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Discovery and characterization of *de novo* sialic acid biosynthesis in the phylum *Fusobacterium*

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Abstract

Sialic acids are nine-carbon backbone carbohydrates found in prominent outermost positions of glycosylated molecules in mammals. Mimicry of sialic acid (N-acetylneuraminic acid, Neu5Ac) enables some pathogenic bacteria to evade host defenses. *Fusobacterium nucleatum* is a ubiquitous oral bacterium also linked with invasive infections throughout the body. We employed multidisciplinary approaches to test predictions that *F. nucleatum* engages in *de novo* synthesis of sialic acids. Here we show that *F. nucleatum* sbsp. *polymorphum* ATCC10953 NeuB (putative Neu5Ac synthase) restores Neu5Ac synthesis to an *Escherichia coli neuB* mutant. Moreover, purified *F. nucleatum* NeuB participated in synthesis of Neu5Ac from *N*-acetylmannosamine and phosphoenolpyruvate *in vitro*. Further studies support the interpretation that *F. nucleatum* ATCC10953 NeuA encodes a functional CMP-sialic acid synthetase and suggest that it may also contain a *C*-terminal sialic acid O-acetylesterase. We also performed BLAST queries of *F. nucleatum* genomes, revealing that only 4/31 strains encode a complete pathway for *de novo* Neu5Ac synthesis. Biochemical studies including mass spectrometry were consistent with the bioinformatic predictions, showing that *F. nucleatum* ATCC10953 synthesizes high levels of Neu5Ac, whereas ATCC23726 and ATCC25586 do not express detectable levels above background. While there are a number of examples of sialic acid mimicry in other phyla, these experiments provide the first biochemical and genetic evidence that a member of the phylum *Fusobacterium* can engage in *de novo* Neu5Ac synthesis.

Key words: carbohydrate biosynthesis, *Fusobacterium*, infection, molecular mimicry, sialic acid

Introduction

Sialic acids are a family of more than 50 structurally distinct naturally occurring nine-carbon carbohydrates present on the surfaces of all vertebrate cells (Angata and Varki 2002). *N*-acetylneuraminic acid (Neu5Ac) is the most common sialic acid in humans. In mammals, accumulating evidence strongly supports a model of sialic acids as part of a molecular definition of self (Varki and Gagneux 2012). Mammalian sialic acid-binding proteins have been shown to act in multiple contexts to limit immune responses upon binding of sialic acid molecules (Varki and Gagneux 2012; Blaum et al. 2015). Indeed, a number of pathogenic bacteria express surface sialic acids as virulence factors. In these bacterial contexts, sialic acids have been shown to shield pathogens from host immune responses by interacting with host sialic acid-binding proteins (Carlin et al. 2009; Chang et al. 2014; Inzana et al. 2012; Ram et al. 1998). For example, roles of bacterial sialic acid mimicry in complement evasion (Factor H) and suppression of neutrophil function (Siglecs) have been described for many different pathogens (Edwards et al.

1980, 1982; Pluschke et al. 1983; Marques et al. 1992; Edwards et al. 1993; Gill et al. 1996; Kahler et al. 1998; Figueira et al. 2007, 2008; Carlin et al. 2009; Ali et al. 2014; Chang et al. 2014; Inzana et al. 2012; Kline et al. 2011; Lewis et al. 2015; Platt et al. 1994; Ram et al. 1998; Takahashi et al. 1999). Two striking examples of bacterial sialic acid mimicry are the perinatal pathogens Group B *Streptococcus* (GBS) and *Escherichia coli* K1. These bacteria are the leading causes of sepsis and meningitis in newborns in the United States (Simonsen et al. 2014). Despite being distantly related, these two bacteria share the ability to survive and proliferate in blood, a host environment in which most pathogens rapidly succumb to host defenses (Weiman et al. 2009). In both GBS and *E. coli* K1, a capsular polysaccharide displaying sialic acid residues is essential for bloodstream survival and progression to meningitis (see references cited above).

