁻³²P]ATP was removed using a nucleotide removal column (Qiagen). EMSA experiments were performed as follows. Briefly, reaction mixtures (19 μl) containing labeled specific and nonspecific probes (0.25 nM each), 25 ng/ml of poly(2'-deoxyinosinic-2'-deoxycytidylic acid), and 10 μl of 2X binding buffer (20 mM Tris·HCl [pH 7.5], 200 mM KCl, 2 mM EDTA, 2 mM DTT, 1% glycerol, and 200 mg/ml of bovine serum albumin) were incubated for 5 min at 25°C. NanR-maltose binding protein (NanR-MBP) was added at various concentrations in a final reaction volume of 20 μl and incubated at 25°C for 15 min. Samples were immediately subjected to electrophoresis on a 5% nondenaturing polyacrylamide gel (10 mM Tris·HCl [pH 7.5], 380 mM glycine, 1 mM EDTA). In some experiments, Neu5Ac (0 to 5 mM), mannose-N-acetylnearcuminic acid (ManNac) (0 to 25 mM), glucose (0 to 25 mM), or ManNac-6P (see below) was included in the binding reactions. Imaging and data analyses (apparent equilibrium constant Kᵦₑᵦ calculations) were performed using an FLA-7000 phosphor-imager and MultiGauge, version 3.0, software (Fujifilm).

**Preparation of ManNac-6P.** Briefly, NanK-His₆ (1 mg of protein) was incubated for 30 min at 37°C in a total volume of 2 ml containing 20 mM ATP, 60 mM Tris·HCl (pH8.0), 20 mM MgCl₂, and 4 mM ManNac. ManNac kinase activity was detected by resolving the reaction by thin-
layer chromatography (TLC) on precoated silica gel 60 TLC plastic sheets (Merck) using a solvent mixture of n-propanol, 1 M sodium acetate (pH 5.0), and water at a 7:1:2 ratio as described by Ringenberg et al. (30). ManNAc-6-phosphate (ManNAc-6P) product generation was visualized by developing the plates with diphenylamine (DPA) reagent (Carolina Biological Supply) and heating to 100°C for 10 min. In TLC, ManNAc migrated faster and reached 80% of the solvent front, while the phosphorylated product (ManNAc-6P) only reached 20% of the solvent front. This reaction mix was passed through a 10-kDa-cutoff filter (Amicon) to remove proteins, and the filtrate (solute containing fraction) was used for EMSA experiments.

RESULTS

S. aureus can use Neu5Ac as a carbon source. Preliminary bioinformatic studies indicated that S. aureus strains may possess some of the genes needed to scavenge bioavailable Neu5Ac (31). In our examination of the S. aureus chromosome, using USA300 strain FPR3757 as a reference (32), we identified a cluster of five open reading frames (ORFs) on the core genome with sequence similarity to known Neu5Ac catabolic genes from nontypeable H. influenzae (1A). Based on homology to their counterparts, we hypothesized that the nanT locus (ORF SAUSA300_0315) and nanT (ORF SAUSA300_0314) genes that appeared to be cotranscribed in a two-gene operon (Fig. 1A). Based on homology to their H. influenzae counterparts, we hypothesized that the nanT gene encodes a Neu5Ac transporter and nanA encodes the Neu5Ac lyase enzyme, which is the first step in the catabolic pathway (Fig. 1B). Upstream of the nanA genes, we identified three additional ORFs that appeared related to the Neu5Ac catabolic cluster. Two of these ORFs included putative nanK (ORF SAUSA300_0316) and nanE (ORF SAUSA300_0318) genes. We hypothesized that the nanK gene encodes a kinase that can phosphorylate the end product of the NanA reaction, ManNAc, and that the nanE gene encodes an epimerase that converts ManNAc-6P into N-acetylgalactosamine-6-phosphate (GlcnAc-6P) (Fig. 1B). Finally, the bioinformatic analysis revealed the presence of an RpiR family transcriptional regulator (ORF SAUSA300_0317), and we have renamed this putative regulator NanR.

