

Influenza Promotes Pneumococcal Growth during Coinfection by Providing Host Sialylated Substrates as a Nutrient Source

Steven J. Siegel,¹ Aoife M. Roche,¹ and Jeffrey N. Weiser^{1,2,*}

¹Department of Microbiology

²Department of Pediatrics

University of Pennsylvania, Philadelphia, PA 19104, USA

*Correspondence: weiser@mail.med.upenn.edu

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SUMMARY

Much of the mortality attributed to influenza virus is due to secondary bacterial pneumonia, particularly from *Streptococcus pneumoniae*. However, mechanisms underlying this coinfection are incompletely understood. We find that prior influenza infection enhances pneumococcal colonization of the murine nasopharynx, which in turn promotes bacterial spread to the lungs. Influenza accelerates bacterial replication in vivo, and sialic acid, a major component of airway glycoconjugates, is identified as the host-derived metabolite that stimulates pneumococcal proliferation. Influenza infection increases sialic acid and sialylated mucin availability and enhances desialylation of host glycoconjugates. Pneumococcal genes for sialic acid catabolism are required for influenza to promote bacterial growth. Decreasing sialic acid availability in vivo by genetic deletion of the major airway mucin Muc5ac or mucolytic treatment limits influenza-induced pneumococcal replication. Our findings suggest that higher rates of disease during coinfection could stem from influenza-provided sialic acid, which increases pneumococcal proliferation, colonization, and aspiration.

INTRODUCTION

Even before the discovery of its viral cause, bacterial infections have been recognized as an important complication of influenza (McCullers, 2006). Much of the mortality attributed to this virus during both seasonal and pandemic influenza seasons is actually from secondary bacterial pneumonia, particularly due to *Streptococcus pneumoniae*, the pneumococcus (McCullers, 2006).

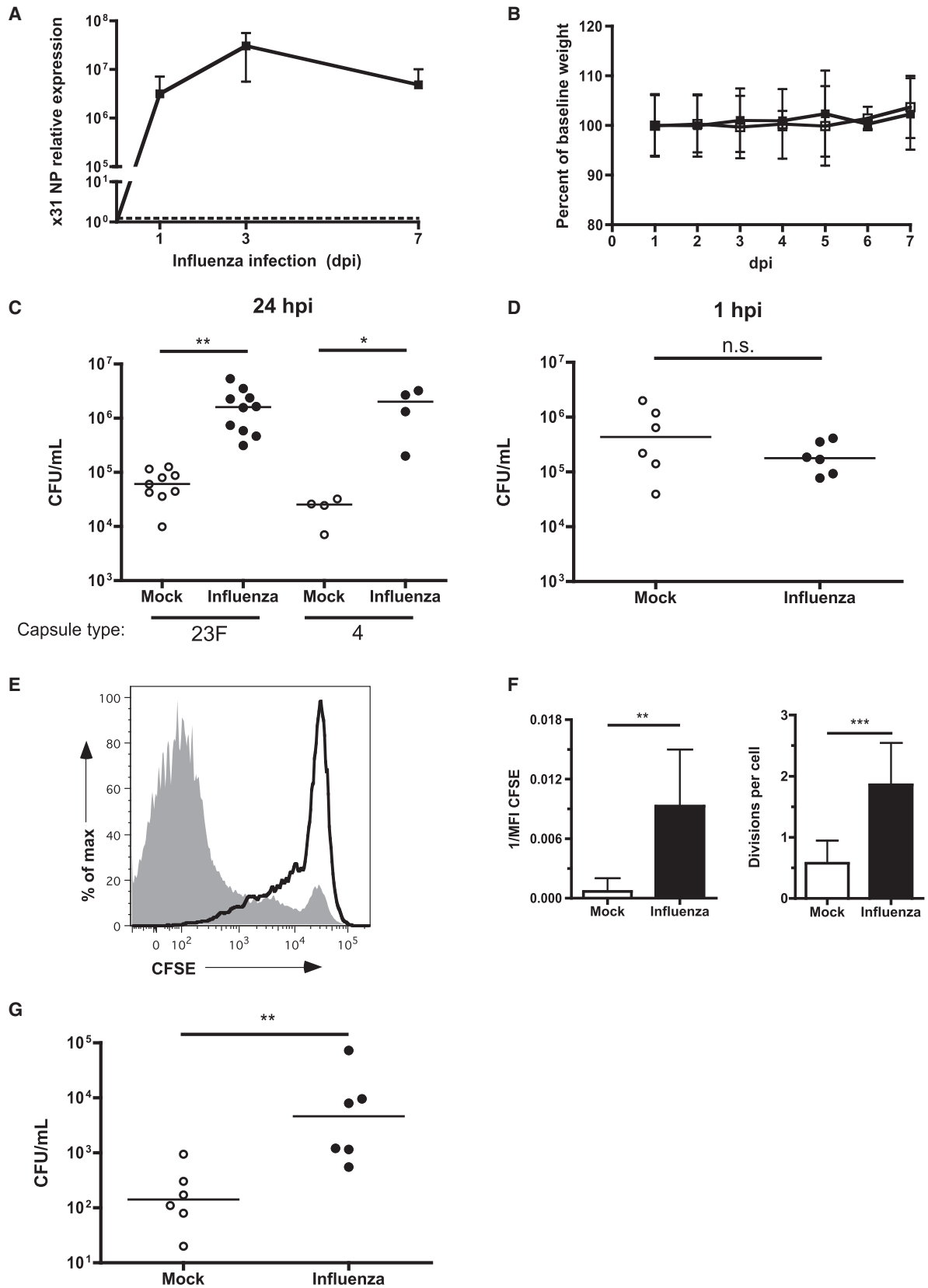
All pneumococcal disease begins with asymptomatic colonization of the nasopharynx (Bogaert et al., 2004). Though carriage is the prerequisite to invasive disease, including pneumonia, most studies of influenza-pneumococcal interaction have relied on directly inoculating bacteria into the lung, bypassing this first, conserved step in pathogenesis (McCullers, 2006; Metzger and Sun, 2013). However, there is evidence that influenza predisposes the host to acquiring pneumococcal colonization (Grijalva

et al., 2014). Pneumococcal colonization in adults and children is temporally associated with viral upper respiratory tract (URT) infections, including influenza, (Kim et al., 1996; Vu et al., 2011) and experimental influenza infection leads to higher pneumococcal loads in the human nasopharynx (Wadowsky et al., 1995). Additionally, the URT is the initial site of replication for both pneumococci and influenza (Bogaert et al., 2004; Matrosovich et al., 2004).

