Article

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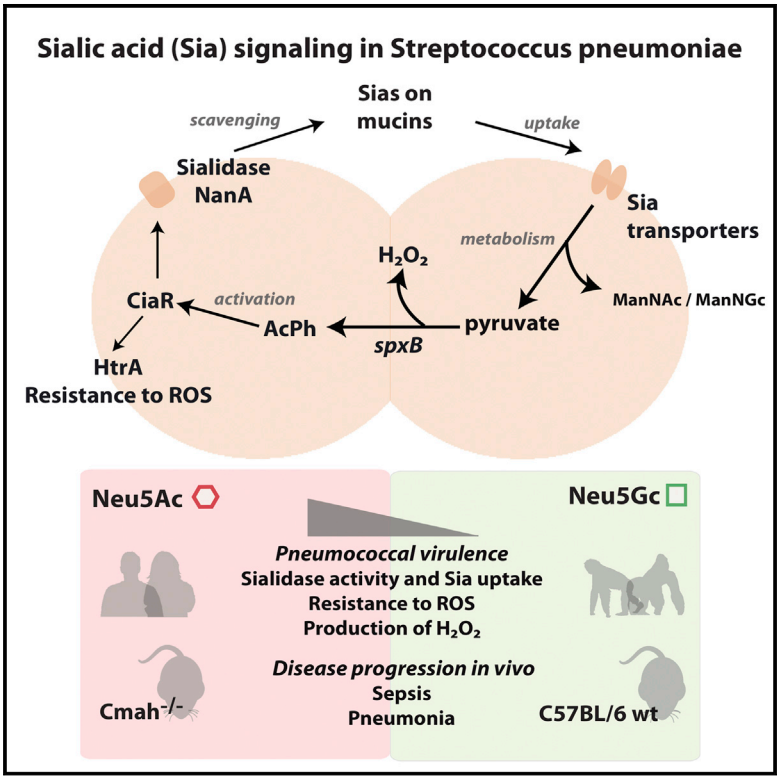
Streptococcus pneumoniae Senses a Human-like Sialic Acid Profile via the Response Regulator CiaR

Graphical Abstract



Highlights

- Intranasal infection with pneumococci progresses faster in Cmah$^{-/-}$ than wild-type mice
- The sialidase NanA and transporter SatABC are upregulated by Neu5Ac compared with Neu5Gc
- Activation of the response regulator CiaR controls the expression of NanA and SatABC
- Uptake and metabolic signaling mediate increased virulence by Neu5Ac versus Neu5Gc

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In Brief

S. pneumoniae infects human lungs, which have abundant levels of the sialic acid Neu5Ac because of inactivation of a hydroxylase. Hentrich et al. show that the pneumococcal response regulator CiaR responds to Neu5Ac, resulting in the upregulation of genes involved in sialic acid metabolism and signaling and leading to increased virulence.

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Streptococcus pneumoniae Senses a Human-like Sialic Acid Profile via the Response Regulator CiaR

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SUMMARY

***Streptococcus pneumoniae* is a human-adapted pathogen that encounters terminally sialylated glycoconjugates and free sialic acid (Sia) in the airways. Upon scavenging by the bacterial sialidase NanA, Sias serve as carbon sources for the bacteria. Unlike most animals in which cytidine-monophosphate-*N*-acetylneuraminic acid hydroxylase (CMAH) converts Sia *N*-acetylneuraminic acid (Neu5Ac) into *N*-glycolylneuraminic acid (Neu5Gc), humans have an inactive CMAH, causing an absence of Neu5Gc and excess Neu5Ac. We find that pneumococcal challenge in *Cmah*$^{-/-}$ mice leads to heightened bacterial loads, virulence, and NanA expression. In vitro, NanA is upregulated in response to Neu5Ac compared with Neu5Gc, a process controlled by the two-component response regulator CiaR and requiring Sia uptake by the transporter SatABC. Additionally, compared with Neu5Gc, Neu5Ac increases pneumococcal resistance to antimicrobial reactive oxygen species in a CiaR-dependent manner. Thus, *S. pneumoniae* senses and responds to Neu5Ac, leading to CiaR activation and increased virulence and potentially explaining the greater susceptibility in humans.**

INTRODUCTION

Streptococcus pneumoniae (the pneumococcus) is a Gram-positive bacterium adapted to the human upper respiratory tract, especially predominant in pre-school-age children and those attending day care centers. *S. pneumoniae* causes diseases ranging from milder respiratory tract infections such as sinusitis and otitis media to severe diseases like pneumonia, septicemia, and meningitis. Pneumococcal infections are a major threat to human health, causing about 11% of all deaths among children below the age of 5 (O'Brien et al., 2009).

When entering the human nasopharynx, *S. pneumoniae* encounters mucus, which contains glycoconjugates displaying sialic acids (Sias) as terminal monosaccharides. In the nasopharynx, glucose is scarce as a carbon source. Therefore, colonizing pneumococci need to acquire carbohydrates from glycoconjugates, a process that involves removal of terminal Sias, which themselves can be used as a carbon source for the bacteria (Burnaugh et al., 2008; King et al., 2004; Traving and Schauer, 1998). In *S. pneumoniae*, the genes involved in Sia retrieval, uptake, and metabolism, are encoded in the *nanAB* locus (King et al., 2004; Vimr et al., 2004) and, in 51% of all pneumococci, also in the *nanC* locus (Pettigrew et al., 2006). The *nanAB* locus contains genes encoding sialidases, such as NanA, and the main Sia transporter SatABC (King et al., 2004; Marion et al., 2011). Furthermore, the locus contains catabolite repression elements (cres), leading to transcriptional inhibition of genes within the locus in the presence of glucose (Afzal et al., 2015; Gualdi et al., 2012). NanA scavenges Sias from host glycoconjugates (King et al., 2006; Tong et al., 2002) and is a known pneumococcal virulence factor promoting colonization of the nasopharynx and lungs (Orihuela et al., 2004), invasion of the brain endothelium (Uchiyama et al., 2009), cytokine release in human monocytes, and neutrophil extracellular trap (NET) formation of human neutrophils (Chang et al., 2012). Thus, NanA plays an important role in the activation of the innate immune response. Besides Sia removal, Sia uptake by SatABC promotes pneumococcal virulence (Marion et al., 2011). The most abundant Sias in mammals are Neu5Ac (*N*-acetylneuraminic acid) and Neu5Gc (*N*-glycolylneuraminic acid). The enzyme CMP-Neu5Ac hydroxylase (CMAH) converts CMP-Neu5Ac to CMP-Neu5Gc in most mammals by addition of a single oxygen atom (Shaw and Schauer, 1989). Although humans have a fixed exon deletion in the gene encoding *CMAH* and therefore only synthesize Neu5Ac, most other mammals, including Old World primates, tend to have a predominance of Neu5Gc on their cell surfaces and secreted glycoconjugates (Chou et al., 1998; Muchmore et al., 1998). Recently, ferrets and New World monkeys have been shown to have independent inactivation of CMAH in their evolution (Ng et al., 2014; Springer et al., 2014). Microbes adhere to Sias during colonization and infection (Traving and

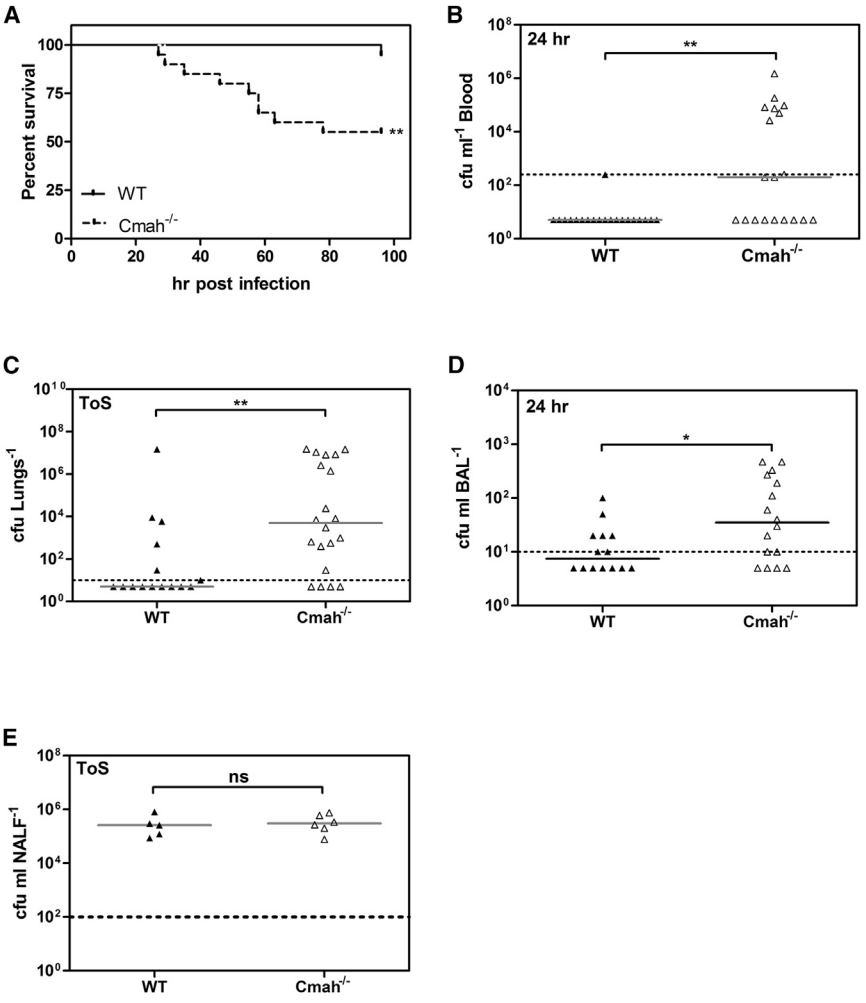

Figure 1. Pneumococcal Disease Progresses Faster in Human-like *Cmah*$^{-/-}$ compared with C57BL/6 Wild-Type Mice after Intranasal Challenge

Mice were infected intranasally with *S. pneumoniae* TIGR4 in 20 μl PBS.

(A) Survival rates were monitored over the course of the experiments.

(B) Bacterial counts in the blood of WT and *Cmah*$^{-/-}$ mice were measured 24 hr p.i. by plating.