Fusobacterium nucleatum is a Gram-negative obligate anaerobe that ubiquitously colonizes periodontal spaces between gums and teeth. *F. nucleatum* overgrows in active sites of periodontal (gum) disease (Moore and Moore 1994). Periodontal disease is characterized by chronic inflammation and periodontal "pockets" that become infected and expose underlying connective tissues and blood capillaries to oral bacteria (Inaba and Amano 2010). *F. nucleatum* is also a common cause of extra-oral infections, most commonly bacteremia and abscesses (skin, brain, pleura, etc.) (Denes and Barraud 2016). A less appreciated site of *F. nucleatum* infection is the reproductive tract; the organism can cause both vaginal (Hillier et al. 1993; Hitti et al. 2001; Holst et al. 1994) and intrauterine infections (Altshuler and Hyde 1985; Chaim and Mazor 1992; Digiulio et al. 2010). In fact, *F. nucleatum* is one of the most frequently isolated organisms from amniotic fluid of pregnant women who experience preterm labor (Chaim and Mazor 1992; Hill 1998; Bearfield et al. 2002; Hitti et al. 2001), and has also been associated with spontaneous miscarriage and stillbirth (Altshuler and Hyde 1985; Chaim and Mazor 1992; Han et al. 2010). Periodontal disease, which is thought to affect ~40% of reproductive age women (Boggess 2008), has also been linked with increased risks of pregnancy complications such as preterm birth and low birth weight (Offenbacher et al. 1996; Dasanayake 1998; Pitiphat et al. 2008; Guimaraes et al. 2010; Saddki et al. 2008).

The idea that *F. nucleatum* may be capable of disseminating from sites in the mouth through the bloodstream is supported by several lines of investigation. The hematogenous route of *F. nucleatum* infection has been modeled in pregnant mice by administering bacteria into the bloodstream through the tail vein and monitoring bacterial titers in tissues, including the placenta, at later time points (Han et al. 2004). These studies demonstrated that bacteria administered intravenously make their way to placental tissue, proliferate there, and then invade into amniotic fluid and fetal tissues, eventually resulting in fetal resorption and stillbirth (Han et al. 2004; Liu et al. 2007). Further evidence for the hematogenous route of dissemination from oral to uterine tissue has come from analysis of bacterial isolates from oral and amniotic fluid sources in the same individual (Han et al. 2010). Another study developed primer sets capable of distinguishing between the five subspecies of *F. nucleatum* and performed PCR using these primer sets on DNA isolated from different body sites of 10 women with localized periodontal pockets who delivered preterm (Gonzales-Marin et al. 2013). This study identified the subspecies *polymorphum* in both aspirated amniotic fluid and matched maternal oral samples, but not from maternal vaginal samples, strongly implicating this specific subspecies as a likely culprit for neonatal *F. nucleatum* infections of oral origin. Pioneering work by the Han lab utilized the animal model (described above) to demonstrate that the protein FadA is required for *F. nucleatum* localization to and proliferation in the placenta and its subsequent invasion into amniotic fluid and fetal tissue (Ikegami et al. 2009). More recently, the adhesin Fap2 has also been shown to be important for bacterial interactions with host carbohydrates, immunomodulation and colonization of the placenta following bloodstream administration (Kaplan et al. 2010; Coppenhagen-Glazer et al. 2015; Gur et al. 2015).