In addition to focusing on the lower respiratory tract, studies of the interactions between influenza and pneumococci have historically emphasized influenza-induced epithelial damage that alters lung architecture and promotes bacterial adherence (Metzger and Sun, 2013; Olitsky and Gates, 1921). More recent studies have observed in vitro that influenza neuraminidase can expose receptors for bacterial adherence by removing terminal sialic acid residues from host glycoconjugates and in vivo that neuraminidase inhibitors protect from postinfluenza pneumonia (McCullers, 2004; Peltola and McCullers, 2004). However, viral strains that do not cause pathologic changes in the epithelium can still lead to secondary bacterial pneumonia in animal models, implying that tissue damage is not necessary for influenza to promote bacterial disease (Metzger and Sun, 2013).

Other work has focused on defects in antibacterial immunity directed by prior viral infection. Influenza influences the immune response to secondary bacterial challenge in murine models of coinfection (McCullers, 2006; Metzger and Sun, 2013). Different groups have demonstrated changes in neutrophil recruitment to the lungs, alveolar macrophage function, and macrophage recruitment to the nasopharynx during postinfluenza challenge (Nakamura et al., 2011; Shahangian et al., 2009; Sun and Metzger, 2008). These immune effects are general, but only a small subset of opportunistic bacterial pathogens cause the vast majority of postinfluenza pneumonia, and chief among these is the pneumococcus (Klugman et al., 2009; McCullers, 2006; Metzger and Sun, 2013). The predominance of pneumococci in postinfluenza disease suggests that this bacterium is particularly able to take advantage of the influenza-infected environment. We hypothesized that influenza infection predisposes the host to rapid pneumococcal growth in the nasopharynx by providing a nutrient source for replicating bacteria.

Here, we show influenza promotes pneumococcal proliferation during colonization in a mouse model of coinfection, that this rapid bacterial growth is dependent on acquisition of the host metabolite sialic acid, that sialylated airway mucins are required for this effect, and that both influenza and



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pneumococcal neuraminidases contribute to the release of sialic acid from host substrates *in vivo*.

RESULTS

Influenza Promotes Pneumococcal Colonization, Growth, and Aspiration

To determine whether influenza infection could promote bacterial colonization of the URT, mice were intranasally inoculated with influenza A virus or PBS (mock). This procedure was carried out using a small volume in unanesthetized mice to avoid direct introduction into the lower respiratory tract. The x31 influenza strain replicated efficiently in the URT, as measured by quantitative RT-PCR (qRT-PCR) viral titers in nasal lavages, which peaked at 3 days postinoculation (Figure 1A). This infection did not cause weight loss or other signs of systemic disease (Figure 1B). Seven days after inoculation, we challenged the mice with pneumococci 24 hr before obtaining nasal lavages to measure bacterial colonization density. Influenza infection promoted >25-fold higher density of colonizing pneumococci with clinical isolates of two different serotypes, 23F (strain P1121) and 4 (strain TIGR4) (Figure 1C).

To address whether this difference in colonization density was due to influenza promoting increased retention of the bacterial inoculum, we challenged mock- and influenza-infected mice for <1 hr before measuring bacterial density in nasal lavages. In contrast to observations at 24 hr postinoculation, there was no difference in bacterial load 1 hr postchallenge, suggesting that initial adherence to the epithelium was not responsible for the effect of prior influenza infection on colonization (Figure 1D).

We next tested whether influenza promoted bacterial growth, rather than or in addition to inhibiting antibacterial immunity in the nasopharynx. To specifically measure bacterial replication *in vivo*, we developed a flow cytometric assay using carboxy-fluorescein diacetate succinimidyl ester (CFSE), a dye used to track eukaryotic cell division. Each round of cell division yields bacteria with less CFSE fluorescence, which we measured in individual cells using flow cytometry (Parish, 1999). We validated the assay *in vitro* and *in vivo* to demonstrate that only dividing bacteria progressively lost CFSE fluorescence (Figure S1 available online). This assay was applied to influenza coinfection by inoculating CFSE-labeled pneumococci into mice that had been previously influenza or mock infected. Eight hours later, we recovered the colonizing pneumococci in nasal lavages, stained the lavage with capsule-type-specific antibody to distin-

guish pneumococci from other particles, and measured CFSE fluorescence per cell. Pneumococci colonizing mice that had been previously infected with influenza had substantially less CFSE fluorescence per cell than did those colonizing mock-treated mice (Figure 1E). To quantify this growth effect, we measured the median fluorescence intensity (MFI) of CFSE per bacterium in each condition. As replication occurred, the MFI decreased and 1/MFI increased (Figure 1F). Additionally, we calculated the division index, the number of divisions per cell (Roederer, 2011). Bacteria colonizing influenza-infected mice underwent more divisions than those in mock-infected mice (Figure 1F). Over 8 hr, influenza promoted 3.7-fold more divisions per bacterial cell compared to PBS treatment, an effect predicted to increase bacterial numbers by 13-fold ($2^{3.7}$). This corresponded to the 12.1-fold increase in colonization density actually observed, a further validation for this assay and demonstrating the importance of bacterial growth in mediating increased colonization during coinfection.

Pneumonia generally begins with aspiration of upper airway contents into the lungs, and clinical studies have noted that pneumococcal pneumonia is associated with higher density of concurrent colonizing pneumococci in the nasopharynx (Albrich et al., 2012; Vu et al., 2011). We hypothesized that the increased bacterial growth stimulated by influenza could increase the likelihood of aspiration of pneumococci into the lungs. To test this possibility, we measured bacterial loads in the bronchoalveolar lavage fluid (BAL) of mock- and influenza-treated mice 24 hr after establishing pneumococcal colonization of the URT. Influenza-infected mice had a higher bacterial burden in the lower respiratory tract compared to mock-treated mice (Figure 1G). Colonization density in the URT correlated with bacterial load in the BAL (Spearman correlation coefficient, $r = 0.6$, $p < 0.05$).

Influenza Increases Sialic Acid Availability in the Nasopharynx

We next sought to determine the nutrient(s) influenza was providing to colonizing pneumococci. Pneumococci have complex growth requirements and can catabolize >30 different carbohydrates as carbon sources (Buckwalter and King, 2012). To assess the availability of possible nutrient sources from inflammation and epithelial damage following influenza infection, we measured the total protein concentration of nasal lavages obtained from mice 7 days after influenza or mock infection and found no significant difference between these mice (Figure 2A). We measured the total reducing sugar content of nasal lavages

Figure 1. Influenza Promotes Pneumococcal Colonization, Growth, and Aspiration

(A) Mice were infected with influenza for the indicated number of days, nasal lavages with RLT RNA lysis buffer were obtained, and qRT-PCR was performed to measure the relative expression of x31 viral nucleoprotein (NP).

(B) Mice given PBS (mock, open symbols) or influenza (closed symbols) were weighed daily for 7 days, and the percent change in weight from baseline graphed.