Interestingly, the nan gene clusters in other bacterial genomes, such as those of E. coli and H. influenzae, are frequently associated with nagA and nagB (14, 33). However, in S. aureus, the nagAB genes are located elsewhere (~370 genes away) in the chromosome (ORF SAUSA300_0686-7 compared with SAUSA300_0314-8). The nagAB genes encode enzymes that convert the Neu5Ac breakdown product, GlcNAc-6P, to fructose-6P and incorporate it into central metabolic pathways (34). Additionally, the S. aureus nan gene cluster does not have a canonical operonic structure; instead, the genes appear to be organized as four separate transcripts (Fig. 1A).

To investigate Neu5Ac catabolism in S. aureus, we developed a carbon-limiting defined medium. By comparing growth yields in media supplemented with glucose, Neu5Ac, or no addition, it was apparent that S. aureus has the capacity to utilize Neu5Ac as a carbon source (Fig. 2A). Although the growth rates with Neu5Ac supplementation were lower than achieved with glucose, Neu5Ac-supplemented growth was accomplished by central metabolic pathways. The unusual molecular arrangement of the genes is also maintained. Notably, this gene cluster is absent in all of the sequenced strains of S. epidermidis. Also of interest is that S. lugdunensis has the nagAB genes adjacent to the nan cluster. To test whether the presence of the locus correlated with growth on Neu5Ac, we tested these different species to determine if they contained functional catabolic pathways (Fig. 2B). S. lugdunensis, S. saprophyticus, and S. intermedius were able to utilize Neu5Ac, but S. carnosus was not. As expected, none of the three S. epidermidis strains tested (1457, RP62a, and ATCC 12228) catabolized Neu5Ac, given that these strains lacked the nan genes. Additionally, a diverse set of S. aureus isolates was tested to assess the conservation of Neu5Ac utilization across lineages. This strain set included representatives of USA100 (BK19296), USA200 (UAMS-1 and MRSA252), USA400 (MW2), and USA600 (BK21157) and laboratory strains COL, Newman, and HG001. All
of these strains were able to catabolize Neu5Ac in a manner similar to that of AH1263 (data not shown).

**nanA, nanT, and nanE are necessary for Neu5Ac utilization as a carbon source.** To investigate the biological function of the genes in the *S. aureus* nan locus, markerless deletion mutants were constructed for all five nan genes. As a genetic host, we used AH1263, the CA-MRSA isolate LAC, and a member of the USA300 lineage (35). These deletion mutants were tested for their ability to utilize Neu5Ac as a carbon source (Fig. 3). Utilization of Neu5Ac is apparent in the LAC wild type (LAC-WT) by the inability to utilize Neu5Ac as a carbon source (Fig. 3). Utilization of Neu5Ac in the locus is induced by Neu5Ac. Surprisingly, the presence of Neu5Ac inhibited the growth of the nanA mutant, as shown with nanA-ΔH11001 (WT) and strains with deletions in the nanE, nanT, nanK, and nanA genes in the nan locus (Fig. 3). For the nanK mutant, as shown with nanK-ΔH11001, the CA-MRSA isolate LAC, and a member of the USA300 lineage (35). These deletion mutants were tested for their ability to utilize Neu5Ac as a carbon source (Fig. 3). Utilization of Neu5Ac is apparent in the LAC wild type (LAC-WT) by the inability to utilize Neu5Ac as a carbon source (Fig. 3). Utilization of Neu5Ac in the locus is induced by Neu5Ac. Surprisingly, the presence of Neu5Ac inhibited the growth of the nanA mutant, as shown with nanA-ΔH11001 (WT) and strains with deletions in the nanE, nanT, nanK, and nanA genes in the nan locus (Fig. 3).