(C) Bacterial numbers in the lungs of mice were determined by plating lung homogenates 96 hr p.i. or at ToS.

(D) Bacterial counts in BALF of WT and *Cmah*$^{-/-}$ mice were measured 24 hr p.i. by plating.

(E) Bacterial counts in NALF of WT and *Cmah*$^{-/-}$ mice were measured 96 hr p.i. or at ToS by plating. Dotted lines indicate the detection limits. Horizontal bars represent medians. For survival rates, log-rank Mantel-Cox test and for CFU counts, Mann-Whitney test were used for statistical analysis. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; ns, non-significant. The graph represents two independent experiments with at least 10 mice/group. See also Figure S1.

Schauer, 1998), and many virulence factors are adapted to preferentially engage Neu5Ac or Neu5Gc, depending on the host species. *Plasmodium falciparum* and *Salmonella* Typhi, causative agents of malaria and typhoid fever, respectively, were shown to optimize specific virulence mechanisms to the human Sia condition. The toxin of *S.* Typhi as well as the merozoite of *P. falciparum* each recognize Neu5Ac, with absent or weak binding to Neu5Gc (Deng et al., 2014; Martin et al., 2005).

Bacteria sense and respond to environmental changes with the help of two-component systems (TCSs) consisting of a membrane-bound histidine kinase (HK) and a cytoplasmic response regulator (RR). The HK senses the signal, is autophosphorylated, and transfers phosphate to the RR, which, in turn, changes its conformation and acts as a transcriptional regulator (Stock et al., 1989). To date, 13 TCSs and a single RR have been identified in *S. pneumoniae*, of which several have been associated with virulence regulation (Throup et al., 2000). TCS05, also known as CiaRH, was identified in a screen for spontaneous cefotaxime-resistant mutants (Guenzi et al., 1994). CiaRH is involved in competence, cefotaxime susceptibility, autolysis, bacteriocin production, and resistance to oxidative stress and plays a role in pneumococcal virulence (Dagkessamanskaia et al., 2004; Ibrahim et al., 2004a; Mascher et al., 2003, 2006; Throup et al., 2000). The RR CiaR directly controls a number of promoters and genes, including the high-temperature requirement A gene *htrA*, enhancing resistance to oxidative stress, and has been shown to affect the transcription of several small non-coding RNAs with different downstream effects (Halfmann et al., 2007; Marx et al., 2010). Phosphorylation of CiaR is required for its regulatory activity, but internal acetyl phosphate (AcPh) may serve as phosphor donor besides CiaH (Marx et al., 2015).

In this study, we show that TIGR4 exhibits a differential response to the form of Sia abundant in humans, Neu5Ac, compared with Neu5Gc, by upregulating the sialidase NanA, the main sialic acid transporter SatABC, and HtrA in response to Neu5Ac in contrast to Neu5Gc. This response is dependent on the RR CiaR and leads to bacterial resistance to oxidative stress. Furthermore, mice with a human-like defect in the *Cmah* gene showed faster pneumococcal disease progression than C57BL/6 wild-type (WT) mice after intranasal but not after intravenous challenge.

RESULTS

Pneumococcal Disease Progresses Faster in Human-like *Cmah*$^{-/-}$ Compared with C57BL/6 Wild-Type Mice after Intranasal Challenge with *S. pneumoniae* TIGR4

To determine whether the human-adapted pathogen *S. pneumoniae* is more virulent in response to Neu5Ac than Neu5Gc, we made use of Neu5Gc-deficient *Cmah*$^{-/-}$ mice, which have a human-like excess of Neu5Ac on the surface of their cells. Using an intranasal challenge with the pneumococcal strain TIGR4 of 3×10^6 colony forming units (CFUs) per animal, disease progressed significantly faster in *Cmah*$^{-/-}$ compared with WT mice. We observed a drastically lower survival rate

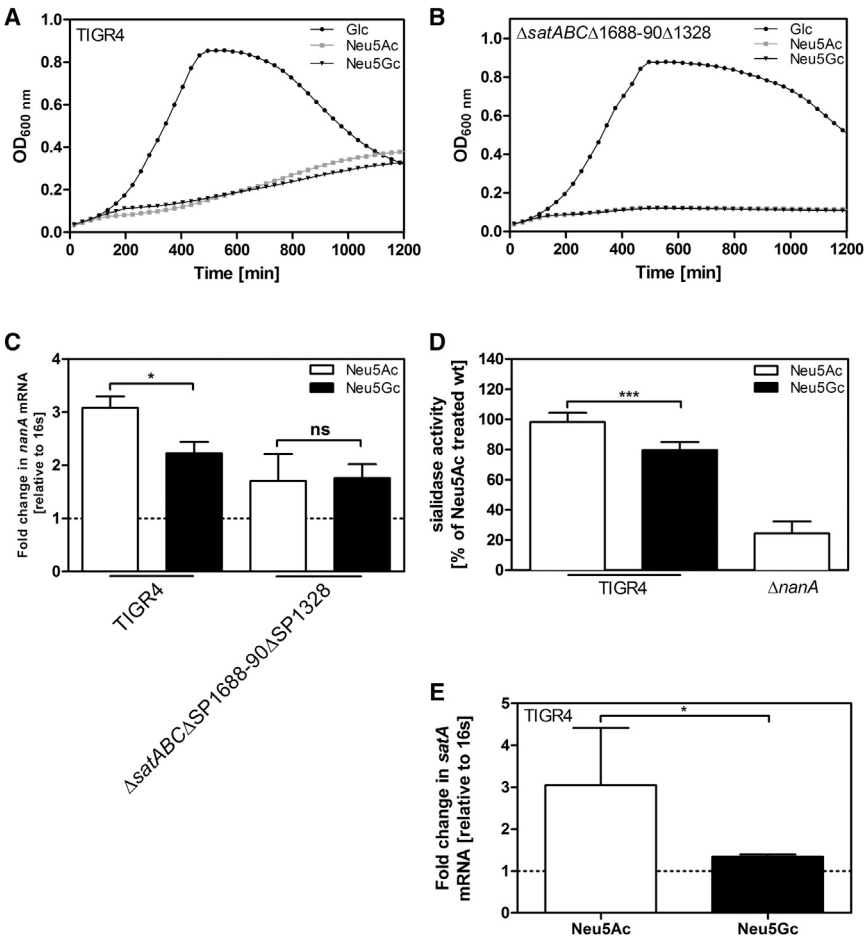

Figure 2. *S. pneumoniae* Senses Sialic Acid, Leading to an Upregulation of Sialidase NanA, and the Main Sialic Acid Transporter SatABC in Response to the Sialic Acid Neu5Ac Compared with Neu5Gc

(A and B) TIGR4 (A) and TIGR4Δ*satABC*ΔSP1688-90ΔSP1328 (B) were grown on glucose, Neu5Ac, or Neu5Gc (12 mM). Optical density at 600 nm was monitored over time.

(C) TIGR4 and TIGR4Δ*satABC*ΔSP1688-90ΔSP1328 were grown on glucose until OD_{620} = 0.45. Cells were washed with PBS and re-suspended in C+Y medium containing either 12 mM Neu5Ac or Neu5Gc or no supplemented carbohydrate. After incubation for 1.5 hr, RNA was isolated, cDNA was synthetized, and qPCR was performed to determine mRNA levels of the pneumococcal sialidase NanA. 16s was used as an endogenous control. The fold change of transcription was normalized to bacteria incubated in monosaccharide-free C+Y medium (dotted line).

(D) The sialidase activity of TIGR4 incubated in C+Y medium containing either 12 mM Neu5Ac or Neu5Gc was determined using 2-O-(p-nitrophenyl)-α-d-N acetylneuraminic acid (pNANA). Values were normalized to Neu5Ac-treated TIGR4.

(E) TIGR4 was incubated as described above. RNA was isolated, cDNA was synthetized, and qPCR was performed to measure mRNA levels of SatA, the substrate binding protein of the main Sia transporter, SatABC. 16s was used as an endogenous control. The fold change of transcription was normalized to bacteria incubated in monosaccharide-free C+Y medium (dotted line).

The data in (A) and (B) are represented as the mean of two independent experiments and in (C)–(E) as the mean ± SEM from at least three independent experiments. Mann-Whitney Test was used for statistical analysis. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. See also Figure S2.

(Figure 1A) and a significantly higher rate of septicemia in *Cmah*$^{-/-}$ compared with WT mice 24 hr post-infection (p.i.) (Figure 1B). Moreover, 96 hr p.i. or at the time of sacrifice (ToS), the bacterial burden was higher in the lungs of *Cmah*$^{-/-}$ compared with WT mice (Figure 1C). Already at 24 hr p.i. we observed higher bacterial numbers in the bronchial lavage (BALF) of *Cmah*$^{-/-}$ compared with WT mice (Figure 1D), even though the nasopharyngeal colonization (NALF) was similar between the mice at 96 hr p.i. or at ToS (Figure 1E).

We infected C57BL/6 WT and Cmah$^{-/-}$ mice intravenously with TIGR4 and found no differences in disease progression between the mice (Figure S1), excluding a general immune deficiency of the Cmah$^{-/-}$ mice.