(C) Mice were infected with influenza or PBS, followed 7 days later by intranasal inoculation with bacteria of the indicated serotype. After 24 hr postbacterial inoculation (hpi), nasal lavages were obtained and plated for quantitative culture of colonizing pneumococci.

(D) Mice were inoculated with influenza or PBS, followed 7 days later by challenge with strain P1121. Within 1 hr, nasal lavages were obtained and plated for quantitative culture (CFUs).

(E) After 7 days of mock (black line) or influenza (gray shaded) infection, mice were inoculated with CFSE-labeled pneumococci for 8 hr. Nasal lavages were obtained, fixed, and stained for pneumococcal capsule, and flow cytometry was performed to compare bacterial replication.

(F) The MFI of CFSE per bacterial cell was calculated for each condition and displayed as 1/MFI, or number of divisions per cell (division index).

(G) Mock- or influenza-infected mice were challenged 7 days later with WT pneumococci for 24 hr. Bronchoalveolar lavages were obtained and plated for quantitative culture. Horizontal lines indicate median values, and data in (F) are represented as mean \pm SD. n.s., not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. All experiments were performed at least twice, with four to eleven mice per group. See also Figure S1.

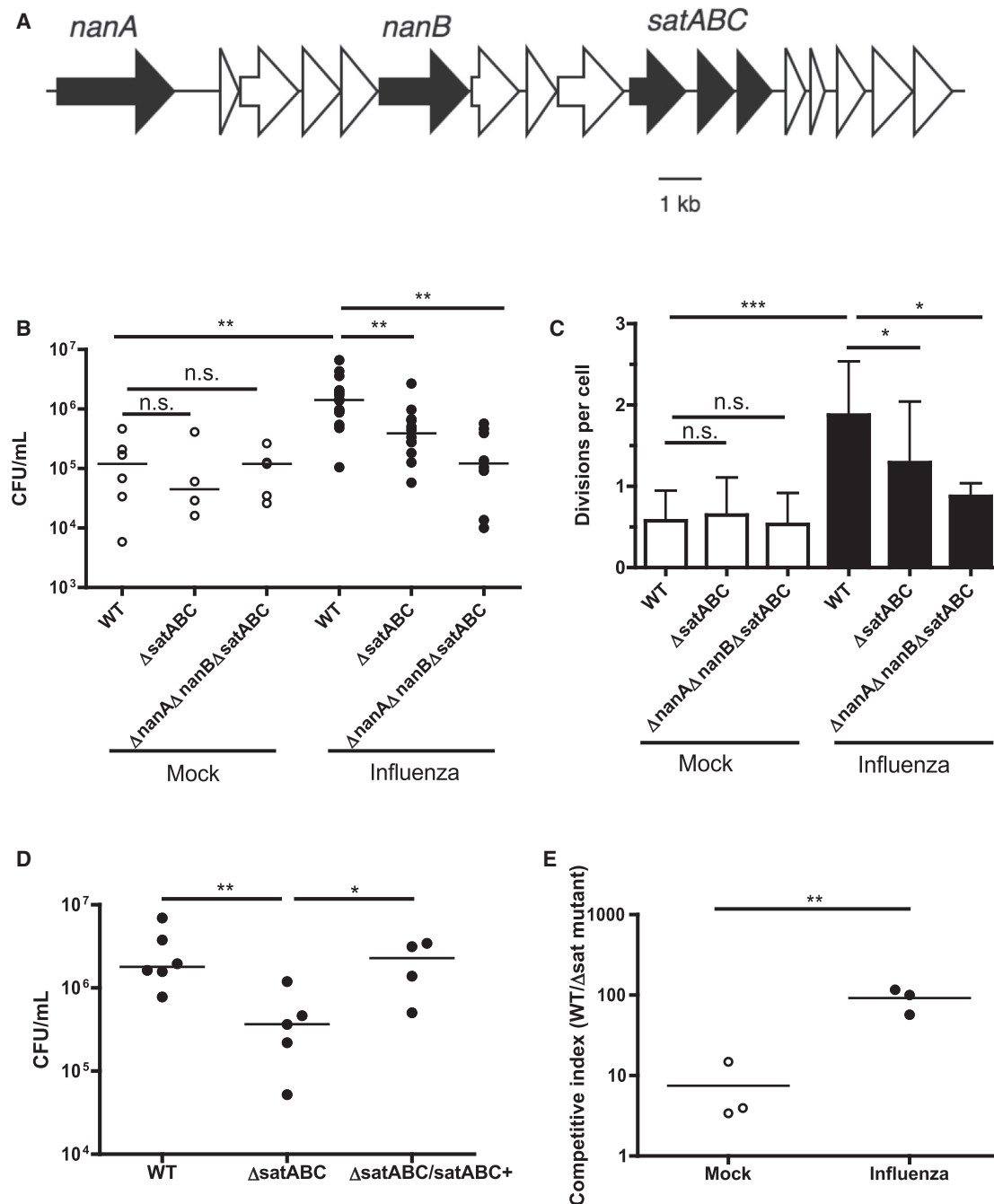


Figure 3. Pneumococci Exploit Host Glycoconjugates for Growth during Coinfection

(A) The genetic locus in strain P1121 containing the neuraminidase (*nanA* and *nanB*) and sialic acid transport (*satABC*) genes is displayed.

(B) Seven days after mock (open symbols) or influenza infection (closed symbols), mice were inoculated with CFSE-labeled pneumococci of the indicated genotypes for 8 hr. Nasal lavages were obtained and plated for quantitative culture.

(C) Lavages were fixed and stained for pneumococcal capsule, and flow cytometry was performed to measure bacterial replication (division index).

(D) Mice were infected with influenza for 7 days, followed by 24 hr of colonization with the indicated bacterial strains. Nasal lavages were plated for quantitative culture.

(E) Seven days after mock or influenza infection, mice were given a mixed inoculum of sialic-acid-catabolizing and $\Delta satABC$ pneumococci. One day later, nasal lavages were obtained and plated for quantitative culture. Colonies from the plated inoculum and the lavages were patched onto antibiotic-containing media to determine the relative advantage in vivo of sialic acid catabolism and calculate competitive indices. CI > 1 indicates outcompetition of the streptomycin-resistant $\Delta satABC$ pneumococci and was defined as the ratio of WT/mutant bacteria in the output (nasal lavage) divided by the ratio of WT/mutant bacteria in the input (inoculum). Data in (C) are represented as mean \pm SD. Horizontal lines indicate median values. n.s., not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Experiments were performed at least twice, with 3–15 mice per group.