We previously applied phylogenetic methods to survey available microbial genomes for sialic acid biosynthetic pathways (Lewis et al. 2009). This approach revealed a clade of sialic acid biosynthetic machinery encoded by distantly related perinatal pathogens including GBS, *E. coli* K1, and *F. nucleatum* (Lewis et al. 2009). The importance of sialic acids in the pathophysiology of bloodstream infections caused by GBS and *E. coli* K1 is well known. Although not well studied, the possibility of sialic acid metabolism in *F. nucleatum* is beginning to attract more attention in the scientific community (Gangi Setty et al. 2014; Yoneda et al. 2014). However, despite some promising initial studies, rigorous biochemical and genetic experiments establishing that members of this genus encode active sialic acid biosynthetic enzymes and engage in *de novo* sialic acid biosynthesis have not yet been undertaken. Here we perform a series of such experiments using *F. nucleatum* sbsp. *polymorphum* ATCC10953. Together, the data demonstrate that this strain encodes active enzymes involved in the *de novo* biosynthesis of sialic acids. Furthermore, high performance liquid chromatography (HPLC) and mass spectrometry showed that this strain does in fact produce high levels of Neu5Ac. These experiments provide the first conclusive evidence that some members of the genus *Fusobacterium* can synthesize sialic acids *de novo* and suggest that *F. nucleatum* may employ these molecules to evade the immune system and disseminate through the bloodstream to cause disease at sites distant from the mouth.

Results

F. nucleatum neuB (from ATCC10953) encodes a functional Neu5Ac synthase

The putative pathway for sialic acid biosynthesis (homologs of NeuC, NeuB and NeuA) in *F. nucleatum* is encoded in a gene cluster containing a variety of other annotated carbohydrate-active enzymes (see Figure 1 and Table I). To examine the functional activity of the putative *F. nucleatum* sialic acid synthase (NeuB homolog), parallel genetic and biochemical approaches were employed. Chromosomal alterations in *Fusobacterium* have been reported in strains ATCC 12230, ATCC25586, and ATCC23726 and are not currently feasible in ATCC10953 due to its resistance to transformation. Instead, we utilized *E. coli* as a facile genetic system to examine the potential function of *F. nucleatum* NeuB. UTI89 is a uropathogenic strain of *E. coli* that synthesizes a K1 capsule consisting of an α2-8-linked homopolymer of sialic acid. We constructed a *neuB* deletion in *E. coli* UTI89 (*E. coli* Δ*neuB*) and used this strain to examine whether *F. nucleatum neuB* could restore its ability to synthesize sialic acid (Neu5Ac). Sialic acids were hydrolyzed with mild acid, fluorescently derivatized, resolved by HPLC with fluorescence detection, and compared to biological and chemically synthesized sialic acid standards. Analysis of *E. coli* Δ*neuB* showed the expected lack of sialic acid synthesis (Figure 2A). However, complementation of this strain with plasmids containing either *E. coli neuB* or *F. nucleatum neuB*

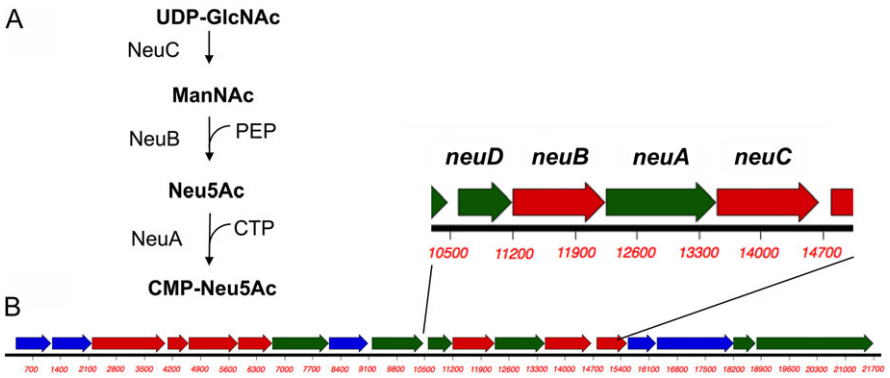

Fig. 1. A putative sialic acid biosynthetic pathway in *F. nucleatum* subsp. *polymorphum*. (**A**) Proposed pathway of Neu5Ac biosynthesis in *F. nucleatum* ATCC10953. (**B**) The putative gene cluster encoding homologs of Neu5Ac biosynthetic enzymes and other predicted carbohydrate-active enzymes. Please see Table I for more detailed information. This figure is available in black and white in print and in colour at *Glycobiology* online.

resulted in the restoration of sialic acid (Neu5Ac) biosynthesis (Figure 2A). This experiment demonstrates that *F. nucleatum* NeuB is able to function within the context of the *E. coli* sialic acid biosynthesis machinery to restore sialic acid synthesis in a strain devoid of the endogenous *E. coli* sialic acid synthase.