S. pneumoniae Senses Sialic Acid, Leading to an Upregulation of the Sialidase NanA and the Main Sialic Acid Transporter SatABC in Response to the Sialic Acid Neu5Ac Compared with Neu5Gc

Next we studied whether there are differences in gene regulation between bacteria grown on Neu5Ac or Neu5Gc. We hypothesized that Neu5Ac preferentially activates the established virulence factor NanA, given the higher pneumococcal virulence in *Cmah*$^{-/-}$ compared with WT mice. We cultured pneumococcal strain TIGR4 in C+Y medium supplemented with 12 mM glucose (Glc), Neu5Ac, or Neu5Gc. Besides being able to grow on Neu5Ac (Marion et al., 2011), TIGR4 (as well as its streptomycin-resistant derivative TIGR4 Sm^r) could use Neu5Gc as the sole carbon source. Pneumococcal growth rates on either Sia were lower than on glucose but similar to one another (Figure 2A). Bacterial growth required Sia transport because a mutant lacking all three known Sia transporters, SatABC, SP1688-90, and SP1328, showed strongly reduced growth on both Neu5Ac and Neu5Gc (Figure 2B). Transcription of sialidase NanA was increased in TIGR4 (and in TIGR4 Sm^r) after incubation with Neu5Ac compared with Neu5Gc. The mutant deficient in sialic transport, TIGR4Δ*satABC*ΔSP1688-90ΔSP1328, showed no differential transcription of NanA in response to Neu5Ac or Neu5Gc (Figure 2C). In coherence with these results, total sialidase activity was higher in TIGR4 cultured on Neu5Ac than on Neu5Gc. A mutant lacking NanA had residual sialidase activity, probably because of the activity of the sialidases NanB and NanC (Figure 2D). Furthermore, transcription of the substrate binding protein *satA* of the main sialic acid transporter SatABC was upregulated in response to Neu5Ac compared with Neu5Gc (Figure 2E). Because of catabolite repression (Afzal et al., 2015; Gualdi et al., 2012), *nanA* and *satA* were more upregulated in response

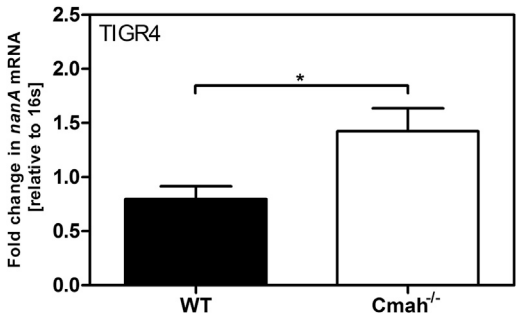

Figure 3. *S. pneumoniae* Senses Sialic Acid In Vivo in the Nasopharynx of Mice

Mice were infected intranasally with *S. pneumoniae* TIGR4 in 20 μl PBS. Nasopharyngeal lavages in PBS were obtained 6 hr p.i. Bacteria were spun down and resuspended in RLT buffer (QIAGEN RNeasy lysis buffer). RNA was isolated, and cDNA was synthetized. The transcription of the sialidase *nanA* was monitored by qPCR. 16s was used as an endogenous control. Data are presented as mean ± SEM of three independent experiments. Fold change of transcription was normalized to the value of bacteria isolated from the nasopharynx of *Cmah*$^{-/-}$ mice. Mann-Whitney test was used for statistical analysis. *$p < 0.05$.

to Sia after normalization to glucose treatment compared with treatment without monosaccharide (Figures S2A and S2B).

S. pneumoniae Senses Sialic Acid In Vivo in the Nasopharynx of Mice

To study whether Sia sensing also occurs in vivo, we isolated pneumococci from the nasopharynx of mice 6 hr p.i., and *nanA* mRNA levels were determined from these bacteria. We observed an upregulation of *nanA* transcription in *Cmah*$^{-/-}$ compared with WT mice after challenge with TIGR4 (Figure 3). To exclude unspecific primer binding to host RNA, we performed the same experiment with TIGR4Δ*nanA* and did not detect any *nanA* transcription in these samples (data not shown).

Intracellular Signaling of Sialic Acid Leads to Higher Transcription of the Two-Component System CiaRH in Response to Neu5Ac Compared with Neu5Gc

Because strain TIGR4 signals differentially in response to Neu5Ac and Neu5Gc, we performed qPCR to compare the transcription rates of all pneumococcal RRs in response to Neu5Ac or Neu5Gc in relation to no carbohydrates added. Of all known RRs, only CiaR was upregulated significantly higher in response to Neu5Ac compared with Neu5Gc (Figure 4A). CiaR is co-transcribed with its cognate HK CiaH, which showed similar transcriptional response patterns to the different sugars (data not shown). In TIGR4Δ*satABC*ΔSP1688-90ΔSP1328, lacking all three known Sia transporters, *ciaR* transcription was downregulated in comparison with incubation without sugar. Moreover, we did not observe any difference in *ciaR* mRNA levels after treatment with either of the Sias (Figure 4B), suggesting that *ciaR* upregulation in response to the two Sias requires transport. It has been demonstrated that phosphorylation of CiaR is required for its regulatory effects (Halfmann et al., 2011). The experiments described above therefore suggest that the level of CiaR phosphorylation might be higher after exposure to Neu5Ac compared with Neu5Gc and that this increased phosphorylation requires Sia transport. It has been shown that, after Sia uptake, Neu5Ac and Neu5Gc are metabolized to N-acetylmannosamine (ManNAc) or N-glycolylmannosamine (ManNGc), respectively, and pyruvate (Hopkins et al., 2013; Vimr and Troy, 1985). Subsequently, pyruvate is catabolized to AcPh and hydrogen peroxide (H_2O_2) by the SpxB pyruvate oxidase (Spellerberg et al., 1996; Figure 4C). Because AcPh can act as a phosphor donor to activate CiaR, we measured the transcriptional response of *spxB* for TIGR4 growing on either of the two Sias. We found a higher expression of *spxB* and also higher amounts of H_2O_2, and AcPh with Neu5Ac than with Neu5Gc (Figures 4D–4F). Furthermore, we found the transcriptional level of *nanA* to be decreased in a *spxB*-deficient mutant compared with TIGR4 WT (Figure 4G), suggesting that *spxB* transcription and internal AcPh affect the transcription of *nanA*.

The Response Regulator CiaR Controls Transcription of the Sialidase NanA and the Main Sialic Acid Transporter SatABC and Is Required for Normal Pneumococcal Growth on Sialic Acid

To determine whether CiaR regulates the transcription of genes involved in Sia metabolism, we performed qPCR to measure *nanA* and *satA* transcription in the TIGR4Δ*ciaR* and TIGR4Δ*ciaRH* mutants. Both mutant strains failed to significantly upregulate *nanA* or *satA* in response to Neu5Ac relative to Neu5Gc. Thus, transcription of the sialidase gene *nanA* and total sialidase activity were similar between the two Sia conditions and upregulated in contrast to bacteria treated without supplemented carbohydrate (Figures 5A–5C). Consequently, the preferential upregulation of *nanA* and *satA* in TIGR4 bacteria exposed to Neu5Ac compared with Neu5Gc is CiaR-dependent. To test whether the RR CiaR affects the growth of TIGR4 on Sia, we cultured TIGR4Δ*ciaR* or TIGR4Δ*ciaRH* on glucose, Neu5Ac, or Neu5Gc. We observed growth defects of TIGR4Δ*ciaR* and TIGR4Δ*ciaRH* on either Sia compared with wild-type TIGR4 (Figures 5D and 5E). Noteworthy is that the growth rate of TIGR4Δ*ciaR* on galactose was also significantly reduced in comparison with WT TIGR4 (data not shown). In contrast, the growth rate on glucose did not differ between TIGR4 and the two mutant strains (Figures 5D and 5E). Deletion mutations of other RRs did not affect growth on glucose or Sia (Figure S3).

Upregulation of *ciaRH* and *htrA* in Response to Neu5Ac Mediates Higher Resistance to Oxidative Stress Compared with Incubation with Neu5Gc

CiaRH is known to mediate resistance to oxidative stress via the high-temperature requirement A, HtrA (Ibrahim et al., 2004a, 2004b). Therefore, we determined the transcriptional level of *htrA* and the survival rate of TIGR4 in response to H_2O_2. Treatment with Neu5Ac resulted in significantly increased transcription of *htrA* and higher numbers of recovered bacteria in response to H_2O_2 than treatment with Neu5Gc (Figures 6A and 6B). In contrast, survival rates were similar after treatment with Neu5Ac or Neu5Gc of the deletion mutant of the TCS CiaRH (TIGR4Δ*ciaRH)*.

Sialidase NanA, the Main Sialic Acid Transporter SatABC, and the Response Regulator CiaR Contribute to the Differential Increase in Pneumococcal Virulence between *Cmah*$^{-/-}$ and WT Mice

Because we observed a more severe pneumococcal disease outcome in *Cmah*$^{-/-}$ compared with WT mice, we asked

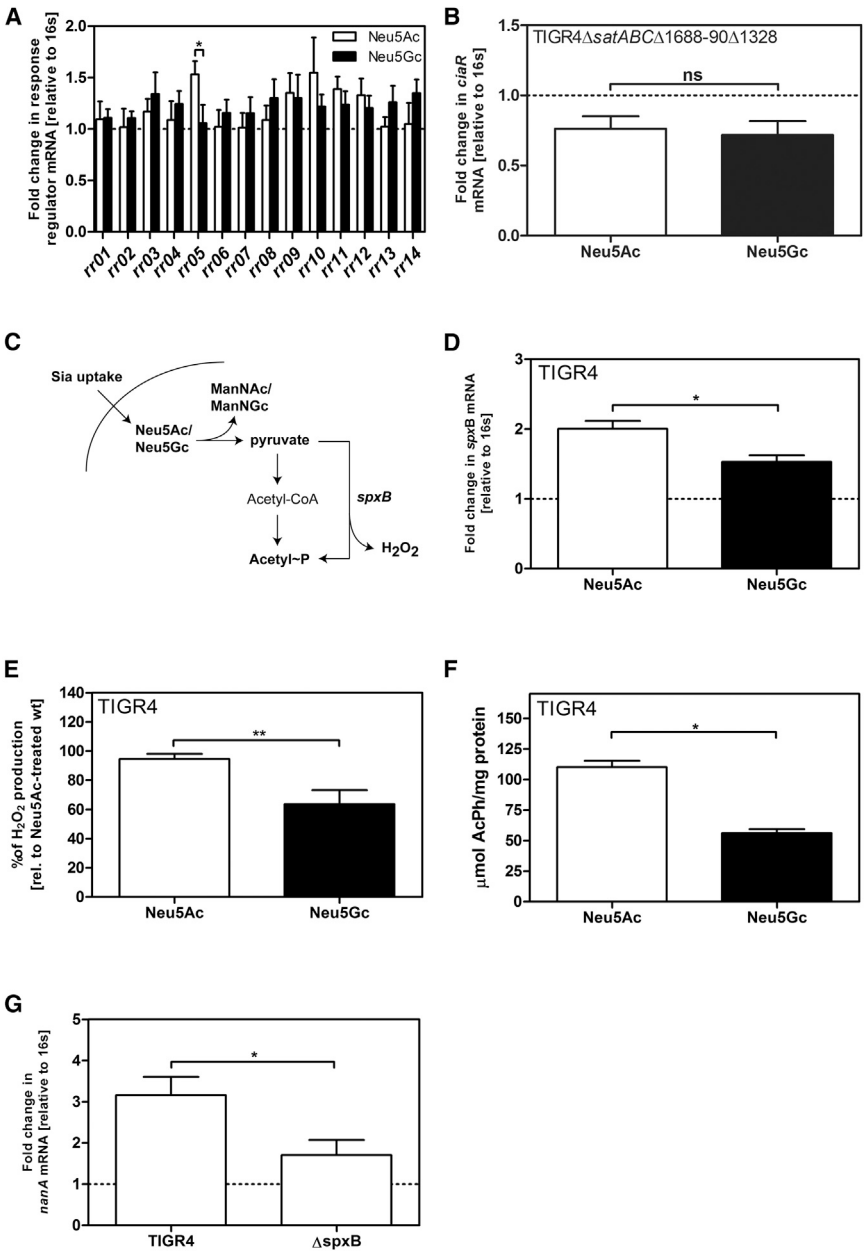

Figure 4. Intracellular Signaling of Sialic Acid Leads to Higher Transcription of the Two-Component System CiaRH in Response to Neu5Ac Compared with Neu5Gc