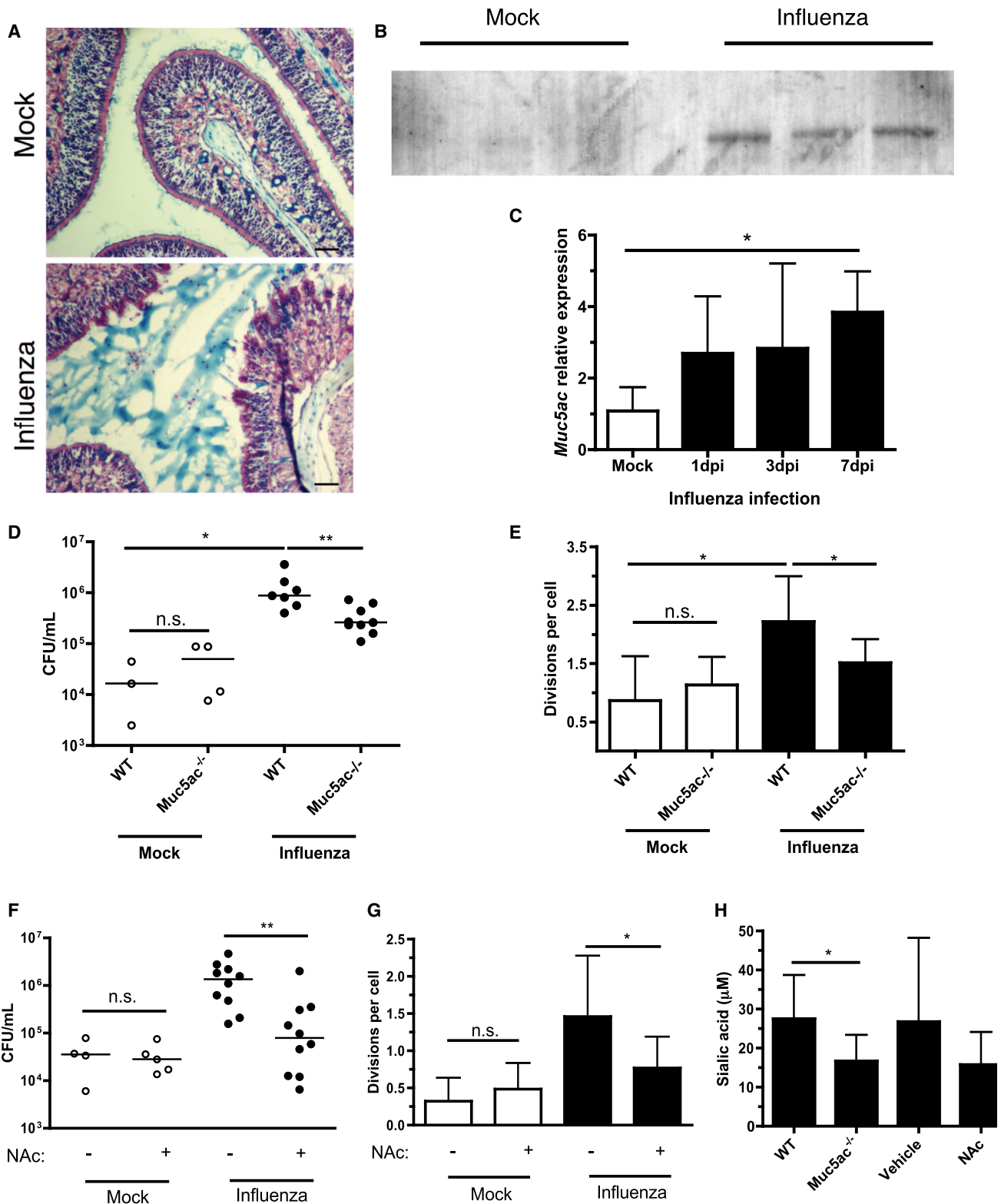


Figure 4. Sialylated Airway Mucins Are Required for Influenza-Induced Pneumococcal Growth

(A) Mouse heads were obtained after 7 days of mock or influenza infection, fixed, decalcified, and sectioned. URT sections were stained with Alcian blue and nuclear fast red. Scale bars, 50 μm.

(B) Nasal lavages were obtained from mice infected with influenza or PBS for 7 days and then analyzed by western blot for the presence of Muc5ac.

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This was accompanied by increased *Muc5ac* transcription in the URT that was maximal at 7 dpi (Figure 4C). These results correlated with the availability of total sialic acid, which was also maximal at 7 dpi (Figure 2D). To test whether mucins were a major source of sialic acid during influenza infection, we first examined *Muc5ac*^{-/-} mice. There was no defect in pneumococcal colonization or growth in *Muc5ac*^{-/-} mice in the absence of influenza, but the influenza-mediated increases in bacterial load (Figure 4D) and replication (Figure 4E) were attenuated in mice that lack this airway mucin. These decreases in growth and bacterial density in *Muc5ac*^{-/-} mice were not due to a less robust influenza infection, as determined by viral titers (data not shown). To further assess the role of mucins in pneumococcal replication during coinfection, we intranasally treated mock- and influenza-infected WT mice with the mucolytic agent N-acetylcysteine daily until pneumococcal challenge. Mucolytic treatment reduced bacterial load (Figure 4F) and growth (Figure 4G), but only in influenza-infected mice. Both genetic deletion and mucolytic treatment reduced total sialic acid in the influenza-infected nasopharynx, confirming that sialylated mucins are a host-derived source of sialic acid pneumococci exploit during coinfection (Figure 4H).

Influenza and Bacterial Neuraminidases Desialylate Host Substrates

We next examined whether viral and bacterial neuraminidases released sialic acid from host sources for pneumococci to use for growth. We qualitatively measured sialic acid release from the epithelial surface by fluorescence microscopy using a fluorescently labeled lectin from *Erythrina cristagalli*, which binds to galactose residues that are exposed when sialic acid is cleaved (Chang et al., 2012). After 7 days of influenza or mock infection, URT sections from mouse heads were stained with the *E. cristagalli* lectin. Influenza infection promoted increased desialylation of the mucosal surface in the nasopharynx. Bacterial neuraminidase desialylated host surfaces further, as there was additional lectin binding in samples from mice given pneumococci for 24 hr after 7 days of influenza. This effect was specific to the pneumococcal neuraminidases, as the epithelial surface in influenza-infected mice colonized with neuraminidase-deficient (Δ *nanA* Δ *nanB*) pneumococci had less lectin binding, and therefore less desialylation, than did those colonized with WT pneumococci (Figure 5A). As a quantitative measure of desialylation, we used flow cytometry to quantify desialylation on infiltrating leukocytes (>80% neutrophils), which are abundant in the influenza-infected airway and can be easily obtained by nasal lavage (data not shown). This method confirmed the pattern of desialylation observed with fluorescence microscopy (Figure 5B). These findings indicated that both viral and bacterial

neuraminidases contribute to the desialylation in vivo of host substrates during coinfection.