Biochemical experiments were also carried out using purified *F. nucleatum* NeuB. Briefly, *F. nucleatum* NeuB was expressed as a his-tagged enzyme in the *E. coli* MG1655 Δ*nanA* strain LSR4, which lacks both a sialic acid biosynthetic pathway as well as a sialic acid-degrading aldolase. Purified NeuB protein was then used in biochemical experiments to examine whether it is capable of catalyzing the synthesis of Neu5Ac. Experiments used *N*-acetylmannosamine (Man*NAc*) together with either pyruvate (negative control) or phosphoenolpyruvate (PEP), and Neu5Ac production was monitored by 1,2-diamino-4,5-methylenedioxybenzene (DMB) HPLC as described in the methods. These experiments clearly demonstrate that in the presence of enzyme and PEP (but not pyruvate), *F. nucleatum* NeuB acted as a Neu5Ac synthase (Figure 2B).

F. nucleatum neuA encodes a CMP-Neu5Ac synthetase

O-acetylation is a common modification of sialic acids in bacteria and mammals and is known to modulate a number of distinct biological processes involving sialic acids (Varki and Gagneux 2012). O-acetylation of sialic acids also occurs in some of the bacteria that mimic sialic acids, including some strains of *E. coli* and GBS. The WT strain of *E. coli* (UTI89) used in this study had low levels of sialic acid O-acetylation under the conditions tested (Figure 3A). However, HPLC analysis revealed that the *E. coli* Δ*neuB* strain expressing *F. nucleatum* NeuB had an unexpected accumulation of O-acetylation on the sialic acid side chain at the seven- and nine-carbon positions (Figure 3A, see the green trace). Peak assignment was based on (1) retention times of sialic acid standards run in parallel, and (2) mild base treatment, showing elimination of peaks corresponding to *O*-acetyl esters together with a corresponding increase in size of the parent Neu5Ac peak. The accumulation of Neu5,7Ac_2 and Neu5,9Ac_2 observed upon complementation of the *neuB* mutant was very similar to a phenotype we previously reported upon *neuA* deletion in GBS (Lewis et al. 2007). In the *E. coli* UTI89 gene cluster, *neuA* is directly downstream of *neuB* and encodes an N-terminal CMP-sialic acid synthetase domain and C-terminal sialic acid O-acetylesterase domain (Steenbergen et al. 2006; Lewis et al. 2007). Consistent with the observation that the *neuB* and *neuA* genes overlap by one base pair and may be translationally coupled, we hypothesized that the observed increase in O-acetylated sialic acids in the *E. coli* Δ*neuB* + *F. nucleatum neuB* strain may reflect a polar effect of the *neuB* deletion on the expression of downstream *neuA*. This would theoretically lead to *intracellular* accumulation of O-acetylated sialic acids due to a decreased ability of *E. coli* to de-O-acetylate intracellular sialic acids (using the C-terminal NeuA esterase) or to activate them for capsular assembly (using the N-terminal CMP-sialic acid synthetase).