(A) RNA samples of TIGR4 incubated either on no supplemented carbohydrate or Neu5Ac or Neu5Gc (12 mM) for 1.5 hr were isolated, and cDNA was synthetized. The transcription rates of the response regulators RR01, RR02, RR03, RR04, RR05 (CiaR), RR06, RR07, RR08, RR09, RR10, RR11, RR12, RR13, and RR14 were determined.

(B) RNA samples of TIGR4Δ*satABC*ΔSP1688-90ΔSP1328 incubated as described above were isolated, and cDNA was synthetized. The transcription rates of RR05 (CiaR) were determined.

(C) A schematic of the uptake and catabolism of Neu5Ac and Neu5Gc and acetyl phosphate production in *S. pneumoniae*.

(D) RNA samples of TIGR4 incubated as described before were isolated, and cDNA was synthetized. Transcription rates of *spxB* were determined.

(E) Bacteria were incubated as described before. After an incubation with 2,2′-azino-bis (3-ethylbenzthiazoline-6 sulfonic acid) and horseradish peroxidase, optical densities at 560 nm were measured, and hydrogen peroxide concentrations were calculated using a standard curve using known concentrations. Values are normalized to amounts of H_2O_2 produced by TIGR4.

(F) TIGR4 was grown as described above, and production of AcPh was assayed by converting ADP and AcPh to ATP by the acetate kinase of *Bacillus stearothermophilus*. Total ATP was finally measured using an ATP bioluminescence kit and normalized to the protein content of the sample.

(G) RNA samples of TIGR4 and TIGR4Δ*spxB* were incubated on Neu5Ac as described above and isolated, and cDNA was synthetized. The transcription rates of *nanA* were determined.

(A, B, D, and G) qPCR was performed with 16s as an endogenous control. mRNA values were normalized to bacteria incubated in C+Y medium without supplemented sugar (dotted lines).

Data are represented as mean ± SEM of at least three independent experiments. Mann-Whitney test was used for statistical analysis. *$p < 0.05$, **$p < 0.01$. See also Figure S3.

whether NanA, SatABC, or CiaR are required for this differential increase in pneumococcal virulence in vivo. We infected WT and *Cmah*$^{-/-}$ mice with TIGR4 or its isogenic mutants lacking NanA, SatABC, or CiaR. 24 hr p.i., nasopharyngeal colonization did not differ between WT and *Cmah*$^{-/-}$ mice challenged with TIGR4, TIGR4Δ*nanA*, TIGR4Δ*satABC*, or TIGR4Δ*ciaR* (Figure 7A). However, we found that TIGR4Δ*satABC* and especially TIGR4Δ*ciaR* exhibited a marked decrease in nasopharyngeal colonization in both WT and *Cmah*$^{-/-}$ mice. Challenge with wild-type TIGR4 resulted in higher bacterial numbers in the lungs of *Cmah*$^{-/-}$ compared with WT mice, whereas infection with TIGR4Δ*nanA*, TIGR4Δ*satABC*, or TIGR4Δ*ciaR* led to similar numbers in the lungs of WT and *Cmah*$^{-/-}$ mice (Figure 7B). Infection with TIGR4 led to sepsis in *Cmah*$^{-/-}$ but not in WT mice, whereas challenge with TIGR4Δ*satABC* and TIGR4Δ*ciaR* did not result in bacterial invasion into the blood, and TIGR4Δ*nanA* showed low bacteremia levels (Figure 7C). Hence, NanA, SatABC, and CiaR each contribute to the higher pneumococcal virulence in *Cmah*$^{-/-}$ compared with wild-type mice.

DISCUSSION

S. pneumoniae, like other respiratory pathogens, closely interacts with Sias in mucins during infection (Feldman et al., 1992; Traving and Schauer, 1998). Because humans are its natural host (Pan et al., 2014), we hypothesized that *S. pneumoniae* would respond differently upon encountering Neu5Ac, abundant in humans, compared with Neu5Gc, which is mostly present in other animal species. Importantly, we show an upregulation of the pneumococcal sialidase NanA and the main sialic acid transporter SatABC

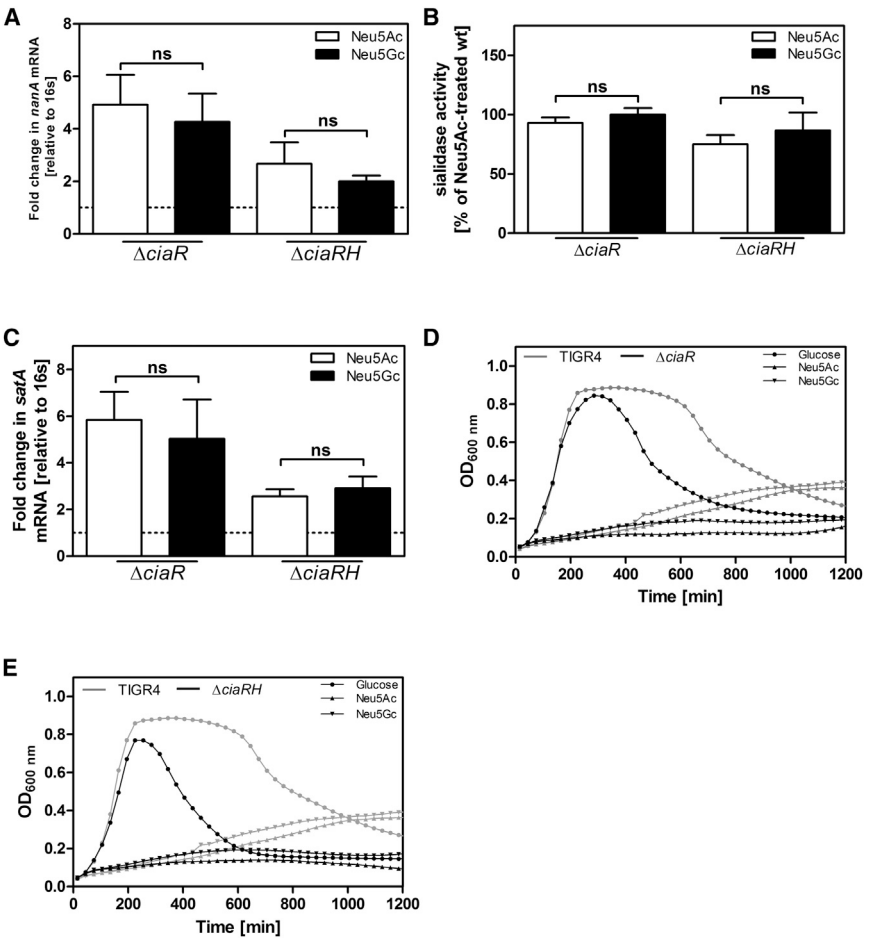

Figure 5. The Response Regulator CiaR Controls Transcription of the Sialidase NanA and the Main Sialic Acid Transporter SatABC and Is Required for Normal Pneumococcal Growth on Sialic Acid

(A) TIGR4Δ*ciaR* and TIGR4Δ*ciaRH* were grown on glucose until OD_{620} = 0.45. Cells were washed with PBS and resuspended in C+Y medium containing either no supplemented carbohydrate or 12 mM Neu5Ac or Neu5Gc. After incubation for 1.5 hr, RNA was isolated, and cDNA was synthetized. The transcription of the sialidase *nanA* was monitored. 16s was used as an endogenous control. Values are presented relative to bacteria incubated in C+Y medium without supplemented carbohydrates (dotted line).

(B) The neuraminidase activities of TIGR4Δ*ciaR* and TIGR4Δ*ciaRH* incubated in C+Y medium containing either 12 mM Neu5Ac or Neu5Gc were determined using pNANA. Values were plotted in relation to Neu5Ac-incubated wild-type bacteria.

(C) TIGR4Δ*ciaR* and TIGR4Δ*ciaRH* were grown on glucose until OD_{620} = 0.45. Cells were washed with PBS and resuspended in C+Y medium containing either no supplemented carbohydrate or 12 mM Neu5Ac or Neu5Gc. After incubation for 1.5 hr, RNA was isolated, and cDNA was synthetized. The transcription of the substrate binding protein *satA* of the main sialic acid transporter SatABC was monitored. 16s was used as an endogenous control. Values are presented relative to bacteria incubated in C+Y medium without supplemented carbohydrates (dotted line).

(A–C) Data are represented as mean ± SEM of three independent experiments. Mann-Whitney test was used for statistical analysis.

(D and E) Mutant strain TIGR4⊿*ciaR* (D) and mutant strain TIGR4⊿*ciaRH* (E) were grown on 12 mM glucose (Glc), Neu5Ac, or Neu5Gc. Optical density at 600 nm was monitored over time. Growth curves of TIGR4 on 12 mM glucose, Neu5Ac, or Neu5Gc were plotted as a reference in each graph. Data are represented as mean of at least two independent experiments.

in response to Neu5Ac compared with Neu5Gc and found that the RR CiaR was involved in the response to Sias. Sia scavenging, uptake, and signaling mediated increased pneumococcal virulence in response to Neu5Ac compared with Neu5Gc after intranasal but not after intravenous challenge.