Sialic Acid Catabolism Contributes to Pneumococcal Colonization in the Absence of Influenza

To assess the role of sialic acid catabolism during pneumococcal colonization, we measured sialic acid in nasal lavages from mice inoculated with pneumococci in the absence of influenza infection. Total sialic acid increased over the duration of colonization, though no free sialic acid was detectable (Figure 6A). Consistent with the slow increase in sialic acid during colonization, we observed that WT pneumococci outcompeted Δ *satABC* and that this outcompetition increased over time, but it was delayed compared to influenza coinfection (Figure 6B). As with influenza infection, pneumococcal colonization induced URT transcription of the sialylated mucin *Muc5ac* (Figure 6C).

Nutrient-Poor Conditions on the Human Mucosal Surface Favor Growth of Sialic Acid Catabolizing Pneumococci

To extend our findings on sialic acid catabolism to human samples, we competed bacterial strains for growth in vitro in human nasal airway surface fluid (hNASF), the natural environment in which pneumococci replicate. There was little competition between WT and Δ *satABC* pneumococci when grown in nutrient broth that contains glucose, (Figure 7A) an in-vitro-preferred carbon source not found in airway secretions (Phillips et al., 2003). The competitive advantage due to sialic acid catabolism increased 15-fold when pneumococci were grown in nutrient-limited hNASF. In contrast, there was no significant advantage for WT over the genetically corrected Δ *satABC*/*satABC*⁺ strain in growth in hNASF (Figure 7A). This effect correlated with the higher levels of sialic acid found in hNASF compared to nutrient broth (Figure 7B). These sialic acid levels could not be directly compared to those found in murine nasal lavages, as the latter are dilutions of the epithelial lining fluid. The total volume of upper airway surface fluid in an adult mouse has been estimated as 3 μ l (Asahi et al., 2002). Assuming the 200 μ l nasal lavage contained the entire surface lining fluid, the lavages are at least a 1:67 dilution of that fluid. The \sim 30 μ M sialic acid concentration measured, therefore, would correspond to >2 mM sialic acid on the mucosal surface during coinfection, a concentration sufficient to promote growth in vitro as a sole carbon source (Figure 7C).

DISCUSSION

Influenza has long been associated with secondary bacterial pneumonia, but how this viral infection primes the host for the

(C) Nasal lavages with RLT RNA lysis buffer were obtained from mice influenza or mock-infected for 7 days. qRT-PCR was performed, and relative expression of *Muc5ac* was measured.

(D) Mice of indicated genotype were mock or influenza infected for 7 days, followed by inoculation with CFSE-labeled pneumococci for 8 hr. Nasal lavages were obtained and plated for quantitative culture.

(E) Lavages were also fixed and stained for pneumococcal capsule, and flow cytometry was performed to measure bacterial replication (division index).

(F and G) WT mice were infected with influenza or mock, followed by daily treatment for 7 days with vehicle (PBS) or 0.5 M N-acetylcysteine (NAC). CFSE-labeled pneumococci were inoculated for 8 hr, then nasal lavages were obtained and used for quantitative culture (F) and flow cytometric analysis of cell division (G).

(H) Total sialic acid content was measured by thiobarbituric acid assay on samples from (D)–(G). Data are represented as mean \pm SD. Horizontal lines indicate median values. *, $p < 0.05$; **, $p < 0.01$. Experiments were performed at least twice, with 3–13 mice per group.

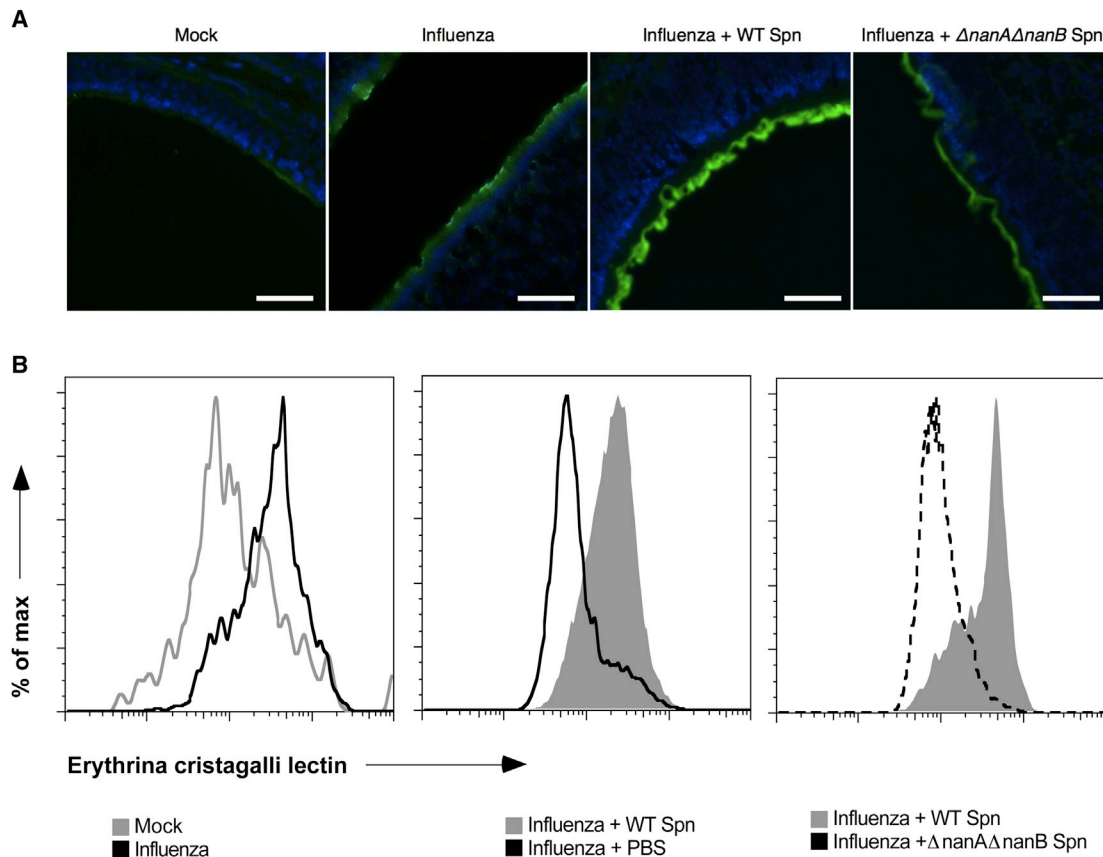


Figure 5. Influenza and Bacterial Neuraminidases Desialylate Host Substrates

(A and B) Mouse heads (A) and nasal lavages (B) were obtained from separate mice after 7 days of influenza or mock infection, followed by 24 hr of colonization with WT or $\Delta nanA\Delta nanB$ pneumococci, or PBS. Heads were fixed, decalcified, and sectioned. URT sections were stained with DAPI and FITC-labeled lectin from *E. cristagalli* that binds galactose residues exposed when sialic acid is removed. All images were taken with the same optical settings. Scale bars, 50 μ m. Lavages were stained with FITC-labeled *E. cristagalli* lectin. Flow cytometry was performed by gating on neutrophils and comparing desialylation. Experiments were performed at least twice, with three to ten mice per group.

rapid development of high bacterial loads has not been fully understood. Here, we demonstrated that influenza infection predisposed mice to increased pneumococcal colonization in the nasopharynx and that faster bacterial replication contributed to this effect. This influenza-mediated growth led to increased aspiration into the lower respiratory tract and was dependent on pneumococcal catabolism of the host sugar sialic acid and host expression of sialylated airway mucins, with both viral and bacterial neuraminidases contributing to the desialylation of host cells in vivo. Sialic acid utilization was important even during pneumococcal colonization in the absence of influenza, but to a lesser extent, consistent with the lower levels of sialic acid present without influenza infection.