To formally test this hypothesis, low molecular weight pools of free sialic acid inside cells were separated from cell-associated capsular sialic acids and examined by DMB-HPLC. These analyses demonstrated a massive accumulation of O-acetylated sialic acids inside the *E. coli* Δ*neuB* + *F. nucleatum neuB* strain, a phenotype that was reversed upon overexpression of *F. nucleatum neuA* (Figure 3B). Overall levels of O-acetylation present in whole cells also plummeted with the addition of plasmid-based *neuA* (Figure 3A). Finally, to examine surface levels of sialic acids in these strains, a commercially available purified sialidase was utilized to remove bound surface sialic acids from cells, followed by HPLC. Such analysis revealed a ~25-fold reduction in surface sialic acid expression in the Δ*neuB* + *F. nucleatum neuB* strain. Simultaneous complementation with *F. nucleatum neuB* and full-length *neuA* resulted in a large increase in the surface sialylation compared to the strain with only *neuB* complementation (Figure 4), consistent with the results of the intracellular sialic acid analysis (Figure 3). These data are consistent with the hypothesized polar effect on the downstream *E. coli neuA* gene in the Δ*neuB* + *F. nucleatum neuB* strain. The change from intracellular accumulation of O-acetylated sialic acids to surface-expressed non-O-acetylated sialic acids strongly suggests that *F. nucleatum neuA* encodes an active bifuctional CMP-Neu5Ac synthetase/sialic acid O-acetylesterase that acts to remove *O*-acetyl modifications prior to CMP-sialic acid activation.

Bioinformatic analysis suggests that *de novo* Neu5Ac synthesis by *F. nucleatum* is strain-specific

To investigate whether *de novo* sialic acid synthesis is conserved among strains and subspecies of *F. nucleatum*, we performed bioinformatic analysis on 31 genomes (as of 15 March 2015) representing all four subspecies of *F. nucleatum* (*animalis, nucleatum, vincentii* and *polymorphum*). Sequenced genomes were systematically queried by BLAST using NeuB, NeuA and NeuC amino acid

Table I. Gene cluster in *F. nucleatum* ATCC10953 encoding a putative biosynthetic pathway for Neu5Ac