To determine whether *S. pneumoniae* exhibits higher virulence in response to Neu5Ac compared with Neu5Gc in vivo, we infected C57BL/6 WT, predominantly having Neu5Gc, and *Cmah*$^{-/-}$ mice, lacking Neu5Gc (Hedlund et al., 2007; Makatsori et al., 1998), intranasally with TIGR4. *Cmah*$^{-/-}$ mice had significantly more bacteria in the lungs and blood than WT mice and a significantly lower survival rate. Already at 24 hr p.i. we observed higher bacterial numbers in the BALF of *Cmah*$^{-/-}$ compared with WT mice, suggesting that pneumococcal growth in the lower airways is higher in a Neu5Ac than in a Neu5Gc environment. This could be due to both enhanced bacterial replication because of a more efficient retrieval and uptake of carbohydrates from glycoconjugates and to increased resistance to host-mediated clearance in this compartment through upregulation of virulence traits in response to Neu5Ac relative to Neu5Gc. These results are in line with earlier studies showing an increased bacterial burden in the lungs of mice after intranasal administration of Neu5Ac compared with Neu5Gc (Trappetti et al., 2009).

We hypothesized that the increased virulence in *Cmah*$^{-/-}$ compared with wild-type mice is caused by differential affinity of pneumococcal factors affecting growth and virulence in the glucose-poor lower airways favoring Neu5Ac compared with Neu5Gc. In general, sialidases are shown to cleave Neu5Ac from glyconconjugates more efficiently than Neu5Gc (Corfield et al., 1981), and a species-specific interaction of pathogens and host structures has been described before; e.g., sialidases of *Salmonella* spp. Also, the pneumococcal sialidase NanC bind stronger to Neu5Ac than to Neu5Gc (Minami et al., 2013; Parker et al., 2012). *S. pneumoniae* de-glycosylates structures on the surface of host cells with the help of the sialidase NanA (King et al., 2004) and can use Sias as a carbon source (Burnaugh et al., 2008; Marion et al., 2011). In TIGR4, genes encoding NanA and other factors involved in Sia uptake and metabolism are encoded in two loci, the *nanAB* and *nanC* loci (King et al., 2004; Vimr et al., 2004). Our in vitro data reveal that *S. pneumoniae* not only grows on Neu5Ac as a sole carbon source (Burnaugh et al., 2008) but can also utilize Neu5Gc in

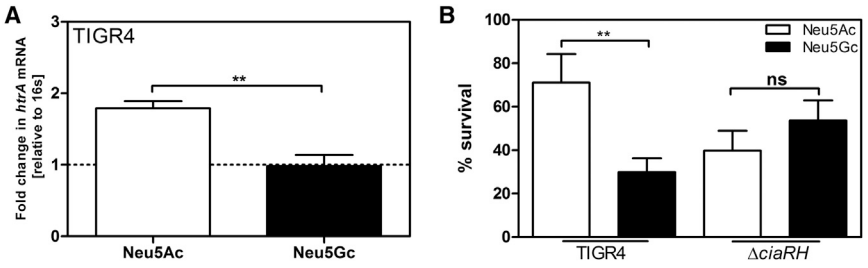

Figure 6. Upregulation of *ciaRH* and *htrA* in Response to Neu5Ac Mediates Higher Resistance to Oxidative Stress Compared with Incubation with Neu5Gc

(A) TIGR4 was grown on glucose until OD_{620} = 0.45. Cells were washed with PBS and re-suspended in C+Y medium containing either no supplemented carbohydrate or 12 mM Neu5Ac or Neu5Gc. After incubation for 1.5 hr, RNA was isolated, and cDNA was synthetized. The transcription of *htrA* was monitored. 16s was used as an endogenous control. Values are presented relative to bacteria incubated in C+Y medium without supplemented carbohydrates (dotted line).

(B) TIGR4 and TIGR4Δ*ciaRH* were grown on glucose until OD_{620} = 0.45. Cells were washed with PBS and resuspended in C+Y medium containing either 12 mM Neu5Ac or Neu5Gc. After incubation for 1.5 hr, 40 mM H_2O_2 was added, and cells were incubated at 37°C for 10 min. Viable bacterial numbers were determined before and after exposure to H_2O_2. The graph represents the percentage of surviving cells.

Values represent the mean from three independent experiments. Mann-Whitney test was used for statistical analysis. *$p < 0.05$.

the same manner. The three known Sia transporters SatABC, SP1688-90, and SP1328 are required for growth on both Sias. Genes within the *nanAB* locus are upregulated in the presence of Neu5Ac (Afzal et al., 2015; Gualdi et al., 2012). Trappetti et al. (2009) suggested that the initial encounter between pneumococci and free Neu5Ac triggers an increased expression of sialidases (e.g., NanA), resulting in further release of free Neu5Ac, therefore leading to a positive feedback loop. Our data reveal an upregulation of NanA and the substrate binding protein SatA of the main sialic acid transporter SatABC in response to Neu5Ac compared with Neu5Gc after Sia uptake in vitro as well as increased pneumococcal sialidase expression in the nasopharynx of *Cmah*$^{-/-}$ compared with WT mice. Subsequently, there are higher amounts of free Sia available that are taken up into the bacteria to a higher extent in *Cmah*$^{-/-}$ compared with WT mice. Also, Siegel et al. (2014) showed that increased desialyation rates of host glycoconjugates promote bacterial growth, which, in turn, enhances translocation of bacteria from the nasopharynx to the lower respiratory tract.

TCS-mediated carbohydrate signaling in bacteria has been described for group B streptococci. TCS16 in this species controls sugar phosphotransferase systems, and a deficient mutant failed to grow on fructose-6-phosphate (Faralla et al., 2014). In our study, several of the 14 known pneumococcal RRs were upregulated in response to Neu5Ac and Neu5Gc in comparison with incubation without supplemented carbohydrate. Among all known RRs in *S. pneumoniae,* RR05, called CiaR, was transcribed significantly higher when TIGR4 was exposed to Neu5Ac relative to Neu5Gc. This differential effect on *ciaR* transcription was not observed in a mutant lacking all three Sia transporters. Instead, we observed a downregulation of *ciaR* in this mutant when exposed to either Neu5Ac or Neu5Gc relative to no carbohydrates added. Subsequently, CiaR might be involved in signaling in response to intracellular Sia or its metabolites, although we cannot exclude that other RRs play a role in the pneumococcal response to sialic acids without being differentially regulated in response to Neu5Ac compared with Neu5Gc.

The TCS CiaRH is well studied and has been shown to affect pneumococcal virulence and competence (Dagkessamanskaia et al., 2004; Throup et al., 2000). Deletion of the RR CiaR led to similar *nanA* and *satA* mRNA levels in response to Neu5Ac or Neu5Gc. Hence, CiaR is required for the differential response to Neu5Ac and Neu5Gc, possibly because of CiaR-regulated small RNAs mediating virulence (Halfmann et al., 2007; Marx et al., 2010). CiaR-mediated effects on gene expression require its phosphorylation (Halfmann et al., 2011). However, not only the cognate sensor CiaH and other TCS sensors may mediate phosphorylation of CiaR. The internal pool of AcPh, the high-energy intermediate of the acetate kinase phosphor transacetylase pathway, can also act as a phosphor donor. Intracellular AcPh levels are affected by the pyruvate oxidase SpxB (Marx et al., 2015). It is interesting to note that pyruvate, the source of AcPh in this pathway, is also a metabolite generated in the pneumococcal catabolism of Neu5Ac (Vimr and Troy, 1985) and probably also of Neu5Gc (Hopkins et al., 2013). Subsequently, SpxB oxidizes pyruvate to H_2O_2 and AcPh. Interestingly, we found that the gene encoding pyruvate oxidase, *spxB*, as well levels of H_2O_2, and AcPh were upregulated in response to Neu5Ac relative to Neu5Gc. Because SpxB produces AcPh (Pericone et al., 2003), we hypothesized that increased intracellular AcPh, because of upregulated SpxB, lead to increased phosphorylation and activation of CiaR and, probably, also other response regulators in the bacterial cell. Marx et al. (2015) showed that a deletion of the *spxB* gene leads to a 2-fold reduction of *ciaR* transcription and its target genes. We found that not only *ciaR* was downregulated (data not shown) but also that *nanA* transcription was reduced in a *spxB* deletion mutant, supporting our hypothesis that CiaR (indirectly) controls transcription of the sialidase NanA.

After phagocytosis of microbes, immune cells like macrophages or neutrophils are known to produce reactive oxygen species (ROSs) (Gee et al., 1970; Rossi and Zatti, 1964), exerting antimicrobial activity (Babior, 1978a, 1978b). Bacteria have evolved different evasion strategies to fight oxidative stress by immune cells. Pneumococcal CiaRH has been shown to regulate the expression of HtrA, which mediates resistance to oxidative stress (Ibrahim et al., 2004b). Hence, we studied *htrA* transcription and bacterial survival in response to oxidative stress after incubation with the two Sias and found an increased transcription of *htrA* as well as higher survival rates when bacteria were incubated with Neu5Ac compared with Neu5Gc.

The RR CiaR, the main sialic acid transporter SatABC, and the sialidase NanA contribute to pneumococcal virulence (Manco et al., 2006; Marion et al., 2011; Marra et al., 2002; Orihuela

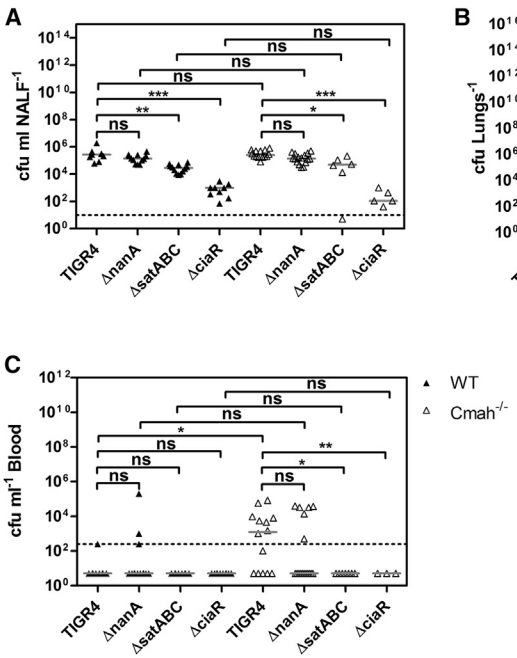

Figure 7. Sialidase NanA, the Main Sialic Acid Transporter SatABC, and the Response Regulator CiaR Contribute to the Differential Increase in Pneumococcal Virulence between *Cmah*$^{-/-}$ and WT Mice

WT and *Cmah*$^{-/-}$ mice were challenged intranasally with *S. pneumoniae* TIGR4, TIGR4Δ*nanA*, TIGR4Δ*satABC*, or TIGR4Δ*ciaR*. Samples were taken 24 hr p.i.