In this study, we challenged mice with pneumococci after 7 days of influenza infection in the URT. At 7 days postinfection, influenza viral titers are declining from a peak reached at 3 to 5 dpi, the same time at which sialic acid levels and sialylated mucin expression in the nasopharynx are increasing. Previous animal studies have indicated this time point correlates with the peak of susceptibility to pneumonia when pneumococci are introduced directly into the lower respiratory tract (McCullers and Rehg, 2002; Sun and Metzger, 2008). The window of

susceptibility to pneumococcal pneumonia is also 7 to 10 days following influenza infection in humans (Shrestha et al., 2013).

Correlations between URT pneumococcal colonization density and pneumonia have been observed in human patients, but it has not been clear whether higher bacterial burdens in the upper airway precede pneumonia or are an effect of lower airway disease (Albrich et al., 2012). We found that influenza, by promoting higher bacterial load in the nasopharynx, led to increased aspiration of pneumococci into the lower respiratory tract, experimentally demonstrating that higher levels of colonization can increase a key step in the early pathogenesis of pneumonia.

Previous studies of the interaction between influenza and pneumococci have focused on epithelial damage or immune dysfunction induced by the viral infection, promoting increased bacterial adherence or decreased bacterial clearance, respectively (McCullers, 2006; Metzger and Sun, 2013). We found no difference in initial adherence of the bacterial inoculum after influenza infection, and further found no increases in total protein or glycan content, suggesting this infection did not cause extensive tissue damage. Using the dilution of CFSE dye, we demonstrated a role for bacterial replication in mediating increased

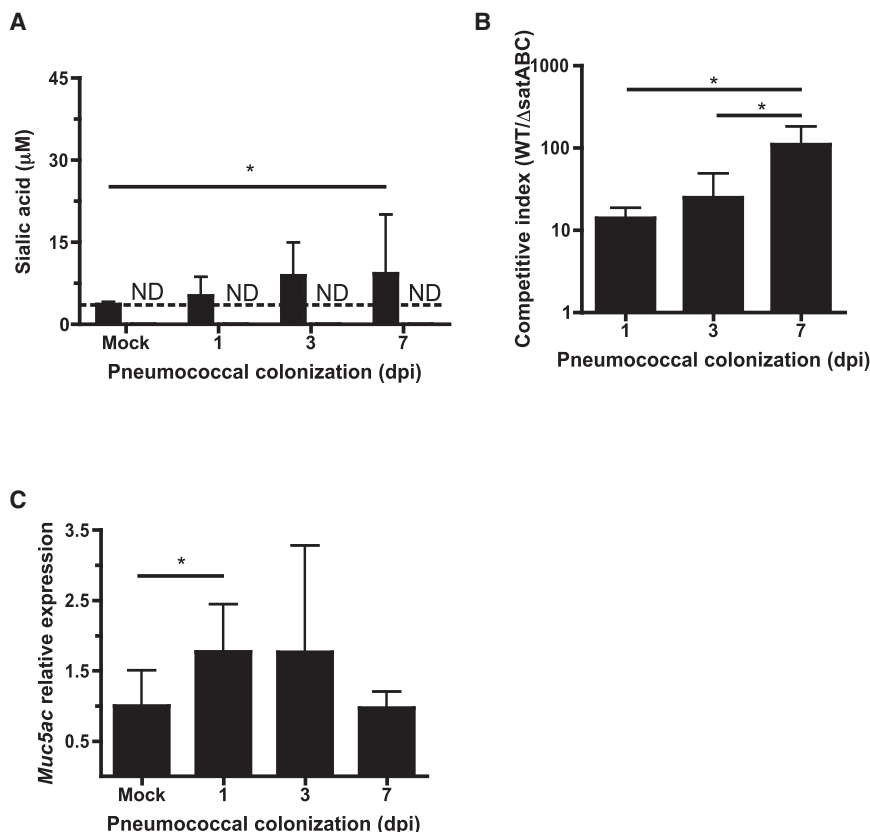


Figure 6. Sialic Acid Catabolism Contributes to Pneumococcal Colonization in the Absence of Influenza

(A) Mice were colonized with pneumococci for the indicated number of days. Sialic acid concentrations in lavages were measured by thiobarbituric acid assay, with or without acid hydrolysis to measure total (solid bars) and free sialic acid (open bars), respectively.

(B) WT and $\Delta satABC$ pneumococci were competed in vivo by colonizing naive mice with mixed inocula. Nasal lavages were plated for quantitative culture and colonies from the inocula and lavages patched onto antibiotic-selective media to determine the relative advantage of sialic acid catabolism in vivo and calculate competitive indices. CI > 1 indicates WT outcompeted $\Delta satABC$ pneumococci.

(C) Nasal lavages were obtained with RLTR RNA lysis buffer from mice colonized with pneumococci for the indicated number of days. qRT-PCR was performed to measure relative expression of *Muc5ac*. Data are represented as mean \pm SD. ND, not detected (below the limit of detection, indicated with a dotted line); *, $p < 0.05$. Experiments were performed at least twice, with 3–20 mice per group.

colonization due to influenza. Previous studies of the interaction between influenza and bacteria have measured bacterial numbers, the sum of bacterial growth and clearance. We measured both bacterial numbers and bacterial growth, allowing us to separate the contribution of bacterial growth from that of immune evasion. Our data are consistent with previous studies demonstrating the importance of viral neuraminidase for the interaction of influenza and pneumococci (Peltola and McCullers, 2004; Peltola et al., 2005). Those studies emphasized neuraminidase removing sialic acid to expose underlying receptors for pneumococci, but the same enzymatic activity would also release free sialic acid into the nasopharynx for pneumococci to utilize.