**Transcriptional reporters indicate that NanR functions as a repressor and the nan locus is induced by Neu5Ac.** We investigated regulation in the nan locus by constructing a transcriptional reporter with the nanA promoter driving sGFP expression (PnanA-sGFP). The resulting plasmid was transduced into LAC-WT and ΔnanR strains, and the constructed strains were grown in unsupplemented medium or medium supplemented with either glucose or Neu5Ac. The PnanA-sGFP reporter showed a clear response to different growth conditions. A marked increase in fluorescence output was seen when Neu5Ac was present compared to that grown in unsupplemented medium (Fig. 4A). However, expression from the reporter was repressed with glucose supplementation. In contrast, expression from the PnanA-sGFP reporter was dysregulated under all growth conditions in the ΔnanR mutant background (Fig. 4). Both in unsupplemented growth medium and in the presence of Neu5Ac, higher levels of sGFP were observed at late time points compared to those of the WT (Fig. 4B). The repressive effect of glucose was also lost in the ΔnanR mutant. Based on these observations, we hypothesized that NanR functions as a transcriptional repressor of nanA and that Neu5Ac serves as an inducer of locus expression. These experiments also suggest that catabolite repression may occur at the nanA promoter.

We also tested regulation at the nanK and nanR promoters using the sGFP reporter. Under different growth conditions, the PnanK-sGFP reporter appeared to be constitutively expressed irrespective of strain background or growth condition (see Fig. S1A and B in the supplemental material). Interestingly, the PnanR-sGFP reporter behaved in a manner similar to that of the nanK promoter with an absence of NanR-dependent regulation (see Fig. S1C and D in the supplemental material). Taken together, the results show that the nanA promoter is responsive to the NanR regulatory action, Neu5Ac induction, and glucose repression, while the nanR and nanK promoters appear to be constitutive under all the conditions tested.

**Identification of nan locus transcriptional start sites.** To further investigate nan locus regulation, 5’ RACE was performed to identify the transcriptional start site for each of the four promoters in the locus (Fig. 5). For the nanAT transcript, the analysis revealed the +1 site to be nucleotide G, which is located 40 bp upstream of the nanA methionine start codon. A potential Pribnow box centered 10 bp upstream of the start site was identified, lending further credence to the 5’ RACE results. For the other transcripts, the start site for nanE was identified as a G nucleotide located 38 bp upstream of the start codon, the nanR start was an A

### TABLE 2 Protein sequence identity of nan gene clusters across staphylococci

<table>
<thead>
<tr>
<th>Organism ORF</th>
<th>NanE</th>
<th>NanR</th>
<th>NanK</th>
<th>NanA</th>
<th>NanT</th>
</tr>
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<tbody>
<tr>
<td>S. carnosus a</td>
<td>80</td>
<td>62</td>
<td>60</td>
<td>79</td>
<td>80</td>
</tr>
<tr>
<td>S. lugdenensis b</td>
<td>81</td>
<td>63</td>
<td>56</td>
<td>83</td>
<td>80</td>
</tr>
<tr>
<td>S. saprophyticus c</td>
<td>82</td>
<td>73</td>
<td>57</td>
<td>82</td>
<td>81</td>
</tr>
<tr>
<td>S. hominis d</td>
<td>76</td>
<td>62</td>
<td>59</td>
<td>83</td>
<td>78</td>
</tr>
<tr>
<td>S. lauenolyticus e</td>
<td>80</td>
<td>63</td>
<td>60</td>
<td>86</td>
<td>81</td>
</tr>
<tr>
<td>S. epidermidis f</td>
<td>NP</td>
<td>NP</td>
<td>NP</td>
<td>NP</td>
<td>NP</td>
</tr>
</tbody>
</table>

a *S. carnosus* TM300, GenBank accession no. NC_012121.1.

b *S. lugdenensis* HKU09-01, GenBank accession no. NC_013893.1.

c *S. saprophyticus* ATCC 15305, GenBank accession no. NC_007350.1.

d *S. hominis* SK119, GenBank accession no. NZ_ACLP01000000.

e *S. haemolyticus* ISC11435, GenBank accession no. NC_007166.1.

f *S. epidermidis* RP62a, GenBank accession no. NC_002976.3, and strain ATCC 12228, GenBank accession no. NC_019303.1.

NP, not present.

![FIG 3 Growth phenotypes of Neu5Ac catabolic pathway mutants. Wild-type AH1263 (WT) and strains with deletions in the nan locus (ΔnanE, ΔnanR, ΔnanK, and ΔnanT) were grown in CLM alone or supplemented with Neu5Ac. The ΔnanE, ΔnanR, ΔnanK, and ΔnanA mutants were also complemented with plasmids containing single genes in CLM with Neu5Ac. Cultures were grown for 24 h, and OD600 readings were obtained. Error bars shown are standard deviations of at least three biological replicates.](http://jb.asm.org/)
nucleotide located 22 bp upstream, and the nanK start was also an A located 44 bp upstream. A schematic of the nan genetic locus, all four transcriptional start sites, and putative −10 boxes in relation to methionine start codons is shown in Fig. 5. Putative −35 boxes are also indicated for all promoters except the nanR gene.