(A) Nasopharyngeal lavages were obtained, and bacterial numbers were determined by plating.

(B) Bacterial counts in the lungs were measured by plating lung homogenates.

(C) Bacterial numbers in the blood were determined by plating.

Dotted lines indicate the detection limits. Horizontal bars represent medians. Kruskal-Wallis with Dunn's multiple comparison test was used for statistical analysis. *p < 0.05, **p < 0.01, ***p < 0.001. Three different experiments were performed, with group sizes between three and ten mice.

et al., 2004; Throup et al., 2000). Consistent with this, TIGR4Δ*satABC* and TIGR4Δ*ciaR* did not cause and TIGR4Δ*nanA* reduced sepsis in WT or Cmah$^{-/-}$ mice in our infection model in contrast to infections with wild-type TIGR4. Challenge with TIGR4 led to a higher bacterial burden in the lungs of *Cmah*$^{-/-}$ compared with wild-type mice at 24 hr p.i. After infection with TIGR4Δ*ciaR*, TIGR4Δ*satABC*, or TIGR4Δ*nanA*, we did not detect any differences in bacterial loads of the lungs between WT and *Cmah*$^{-/-}$ mice 24 hr p.i., suggesting that these genes contribute to the increased pneumococcal virulence observed in response to Neu5Ac compared with Neu5Gc. Bacterial counts in the nasopharynx of WT and *Cmah*$^{-/-}$ mice were similar after infection with TIGR4 or TIGR4Δ*nanA*, whereas deletion of SatABC or the RR CiaR, previously associated with nasopharyngeal colonization (Sebert et al., 2002), led to lower bacterial numbers in the nasopharynx of both WT and *Cmah*$^{-/-}$ mice. TIGR4Δ*satABC* and TIGR4Δ*ciaR* had significant growth defects or failed to grow on Sia, and TIGR4Δ*ciaR* additionally did not grow on galactose (data not shown), which, like Sia, is known to be part of the glycan chain. These data suggest retained catabolite repression in the *ciaR* mutant.

After intravenous challenge with TIGR4, no differences in disease progression were observed between WT and *Cmah*$^{-/-}$ mice. Because the *nanAB* locus contains cres (Afzal et al., 2015; Gualdi et al., 2012), and *ciaR* is known to be repressed in the presence of glucose (Paixão et al., 2015), we hypothesized that these genes specifically mediate increased virulence in response to Neu5Ac in the glucose-free respiratory tract (Kingston et al., 1991) but not in the glucose-rich bloodstream (Han et al., 2008), which possesses very low levels of free Sias because of rapid renal clearance (Banda et al., 2012; Tangvoranuntakul et al., 2003).

In summary, we present findings in pneumococcal host specificity and pathogenesis. We demonstrate that pneumococci are able to sense the Sia Neu5Ac, which is abundant in humans, in a host-specific manner involving the RR CiaR. Our data show upregulation of *nanA*, *satA*, and *ciaR* and subsequent enhanced bacterial burden in *Cmah*$^{-/-}$ compared with WT mice. The increased expression of CiaR led to upregulation of *htrA* and higher resistance to reactive oxygen species and, therefore, decreased bacterial killing by host immune cells. Together, these factors mediate increased virulence in response to Neu5Ac, mainly abundant in humans, compared with Neu5Gc.

EXPERIMENTAL PROCEDURES

Bacterial Strains Used and Growth Conditions

S. pneumoniae TIGR4 (TIGR4, ATCC BAA-334) and its isogenic mutants (Table S1) were grown in C medium with yeast extract (C+Y) (Lacks and Hotchkiss, 1960) until optical density 620 (OD_{620}) = 0.45, washed with PBS, and resuspended in monosaccharide-free C+Y medium supplemented with either 12 mM glucose (Sigma-Aldrich), Neu5Ac (Nacalai), or Neu5Gc (Inalco) or no carbohydrate. The cultures were grown at 37°C in a water bath or in multiwell plates using a Bioscreen instrument (Growth Curves OY) to follow the growth.

Mutant Construction

The bacterial mutants used in this study were constructed by fusion PCR mutagenesis (Karreman, 1998). Upstream and downstream fragments of the target gene as well as the erythromycin cassette were amplified with overlapping regions using the primers listed in Table S2. The erythromycin cassette was fused to the upstream region of the target gene, and, afterwards, the downstream region was added by PCR. The correct fragment was gel-purified and transformed into *S. pneumoniae*. Transformation was carried out by culturing *S. pneumoniae* in C+Y medium until the bacteria reached OD_{620} = 0.15. To 400 μl of the culture, 100 ng μl^{-1} of the competence-stimulating peptide CSP-2 was added. After incubation at 37°C for 15 min, the fusion PCR product was added. The cells were grown at 37°C for 90 min and plated onto blood agar plates containing appropriate antibiotics. Mutants were confirmed by PCR and sequencing.

RNA Isolation, cDNA Synthesis, and Real-Time PCR

RNA from bacteria grown under in vitro conditions or from nasopharyngeal lavages of mice was isolated using the RNeasy MiniKit (QIAGEN) as described in the Supplemental Experimental Procedures.

Sialidase Activity Assay

Sialidase activity was determined as described previously (Manco et al., 2006; Supplemental Experimental Procedures).

H_2O_2 Production and Sensitivity Assay

Pneumococcal production of and sensitivity to H_2O_2 were tested as described previously (Ibrahim et al., 2004a; Pericone et al., 2003; Supplemental Experimental Procedures).

Acetyl Phosphate Production Assay

Pneumococcal production of AcPh was measured as described before (Prüss and Wolfe, 1994). For a detailed description, see the Supplemental Experimental Procedures.

Mouse Challenge

All experiments were performed in accordance with the local ethical committee (Stockholms Norra djurförsöksetiska nämnd). Six- to eight-week-old male C57BL/6 wild-type and *Cmah*$^{-/-}$ mice with a human-like deletion in exon 6 of the *Cmah* gene (Hedlund et al., 2007) were used. The Cmah$^{-/-}$ mice tested negative for a *dock2* mutation, recently shown to be present in some of these mice because of backcrossing into commercially available C57BL/6 mice (Mahajan et al., 2016). Sedation was performed by inhalation of 4% isoflurane or by intraperitoneal injection of 80 mg kg^{-1} ketamine and 5 mg kg^{-1} Rompun (Bayer) according to their body weight. Mice were infected intranasally or intravenously with 10^6 CFUs per mouse in 20 µl or 10^5 CFUs per mouse in 100 µl PBS, respectively. The health status of the mice was controlled regularly, and clinical scores were given. To obtain BALF, lungs were lavaged twice with PBS. To determine bacterial numbers in the BALF, NALF, blood, and lungs (homogenized), samples were plated onto blood agar plates.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism. Mann-Whitney test or Kruskal-Wallis with Dunn's multiple comparison test were performed to compare two groups or more than two groups, respectively.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2016.07.019.

AUTHOR CONTRIBUTIONS

K.H., J.L., and B.H.N. designed the study. K.H., J.L., and A.P. performed the research experiments. K.H., J.L., A.P., and B.H.N. analyzed the data. K.H., V.N., A.V., and B.H.N. wrote the paper.

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Supplemental Information

Streptococcus pneumoniae Senses a Human-like Sialic Acid Profile via the Response Regulator CiaR

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Supplemental Information

Supplemental Methods

RNA isolation, cDNA synthesis and real-time PCR (qPCR), related to Experimental Procedures and to Supplemental Table S2

In order to isolate RNA from bacteria grown under *in vitro* conditions, cells were grown as described above. After incubation at 37°C for 1.5 hr with or without Sia, bacteria were spun down and lysed with 1% Triton X-100. Bacteria isolated from nasopharyngeal lavages of mice were taken up RLT buffer (QIAGEN RNeasy lysis buffer). RNA was isolated using the QIAGEN RNeasy MiniKit following the manufacturer's instruction. The "High Capacity cDNA Reverse Transcription Kit" from Applied Biosystems was used to produce cDNA. To determine gene expression, qPCR was carried out using "iTaq™ SYBR® Green Supermix" and a "CFX Connect™ Real-Time PCR Detection System" (BIORAD). Primers used to amplify control and target genes are listed in Supplemental Table S2.

Sialidase activity assay, related to Experimental Procedure

Bacteria were cultured as described above and lysed with 1% Triton X-100. Sialidase activity was determined as described before (Manco et al., 2006). In brief, bacterial samples and 0.3 mM 2-O-(p-nitrophenyl)-D-N-acetylneuraminic acid (pNP-NANA; Sigma-Aldrich) were mixed 1:1 and incubated statically at 37°C for 2 hr. Ice-cold 0.5 M Na_2CO_3 was added to stop the reaction and absorbance at 405 nm was determined. To determine the activity of the sialidase, a standard curve was prepared using known concentrations of the sialidase of *Arthrobacter ureafaciens* (Sigma-Aldrich). Values were normalized against protein content of the samples (Pierce™ BCA Protein Assay Kit, ThermoFisher Scientific).

H_2O_2 sensitivity and production assay, related to Experimental Procedures

Bacteria were grown as described above and resuspended in monosaccharide-free C+Y medium supplemented with either 12 mM Neu5Ac (Nacalai) or Neu5Gc (Inalco). After an incubation at 37°C for 1.5 hr, pneumococcal sensitivity to H_2O_2 was tested as described previously (Ibrahim et al., 2004). 40 mM H_2O_2 (Sigma-Aldrich) were added and cells were incubated at 37°C for 10

minutes. Viable bacterial numbers were determined before and after exposure to H_2O_2.