Our work has implications for the therapeutic use of neuraminidase inhibitors. Most clinical studies of neuraminidase inhibitor efficacy have focused on early treatment, (Hernán and Lipsitch, 2011; Hsu et al., 2012), but experiments in mice demonstrated survival benefits to neuraminidase inhibitor treatment during coinfection even at later time points when there was no effect on viral replication (McCullers, 2004). Our results may provide a mechanistic understanding of this effect, as inhibiting viral neuraminidase may limit sialic acid release even after the peak in viral replication. Additional studies on the effect of late neuraminidase inhibitor treatment on complications related to bacterial density, such as pneumonia, could be warranted. Bacterial neuraminidases, including pneumococcal NanA and NanB, are not inhibited by clinically used neuraminidase inhibitors at concentrations reached in vivo (Nishikawa et al., 2012). Broader neuraminidase inhibi-

tors that can prevent bacterial acquisition of sialic acid, and underlying sugars may be an important, unexplored therapeutic strategy.

We observed desialylation of the mucosal surface and leukocytes during influenza infection, exacerbated by coinfection with neuraminidase-expressing pneumococci. We found that transcription of *Muc5ac* was upregulated in the nasopharynx during influenza infection as part of the inflammatory response, consistent with a previous report in the lungs of influenza-infected mice (Barbier et al., 2012). We also found that mucus and *Muc5ac* secretion increased during influenza infection and that reducing mucins by genetic knockout of *Muc5ac*, or solubilizing mucus by N-acetylcysteine treatment, decreased influenza-induced pneumococcal growth. Mucins are 50%–90% glycan by mass and are heavily sialylated, particularly at the terminal, most-accessible ends of sugar chains (Angata and Varki, 2002; Rose and Voynow, 2006). The epithelial surface is also heavily sialylated and could serve as an additional source of sialic acid during influenza infection (Lewis and Lewis, 2012). The commensal flora of the nasopharynx could also provide sialic acid (Shakhnovich et al., 2002).

Sialic acid is both necessary and sufficient for pneumococcal colonization in mice (Marion et al., 2011; Trappetti et al., 2009). We noted a competitive disadvantage in vivo that increased over 7 days of colonization for pneumococci that lack the sialic acid transporter *SatABC*, consistent with previous work showing a defect in colonization at 5 days postinoculation (Marion et al., 2011). Increased nasal secretions, reflecting greater sialylated mucin production, have been associated with higher density of pneumococcal colonization in children (Rodrigues et al., 2013). Exogenous sialic acid, but not other amino sugars, has been shown to increase colonization density

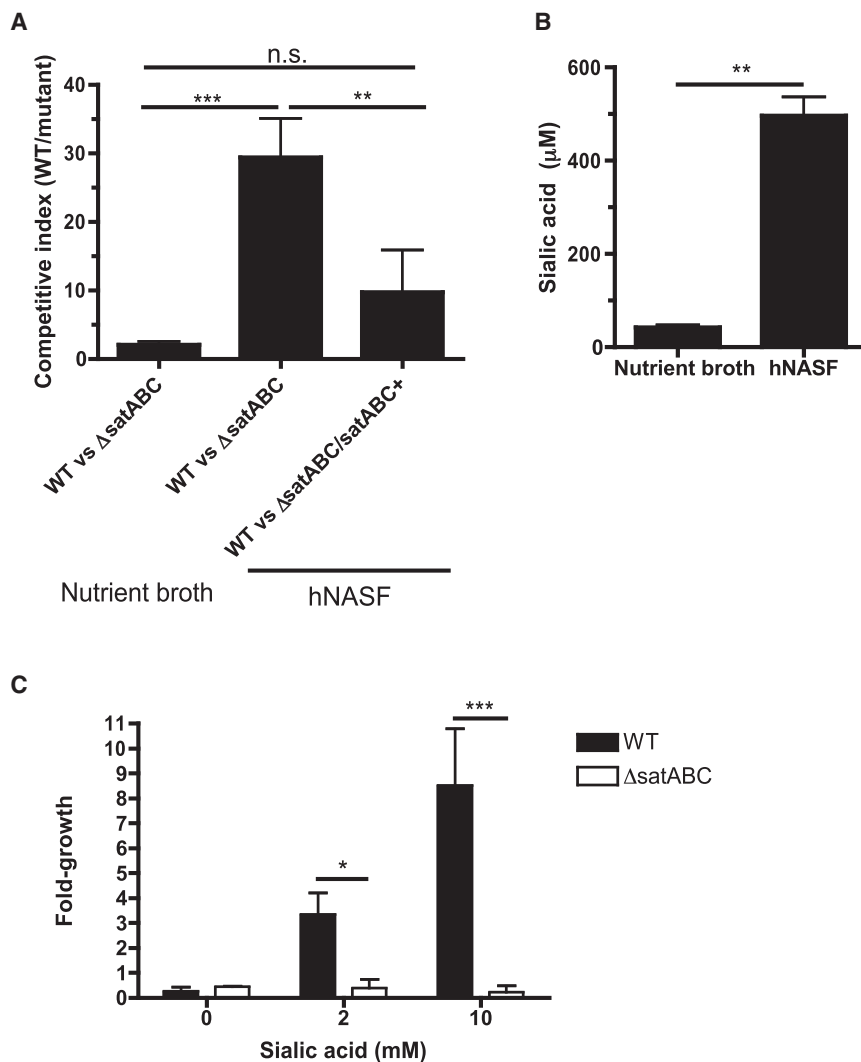


Figure 7. Nutrient-Poor Conditions on the Human Mucosal Surface Favor Growth of Sialic-Acid-Catabolizing Pneumococci

(A) Bacteria of the indicated genotypes were grown for 8 hr in mixed inocula in tryptic soy nutrient broth or in nutrient-limited hNASF. Aliquots were plated at the beginning and end of the growth period, and colonies patched onto antibiotic-selective media to determine the relative advantage of sialic acid catabolism in vivo and calculate competitive indices. CI > 1 indicates WT outcompeted the indicated mutant strain.

(B) Total sialic acid in the growth media used in (A) was measured by thiobarbituric acid assay after acid hydrolysis.

(C) WT (solid bars) and $\Delta satABC$ (open bars) pneumococci were grown for 24 hr in chemically defined medium with the indicated concentrations of sialic acid as the sole carbon source. Data show fold-growth compared to inoculum. Data are represented as mean \pm SD. n.s., not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Experiments were performed at least twice.

aminidase activity is required for providing sufficient amounts of sialic acid.