Northern blot analysis of nan locus. Northern blots were performed in the presence of glucose or Neu5Ac to gain insight into the transcriptional activity occurring at the nan locus. Comparative analyses were performed on the LAC-WT and the ΔnanR, ΔnanE, and ΔnanE mutant strains. Given the complexity of this locus, probes for both the nanT (Fig. 6A) and nanE (Fig. 6B) genes were tested. Comparison of the WT strain shows that both nanAT and nanE are strongly induced in the presence of Neu5Ac but remained undetectable under the glucose supplementation conditions. The size of each transcript matched bioinformatic predictions, with the nanAT transcript being ~2.6 kb (Fig. 6A, transcript i) and nanE being ~0.8 kb (Fig. 6B, transcript iii). The induction of nanAT transcript with Neu5Ac and repression with glucose supported the transcriptional reporter experiments (Fig. 4A).

An assessment of transcriptional activity in the ΔnanR mutant showed that Neu5Ac still functioned as a potential inducer, but the quantity of nanAT and nanE transcripts were markedly lower than with LAC-WT (Fig. 6). The probe was designed to the nanT gene, allowing the smaller fragment of the nanAT transcript to be detectable in the absence of nanA (Fig. 6A, transcript ii). Similar to the case with LAC-WT, no transcriptional activity in the ΔnanE mutant was detected with glucose supplementation for either the nanAT or nanE transcript. For the ΔnanE mutant, the nanT probe was detected in the presence of Neu5Ac (Fig. 6A), and surprisingly, nanT was detected even in the presence of glucose. As expected, the nanE transcript was absent in the nanE mutant (Fig. 6B). To assess the function of the putative regulator NanR, the nanR mutant was also included in the transcriptional analysis. In the presence of inducer Neu5Ac, the ΔnanR strain behaved similarly to LAC-WT, except that the level of nanE transcript was not quite as high (Fig. 6B, transcript iii). Notably, in the presence of glucose, both nanAT and nanE transcripts were detected, confirming the predicted repressive nature of NanR.

The presence of other transcripts in the nanE blots (Fig. 6B, transcripts i and ii) was not expected. These transcripts are larger and are hypothesized to be a read-through product of nanK expression. An extension of the nanK transcript is the most likely explanation, given that a smaller band is detected in the ΔnanR lane, resulting from the deletion within a potential read-through

![FIG 4](http://jb.asm.org) Transcriptional regulation of nanA. The PnanA-sGFP transcriptional reporter was introduced into wild-type (A) and ΔnanR (B) strains. The strains were grown in TSB alone or TSB supplemented with glucose or Neu5Ac. A time course was performed under each medium condition, and GFP fluorescence relative to absorbance (600 nm) was plotted versus the time over a 30-h period. Error bars shown in both panels are standard deviations of at least three biological replicates.

![FIG 5](http://jb.asm.org) Identification of transcriptional start sites in the nan locus. (A) Schematic of the nan locus with four promoters marked. The locations of EMSA and Northern probes are also indicated. (B) nanE, nanR, nanK, and nanAT promoter regions. Transcriptional start sites determined by 5’ RACE are indicated with asterisks. Distance from the methionine (ATG) start codon and the potential −10 region (bold) and −35 region (bold) are indicated. The nanR gene does not possess an obvious −35 region.
nanK transcript. Several initial conclusions can be drawn from these Northern blot data taken together with the transcriptional fusion data. Neu5Ac functions as an inducer of both the nanA and nanK promoters, whereas glucose mediates their repression. Induction is significantly attenuated in the absence of nanA, suggesting that the inducer may be another metabolite in the catabolic pathway. Finally, both the nanA and nanE promoters were repressed in a NanR-dependent manner.