Hydrogen peroxide production rates were studied as described previously (Pericone et al., 2003). Neu5Ac- and Neu5Gc-treated bacteria were incubated at 37°C for 1.5 hr, washed with PBS, resuspended in fresh monosaccharide-free C+Y supplemented with either 12mM Neu5Ac or Neu5Gc and incubated for 30 minutes at 37°C. Bacterial numbers were determined by plating serial dilutions on blood agar plates. After addition of 2,2´-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) and horseradish peroxidase (both Sigma –Aldrich) and an incubation at room temperature for 20 minutes, optical densities at 560 nm were measured and hydrogen peroxide concentrations were calculated with a standard curve using known concentrations.

Acetyl phosphate production assay, related to Experimental Procedures

The amount of acetyl phosphate (AcPh) inside the bacterial cells was determined by adapting the protocol of Pruss and Wolfe (Pruss and Wolfe, 1994). Bacteria were incubated on Neu5Ac or Neu5Gc as described above. 10 ml of culture were spun down at 4000 rpm for 10 min and resuspended in 250 µl of cold 10 mM sodium phosphate (pH 7.5), 10 mM $MgCl_2$ and 1 mM EDTA. 50µl of cold 3 M $HClO_4$ were added and the mixture was incubated for 30 min on ice. The supernatants were neutralized with saturated $KHCO_3$ (250 µl/ml) and centrifuged at 10000 rpm for 2 min. Aliquots of each extract were taken to determine the protein content by using a Micro BCA protein assay (Pierce). 50 mg of powdered activated charcoal (Sigma-Aldrich) were added per ml supernatant in order to remove small compounds like ADP or ATP. The tubes were mixed and incubated on ice for 15 min. To remove the charcoal the samples were filtered through a 0.22-µm-pore-size filter. In order to determine AcPh amounts, 1 mM $MgCl_2$, 30 µM ADP, and 4 µg of acetate kinase/ml were added to 100 µl of the sample. The reactions were incubated at 30°C for 180 min. ATP was then assayed using ATP Bioluminescence Kit (Roche) following the manufacturers' instruction. 100 µl of luciferase reagent were added to the reaction mixtures and the samples were incubated at room temperature for 2 min before the luminescence was measured using a FLUOstar Omega microplate reader. Luminescence data obtained from samples with acetate kinase were subtracted from data obtained from samples without acetate kinase. ATP amounts were determined by comparison to a standard curve with known AcPh amounts (0.2 to 50µM). Finally, the results were normalized to the protein content of the sample.

Supplemental Tables

Table S1, related to Experimental Procedures, Bacterial Strains used and growth conditions. Strains of *S. pneumoniae* used in this study. Description of wild type and mutant pneumococcal strains used with references inserted.

Strain	Relevant Genotype[a]	Reference
***S. pneumoniae* TIGR4**		
ATCC BAA-334		(Tettelin et al., 2001)
TIGR4 Sm^r	Lys56→Thr in RpsL [rpsL(*K56T*)] Sm^r	(Bender and Weiser, 2006)
TIGR4Δ*satABC*	Δ*satABC rpsL* (*K56T*) Sm^r	(Marion et al., 2011)
TIGR4Δ*satABC*Δ1688-90Δ1328	Δ*satABC*Δ1688-90Δ1328 *rpsL* (*K56T*) Sm^r	(Marion et al., 2011)
TIGR4Δ*nanA*	TIGR4 *nanA::erm* Erm^r	This study
TIGR4Δ*satABC*	TIGR4Δ*satABC::erm* Erm^r	This study
TIGR4Δ*ciaR*	TIGR4Δ*ciaR::erm* Erm^r	This study
TIGR4Δ*ciaRH*	TIGR4Δ*ciaRH::erm* Erm^r	This study
TIGR4Δ*rr01*	TIGR4Δ*rr01::erm* Erm^r	This study
TIGR4Δ*rr03*	TIGR4Δ*rr03::erm* Erm^r	This study
TIGR4Δ*rr04*	TIGR4Δ*rr04::erm* Erm^r	This study
TIGR4Δ*rr07*	TIGR4Δ*rr07::erm* Erm^r	This study
TIGR4Δ*rr08*	TIGR4Δ*rr08::erm* Erm^r	This study
TIGR4Δ*rr09*	TIGR4Δ*rr09::erm* Erm^r	This study
TIGR4Δ*rr14*	TIGR4Δ*rr14::erm* Erm^r	This study
TIGR4Δ*spxB*	TIGR4Δ*spxB::erm* Erm^r	(Syk et al., 2014)

[a] Erm^r, resistance to erythromycin, Sm^r, resistance to streptomycin

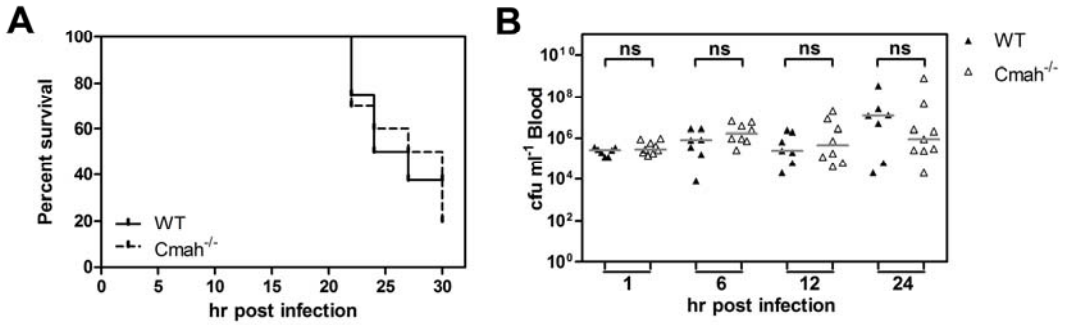
Table S2, related to Experimental procedures, Mutant construction. Primers used in this study. Description of primer sequences used for mutant construction and qPCR.

Primer name	Sequence (5'→3')
Mutant	
NanA-Up-F	TCGATGAATTCCATGGTACCTATCGAGTAGGGTAGTTCTTGCT
NanA-Up-R	TGCAAGTCACACGAACACGAAACTGATCCTCTCTATATCT
Erm_F	TTCGTGTTCGTGTGACTTGCACCATATC
Erm_R	TTATTTCCTCCCGTTAAATAATAG
NanA-Dwn-F	TATTTAACGGGAGGAAATAAAGAGATGGGCAAAGGAG
NanA-Dwn-R	AGGTGACACTATAGAACTCGAGATCGCCCTACTTTTCGTATGCA
Up_SatABC_Rev	GACACTATAGAACTCGAGATTTACCCTATGTTTTG
Erm_SP1684_fwd	GTGCAAGTCACACGAACACGAATTGTATTCTCCTATGTAATAAG
Erm_SP1678_rev	CTATTATTTAACGGGAGGAAATAATACTCTGCGAAAATCTCTTC
Dwn_SatABC_fwd	ATGAATTCCATGGTACCTACCCTACTTAAGATCGAG
RR01 F1	TAGATAGATAAAGGCCAAGTCCAGA
RR01 R1	AAAGGAGGAAAATCACATGTCCAACACCAAGAAAGGAATAGGGTACG
RR01 F3	CCTTCTCACTATTTAGTCATCCAACGTGCATGCGCTTCTCCTTTTCC
RR01 R3	ATAATGGAACCTTGTGGAATGAATA
RR03 F1	TCTTTTTATTGTTGGTTTTCAGCAT
RR03 F3	AAAGGAGGAAAATCACATGTCCAACCACCATTTGGTGGGGCAAGAGG
RR03 R3	CCCTCCATTATCACATAAACAGGTA
RR04 F1	GATATTTCTGCGACTCATTTTGAAC
RR04 R1	CCTTCTCACTATTTAGTCATCCAACTGTCATCTATTATCTCCTATTGGT
RR04 F3	AAAGGAGGAAAATCACATGTCCAACGGTTATGGTTATAACTTCAAGGAG
RR04 R3	AGATGCTCAACAATATGCTCAAGAC
RR05 F1	GTTGCTAAATTGGCTCGTCATAACT
RR05 R1	CCTTCTCACTATTTAGTCATCCAACTATCATGAGAAACTCCTCCTTATT
RR05 F3	AAAGGAGGAAAATCACATGTCCAACACTTTGCGTAGTGTTGGGTATC
RR05 R3	ATCATCTCTCCGAGCTAAGTTCA
RR07 F1	CCAAAAGCAGGTAGTGGATTTAGTA
RR07 R1	CCTTCTCACTATTTAGTCATCCAACATACATTTTCTCCCTTTCTACTCA
RR07 F3	AAAGGAGGAAAATCACATGTCCAACTACCGAAAACAGGTAGAAACTATA
RR07 R3	GGTCCATTTCATAGAAATTTTTGC
RR08 F1	CATGTGATTCCATACGAACTCTTC
RR09 F1	AGGAAAACTTGAAGAATTTTGTGGT
RR09 R1	CCTTCTCACTATTTAGTCATCCAACGGTCATGCTCTGCTCCTTTACC
RR09 F3	AAAGGAGGAAAATCACATGTCCAACCCTCGTCAGTTTAAGAAGGGAG
RR09 R3	ACAGACAAGAAGAGATGTGACACTG
RR14 F1	GAAAGCTATGACTTGATGCAACACT
RR14 R1	CCTTCTCACTATTTAGTCATCCAACCCCCATGGCTGACCTACTTATT
RR14 F3	AAAGGAGGAAAATCACATGTCCAACCGTGGTGTTGGATATACCATGC
RR14 R3	ATACCATTTGCCTCGTACTATATTTC
qPCR	
RT_16s_2_F	CTGCGTTGTATTAGCTAGTTGGTG
RT_16s_2_R	TCCGTCCATTGCCGAAGATTC
RT_nanA_fwd	GACATATTCGAAAGCGGGCGTAACG
RT_nanA_rev	GCGTTCATCTGCACCTGCGATCAAAG
RT_CiaR_fwd	TGACTGCCAAGGAAAGTTTG
RT_CiaR_rev	GTTTGAGAAGGGCCTGAATC
RT_rr01_fwd	CATGCTTCAATCCGTACCCT
RT_rr01_rev	TATTTGAGCATGCAGGCAAC
RT_rr02_fwd	GCTCAAATTCACGATGGGTT

Table S2 Cont.