Obtaining a source of carbon is necessary for bacterial growth in any environment, but how this occurs during colonization of the nutrient-poor mucosal surface has been unclear (Buckwalter and King, 2012). Free sugar is particularly limited in the nasopharynx, the pneumococcal niche, unlike in the intestines or oral cavity (Phillips et al., 2003). In the gut, sialic acid released by the sialidase of one bacterial species can promote sialic acid catabolism-dependent growth of another (Ng et al., 2013). Carbon acquisition

is especially important for the pneumococcus, which devotes more of its genome to sugar transporters than any other sequenced bacterium (Tettelin et al., 2001). This diversity implies that pneumococci may encounter different host sugars in abundance in distinct microenvironments; infection with neuraminidase-expressing viruses such as influenza could provide one such microenvironment. Pneumococci are not the only bacteria that can use sialic acid. Interestingly, the other prominent pathogens in secondary bacterial pneumonia following influenza, *Staphylococcus aureus* and *Haemophilus influenzae*, can also catabolize this host sugar (Olson et al., 2013; Vimr et al., 2000). Sialic acid utilization could also contribute to other common bacterial complications following influenza infection, such as acute otitis media (McCullers, 2006).

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EXPERIMENTAL PROCEDURES

Mice

Six- to eight-week-old C57Bl/6 mice were obtained from Jackson Laboratory. *Muc5ac*^{-/-} mice on a C57Bl/6 background were previously described (Hasnain et al., 2011). Procedures were carried out according to an animal protocol approved by the University of Pennsylvania IACUC.

Influenza Infection

Mice were intranasally inoculated with 2×10^4 TCID₅₀ of influenza A virus, strain HKx31 (H3N2 from A/Hong Kong/1/1968 with the backbone of PR8 virus), diluted into 10 μ l PBS.

Bacterial Strains and Colonization

Pneumococcal strains included TIGR4 (Tettelin et al., 2001) and P1121, (McCool et al., 2002) clinical isolates of capsule types 4 and 23F, respectively. Mutants of P1121 in the sialic acid transporter (*satABC*) were previously made using the Janus cassette to create unmarked and correct mutations (Marion et al., 2011). Mutants in the neuraminidase genes *nanA* and *nanB* were constructed as described previously by introducing antibiotic resistance cassettes (King et al., 2004). Pneumococci were grown in tryptic soy (TS) nutrient broth at 37°C until mid-log phase. For mouse colonization, 10^7 CFU bacteria were inoculated intranasally in a volume of 10 μ l. Mice were sacrificed, and nasal lavages obtained with 200 μ l PBS. Lavages were plated on TS agar supplemented with catalase (5,000 U/plate) (Worthington Biochemical) and 5 μ g/mL neomycin for quantitative culture in 5% CO₂. TS agar was supplemented with 200 μ g/ml streptomycin to distinguish Δ *satABC* pneumococci from WT. For bronchoalveolar lavage, 1 ml PBS was instilled into the lungs intratracheally, withdrawn with a syringe, and used for quantitative culture.

CFSE Staining

Bacteria were resuspended in 1 ml PBS with 1% catalase and 10 μ M carboxy-fluorescein diacetate succinimidyl diester (Molecular Probes). Reactions were incubated at 37°C for 25 min and then washed three times in PBS. Mice were inoculated with CFSE-labeled pneumococci. After 8 hr, mice were sacrificed and nasal lavages obtained and then prepared for flow cytometry.

Flow Cytometry

For the in vivo CFSE assay, nasal lavages were obtained from mice inoculated with CFSE-labeled pneumococci. Samples were fixed and stained with typing serum specific to the capsule type used (Statens Serum Institut), followed by AF647-labeled secondary antibody to rabbit IgG, then analyzed by flow cytometry. For measurement of desialylation, nasal lavages were stained with FITC-labeled lectin from *Erythrina cristagalli* (Vector Labs) and antibodies to identify neutrophils: anti-Ly6G (clone 1A8) and anti-CD11b. Flow cytometry was conducted using FACS Calibur and FACS Canto instruments (Becton Dickinson) and analyzed using FlowJo software (Tree Star).

Protein, Reducing Sugar, and Sialic Acid Measurements

Protein was measured by bicinchoninic acid assay (Pierce). Reducing sugar was measured by tetrazolium blue assay (Jue and Lipke, 1985). Samples were diluted 1:100 in 0.1% tetrazolium blue, 0.05 M NaOH, and 0.5 M potassium sodium tartrate. Samples were boiled for 10 min, and absorbance was measured at 655 nm. Sialic acid was measured by thiobarbituric acid assay (Nakano and Ozimek, 1999; Skoza and Mohos, 1976). To measure total sialic acid, samples were first incubated with 0.1 M H₂SO₄ for 30 min at 80°C. All samples were then incubated with 25 μ M periodic acid/62.5 mM H₂SO₄ for 30 min at 37°C, followed by boiling in 2% sodium arsenite/0.5 M HCl and 6% thiobarbituric acid for 10 min. Absorbance was measured at 550 nm and compared to a standard curve to calculate concentrations in lavage fluid. The specificity of this assay was confirmed by using exogenous neuraminidase instead of acid hydrolysis to enzymatically release sialic acid.

qRT-PCR

RNA was harvested from the URT epithelium by lavage with RLT buffer (QIAGEN) with 1:100 β -mercaptoethanol. RNA was isolated with the RNeasy kit (QIAGEN), and cDNA was reverse transcribed with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). Reactions were carried out with Sybr Green reagents (Applied Biosystems) with 10 ng cDNA and 0.5 μ M primers. Comparisons were made between conditions by the $\Delta\Delta$ C_T method. Primer sequences are in Supplemental Experimental Procedures.

Western Blot

Proteins in nasal lavages were separated by SDS-PAGE on a 10% Tris gel (Bio-Rad) and transferred onto PVDF membrane. Muc5ac was detected by poly-

clonal antibody (Santa Cruz Biotechnology) and rabbit anti-goat secondary antibody conjugated to alkaline phosphatase (Sigma).

Microscopy

Tissue sections of the URT were obtained as previously described (Nelson et al., 2007). Staining and microscopy was performed as described, modified by blocking in 1% gelatin in PBS when using *E. cristagalli* lectin.

Growth in Human Nasal Airway Surface Fluid

Human nasal airway surface fluid was isolated as previously described (Gould and Weiser, 2001). Pneumococci were grown to log phase in TS nutrient broth and diluted to a concentration of 10⁴ CFU/mL in both hNASF and TS. Reactions were supplemented 1:100 with catalase and incubated at 37°C in 5% CO₂ for 8 hr. Samples were plated for quantitative culture at the start and end of the growth period, and colonies were patched onto antibiotic selective media to calculate competitive indices. Pneumococci were grown in chemically defined medium as previously described, (Kloosterman et al., 2006) with sialic acid in place of glucose.

Statistical Analysis

Prism (Graphpad) was used for statistical analysis. Comparisons were made by Mann-Whitney U test for colonization (CFU) data and by Student's t test for all other data. For multiple comparisons, we used Kruskal-Wallis with Dunn's post hoc test or 1-way ANOVA with Newman-Keuls post hoc test, respectively.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.chom.2014.06.005>.

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