NanR binds the nanA and nanE promoter regions. NanR protein was affinity purified and used in electrophoretic mobility shift assays (EMSA) to test promoter binding. For the nanAT promoter region, an ~250-bp region upstream of nanAT was purified, labeled, and incubated with NanR, and a nonspecific control was included in every EMSA to demonstrate the specificity of NanR binding. As shown in Fig. 7A, NanR has the capacity to bind the nanA promoter region, although with less affinity than for the nanE promoter region (Fig. 8), whereas glucose mediates their repression. Induction is significantly attenuated in the absence of nanA, suggesting that the inducer may be another metabolite in the catabolic pathway. Finally, both the nanA and nanE promoters were repressed in a NanR-dependent manner.

FIG 6 Northern blot analysis of nanAT and nanE. Wild-type AH1263 (WT) and ΔnanR, ΔnanA, and ΔnanE mutant strains were grown in TSB supplemented with either glucose or Neu5Ac to an OD_{600} of 1.0. RNA was extracted and hybridized with nanT (A) and nanE (B) DNA probes, respectively. (A) The nanA and nanT genes are present in a single 2.6-kb transcript (i). The size difference of the shorter (ii) transcript indicates the deletion of the nanA gene. (B) The nanE transcript is transcript iii. A second larger nanE-containing transcript is transcript i and is hypothesized to be read-through from the nanK promoter. This longer transcript shifts smaller in the nanR deletion (ii) due to the loss of this gene.

FIG 7 EMSA of nanA and nanE promoter regions. The NanR-MBP protein was purified and used in EMSAs with 32P-labeled probes. (A) NanR binds the nanA promoter in a dose-dependent (6.25 to 200 nM) manner, as evidenced by the appearance of the upper band (Shift). Unshifted nanA promoter probe (P_{nanA}) and a nonspecific competitor probe (Non-Sp) are also shown. (B) NanR binds the nanE promoter in a similar manner, as shown by the appearance of the upper band (Shift). Unbound nanE promoter probe (P_{nanE}) and nonspecific probe (Non-Sp) are also shown.
binding to the nanA probe, as indicated by the loss of NanR/nanA promoter complex formation (Fig. 8). As controls, NanK alone did not disrupt the NanR/nanA complex, nor did reaction mixtures that lacked either NanK or ManNac.

DISCUSSION

Our findings demonstrate that sialic acid (Neu5Ac) can be utilized by many staphylococcal species as a carbon source for growth. Numerous types of members of the normal flora and opportunistic pathogens that colonize the human airway or gut mucosa can grow on Neu5Ac, including H. influenzae, streptococci, V. cholerae, Vibrio vulnificus, Vibrio fischeri, Salmonella enterica, Yersinia enterocolitica, and bifidobacteria (21, 24, 31, 38–41). Taken together, these observations suggest that Neu5Ac utilization is a conserved characteristic in human-adapted bacteria (31), leading us to speculate that the ability to catabolize Neu5Ac is a colonization factor. Additional support for this hypothesis comes from a comparison of S. aureus and S. epidermidis, which predominantly colonize the nares and skin, respectively (42, 43). Our analyses of S. epidermidis growth properties, and the absence of the nan locus (Table 2), indicate that S. epidermidis strains are unable to use Neu5Ac as a carbon source, whereas all S. aureus strains tested possess this ability. The observation that Neu5Ac is abundant on mucosal surfaces supports this hypothesis (44, 45).

Through bioinformatic analyses, we identified a five-gene locus that is conserved in S. aureus, and our studies described herein linked this locus to Neu5Ac utilization. Surprisingly, the genes are assembled in piecemeal fashion and organized into four different transcripts (Fig. 1A). Through genetic studies, we demonstrated that only three of the genes were essential for Neu5Ac catabolism; these included nanT, nanA, and nanE. The essential nature of nanT and nanA is not surprising considering that these encode the Neu5Ac transporter and the first committed catabolic step in the degradation pathway, respectively. The nanE gene encodes an essential epimerase that converts ManNac-6P to GlcNAc-6P, but perhaps more unexpected is that the loss of nanE resulted in deleterious growth effects in the presence of Neu5Ac. Based on studies of enteric pathogens, ManNac-6P accumulation is known to be growth inhibitory (46), and we speculate that the buildup of ManNac-6P also inhibits S. aureus growth. The absence of a phenotype with the nanK mutant is not surprising, as redundant kinase activities are common in bacteria (47).