Primer name	Sequence (5'→3')
RT_rr02_rev	AGCTCTTCTGCGTCGTTCTC
RT_rr03_fwd	TGCCAAAGGCTATATGCTCA
RT_rr03_rev	TCCATATGATTGCGGTGGTA
RT_rr04_fwd	ACGCCGTGAGTATGACCTTT
RT_rr04_rev	ATTTGTCTCGGTCGCACTTT
RT_rr06_fwd	CCAAGACCTCTTGTCCATCC
RT_rr06_rev	AGTTGCAGATGACGAGGAAA
RT_rr07_fwd	GGTCAGTGTGCTGGAAGTCA
RT_rr07_rev	CTGCTGGGCAGCCTTAATAC
RT_rr08_fwd	TCGTTGACGACGAGGTAGAA
RT_rr08_rev	AACAGCTCTAGCGCTTCCAG
RT_rr09_fwd	GCCTCATCTAAATGGCATCC
RT_rr09_rev	TTTGACAGCAGACAAGGCAT
RT_rr10_fwd	TGAAAGCTACAGTGCAAGCC
RT_rr10_rev	TAACACGGTCAAAGGGAACC
RT_rr11_fwd	TTTCGCGATATTAGCTGCCT
RT_rr11_rev	GCTCAGGGCTTCTCTAACCA
RT_rr12_fwd	AGTCGCAAACTCTGATCGAC
RT_rr12_rev	TCCAATATCATACAAGACAACGG
RT_rr13_fwd	CATCAATCCTGCAAATGTGG
RT_rr13_rev	GCCTCAGACACTTCCCTGAC
RT_rr14_fwd	ACGCCGTGAGTATGACCTTT
RT_rr14_rev	ATTTGTCTCGGTCGCACTTT
RT_spxB_fwd	AGCTCAAGGAGCTGTTGGAT
RT_spxB_rev	GAAGTGGACGGTGTTGAGTG
RT_htrA_fwd	ACCAGCCCAAGTGGATATTG
RT_htrA_rev	AGCATCAACCACATGACCAA

Supplemental Figures

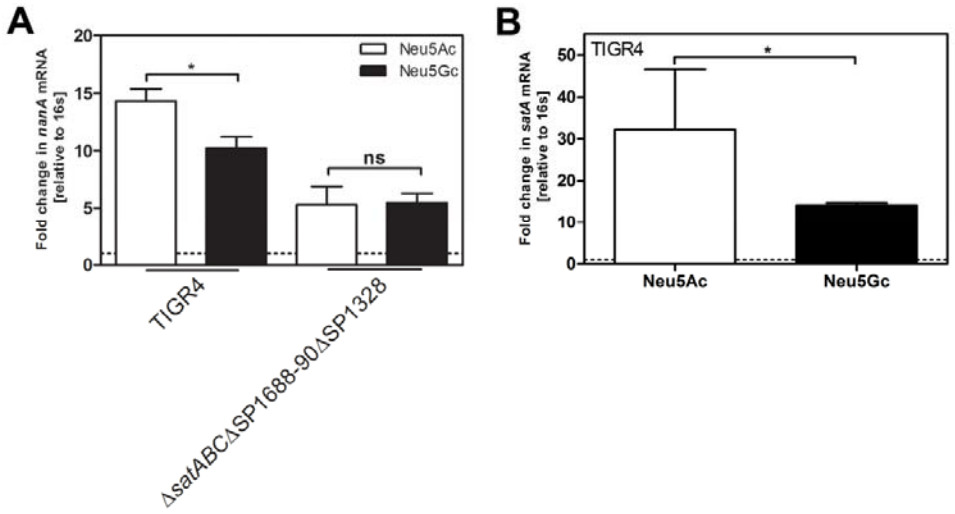


Supplemental Figure S1, related to Figure 1. Pneumococcal disease progresses similar in Cmah$^{-/-}$ and wild-type mice after intravenous challenge.

Mice were infected intravenously with *S. pneumoniae* TIGR4, 10^5 cfu in 100 µl PBS.

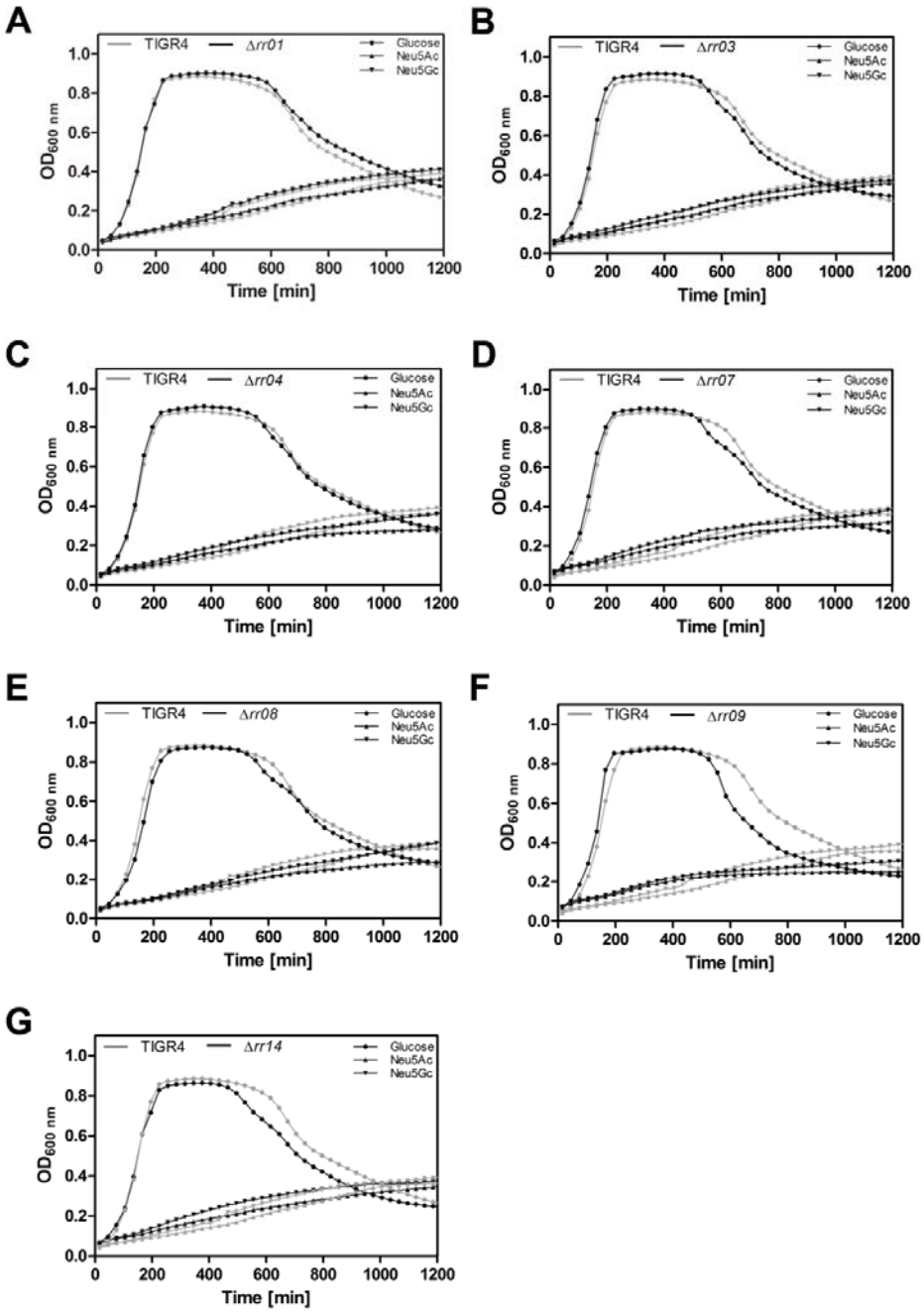
(A) The survival rates of WT and Cmah$^{-/-}$ mice were monitored over the time of the experiment.

(B) Bacterial numbers in the blood of wt and Cmah$^{-/-}$ mice were determined by plating at 1, 6, 12 and 24 hr p.i. (**A-B)** The dotted line indicates the detection limit. Horizontal bars represent medians. For survival rates the Log-Rank Mantel-Cox Test and for cfu counts the Mann-Whitney Test were used for statistical analysis. ns, non-significant. 2 different experiments were performed with group sizes of 4 and 5 mice.



Supplemental Figure S2, related to Figure 2. Catabolite repression leads to an upregulation of sialidase NanA and the main sialic acid transporter SatABC in response to Neu5Ac and Neu5Gc compared to growth on glucose.

(A-B) TIGR4 and TIGR4Δ*satABC*ΔSP1688-90ΔSP1328 were grown on glucose until OD_{620nm}=0.45. Cells were washed with PBS and resuspended in C+Y medium containing either 12 mM Neu5Ac or Neu5Gc. After incubation for 1.5 hr, RNA was isolated, cDNA was synthetized and qPCR was performed to determine mRNA levels of **(A)** the pneumococcal sialidase A (NanA) or **(B)** the substrate binding protein SatA of the main sialic acid transporter SatABC. 16s was used as endogenous control. Fold change of transcription was normalized to bacteria grown on glucose-containing C+Y until they reached OD_{620nm}=0.45 (dotted line). Data in **(A-B)** as mean ± SEM from at least 3 independent experiments. The Mann-Whitney Test was used for statistical analysis. *, $p<0.05$; ns, non-significant.



Supplemental Figure S3, related to Figure 4. Mutants in the response regulators RR01, RR03, RR04, RR07, RR08, RR09 and RR014 do not affect growth on glucose or sialic acid

Strains **(A)** TIGR4Δ*rr01*, **(B)** TIGR4Δ*rr03*, **(C)** TIGR4Δ*rr04*, **(D)** TIGR4Δ*rr07* and **(E)** TIGR4Δ*rr08*, **(F)** TIGR4Δ*rr09* and **G** TIGR4Δ*rr14* were grown on 12 mM glucose (Glc), Neu5Ac or Neu5Gc. **(A-G)** Optical density at 600 nm was monitored over time. Growth curves of TIGR4 on 12 mM glucose, Neu5Ac or Neu5Gc were plotted as a reference in each graph. Data is represented as mean of 2 independent experiments.

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