Based on our reporter fusion and Northern blot observations (Fig. 4 and 6), we reasoned that NanR functions as a DNA binding protein that represses gene expression of nanE and nanAT. The addition of Neu5Ac induces gene expression in the nan locus, indicating that through some mechanism NanR repressive action is relieved. Bioinformatic analyses suggest that NanR is part of the RPR family of transcriptional regulators, which contain both sugar isomerase (SIS) and helix-turn-helix (HTH) domains (48). The presence of the SIS domain leads us to speculate that NanR might respond in some manner to the sugar-like structure of Neu5Ac or a Neu5Ac breakdown product to prevent repression of both nanE and nanAT. Northern analyses provided initial clues regarding the small-molecule induction response. Mutations in nanA, encoding the first enzyme to catabolize Neu5Ac, attenuated Neu5Ac-dependent induction (Fig. 6), suggesting that Neu5Ac itself is not the inducer. The absence of induction was not due to polar effects on the NanT transporter, since nanT is still transcribed (Fig. 6) and nanA mutants were complemented with single-gene-containing plasmids (Fig. 3). Although Neu5Ac-dependent induction was reduced in a nanA mutant, a nanE mutant retained the ability to induce locus expression, even in the presence of glucose (Fig. 6A). In the absence of NanE, the ManNac-6P intermediate should accumulate (Fig. 1B), and we speculated that ManNac-6P bound to NanR and relieved repression. Consistent with this hypothesis, SIS domain-containing proteins are known to interact with phosphorylated sugars (49).

To address the ManNac-6P induction hypothesis, EMSA studies were performed with NanR in the presence of pathway intermediates. Purified NanR can bind specifically to both the nanA probe (Shift). The ManNac-6P reaction mixture (Rxn) was added to the EMSA reactions in increasing concentrations. Higher concentrations of ManNac-6P restored the presence of the unbound nanA promoter probe (PnanA). As controls, the addition of NanK alone, reaction mixture without NanK, or reaction mixture lacking ManNac (as indicated) did not relieve the NanR-dependent interaction with the nanA promoter probe. A nongen probe (Non-Sp) is also shown.

FIG 8 ManNac-6P inhibits NanR DNA binding activity. ManNac-6P was biosynthesized using NanK, ManNac, and ATP. EMSAs were performed using NanA at 75 nM to provide complete binding of the nanA probe (Shift). The ManNac-6P reaction mixture (Rxn) was added to the EMSA reactions in increasing concentrations. Higher concentrations of ManNac-6P restored the presence of the unbound nanA promoter probe (PnanA). As controls, the addition of NanK alone, reaction mixture without NanK, or reaction mixture lacking ManNac (as indicated) did not relieve the NanR-dependent interaction with the nanA promoter probe. A nongen probe (Non-Sp) is also shown.
as a result of NanT transport, due to the basal level of nan locus expression. A portion of the intracellular Neu5Ac is catabolized to ManNAC-6P, which interacts with NanR to generate a protein complex with ManNAC-6P. The altered conformation of the NanR–ManNAC-6P complex is unable to repress transcription of the nanAT and nanE genes, resulting in full expression of the catabolic pathway and catabolism of Neu5Ac to central metabolic intermediates.

In summary, many staphylococcal species have the ability to utilize Neu5Ac as a carbon and energy source. The tight regulation of genes within the nan cluster, and repression of the pathway with glucose, suggests that Neu5Ac may be an attractive alternative energy source that is bioavailable in colonization environments, giving S. aureus a competitive advantage in certain host niches. Based on our findings, this regulation is coordinated by sensing the intracellular pools of ManNAC-6P, which leads to growth inhibition of S. aureus upon accumulation. The ability to utilize Neu5Ac may also have implications for S. aureus pathogenesis by aiding growth, host recognition, and persistence. Whether the presence of this locus is an important determinant of colonization or provides a competitive advantage in this environment remains to be assessed through in vivo studies.

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