Accession number	Gene position	Current NCBI annotation	Comments
WP_005897404[a]	4397–5395	ADP-L-glycero-D-manno-heptose-6-epimerase	Well conserved among many *Fusobacterium* strains
WP_005897403[b]	5413–6228	UDP pyrophosphate phosphatase	56% Identical over 98% coverage with an ortholog from *Leptotrichia trevisanii*
WP_005897401[c]	6870–7766	dTDP-4-dehydrorhamnose reductase	Well conserved among *Fusobacterium*. 50% identical over 98% coverage with ortholog from *L. trevisanii*
WP_005897400[d]	7779–8777	Hypothetical protein	85% Identical over 100% coverage to an uncharacterized protein predicted to be involved in expolysaccharide biosynthesis in *F. nucleatum animalis*
WP_005897399[e]	8777–10,600	UDP-4-dehydro-6-deoxy-2-acetamido-D-glucose 4-reductase	Includes UDP-GlcNAc-inverting 4,6-dehydratase FlaA1 and capsular polysaccharide biosynthesis protein EpsC (cell wall/membrane/envelope biogenesis)
WP_005897398[f]	10,667–11,191	Glycosyltransferase Bacterial sugar transferase	
WP_005897396[g]	11,192–12,421	Capsular polysaccharide biogenesis protein, DegT/DnrJ/EryC1/StrS aminotransferase family	3-Amino-5-hydroxybenzoic acid synthase family
WP_005897395[h]	12,431–13,273	WcaA glycosyltransferase	Involved in cell wall biosynthesis, GT-A type fold
WP_005897394[i]	13,270–14,697	Hypothetical protein	No homology found in bacteria
WP_005897393[j]	14,700–15,680	Possible lipooligosaccharide sialyltransferase	35% Identical over 96% coverage to WfaR in *E. coli* H299 38% Identical over 92% coverage to a lipooligosaccharide sialyltransferase in *Histophilus somni*
WP_005897392[k]	15,754–17,046	MOP/MATE family multidrug/oligosaccharidyl-lipid/polysaccharide flippase	75% Identical over 99% coverage to flippase in *F. nucleatum* 13_3 C: annotated as membrane protein involved in the export of *O*-antigen and teichoic acid (cell wall/membrane/envelope biogenesis)
WP_005897391[l]	17,158–17,775	Shikimate 5-deydrogenase	48% Identical over 99% to *E. coli* UTI89 NeuD, which is often incorrectly annotated as a shikimate 5-dehydrogenase
		NeuD, NnaD sugar *O*-aceyltransferase, sialic acid *O*-acetyltransferase NeuD family	39% Identical over 99% coverage to *S. agalactiae* COH1 NeuD
WP_005897390[m]	17,780–18,823	NeuB, NnaB N-acetylneuraminate synthase	NeuB—enzyme activity demonstrated in the current study
WP_005897387[n]	18,826–20,082	N-acylneuraminate cytidylyltransferase, Region 2–221: CMP-NeuAc synthase, Region 249418: SGNH hydrolase	NeuA—activity demonstrated in the current study
WP_005897384[n]	20,087–21,235	NeuC, NnaA UDP-*N*-acetyl-D-glucosamine 2-epimerase, UDP-hydrolysing	57% Identical over 100% coverage to *E. coli* O104 NnaA (GlcNAc-2-epimerase), 58% identical over 98% coverage to *E. coli* LSPQ A134697 NODE_1 polysialic acid biosynthesis protein P7polyhydroxyalkanoate synthesis repressor PhaR homolog
WP_005897381[o]	21,365–22,141	Dolichyl-phosphate mannose synthase	WcaA, glycosyltransferase involved in cell wall biosynthesis. Above 55% identical over 97% coverage with homologs from *Leptotrichia*, *Roseburia*, *Eubacterium*, *Johnsonella* and *Clostridium*
WP_032842809[p]	22,149–22,865	Dolichyl-phosphate mannose synthase	At least 72% identical over 100% coverage to a *Leptotrichia* homolog
		GT2_BcE_like, likely involved in the biosynthesis of the polysaccharide capsule	At least 56% identical over 96% coverage to homologs from *Mycoplasma*, *Johnsonella* and *Clostridium*
WP_032842808[r]	22,875–24,806	Hypothetical protein	22% Identical over 97% to SpaG of *Spirochaeta aurantia* LGLA biosynthetic
WP_005897376[s]	24,781–25,350	Phosphatase β-phosphogluco-mutase family hydrolase	HAD_like Haloacid dehalogenase-like hydrolases
WP_005897374[t]	25,369–28,284	Hypothetical protein	No significant homology to any protein in the database
WP_032842709[u]	29,595–30,395	UTP-glucose-1-phosphate uridylyltransferase catalyses the synthesis of UDP-glucose	

Continued

Table I. Continued

Accession number	Gene position	Current NCBI annotation	Comments
WP_005897365[v]	32,653–34,050	Alanine ABC transporter, DltB D-alanyl-lipoteichoic acid acyltransferase DltB, MBOAT superfamily [cell wall/membrane/envelope biogenesis]	98% Identical over 58% coverage to alginate O-acetyltransferase AlgI in *F. nucleatum animalis* ATCC 51191
WP_005897360[w] (last ORF in this orientation)	34,110–35,312	RfbB dTDP-D-glucose 4,6-dehydratase	At least 69% identical over 99% coverage in *Leptotrichia* and *Bacteroides*

[a]NCBI protein database = NCB accession # WP_005897404.
[b]NCBI protein database = NCB accession # WP_005897403.
[c]NCBI protein database = NCB accession # WP_005897401.
[d]NCBI protein database = NCB accession # WP_005897400.
[e]NCBI protein database = NCB accession # WP_005897399.
[f]NCBI protein database = NCB accession # WP_005897398.
[g]NCBI protein database = NCB accession # WP_005897396.
[h]NCBI protein database = NCB accession # WP_005897395.
[i]NCBI protein database = NCB accession # WP_005897394.
[j]NCBI protein database = NCB accession # WP_005897393.
[k]NCBI protein database = NCB accession # WP_005897392.
[l]NCBI protein database = NCB accession # WP_005897391.
[m]NCBI protein database = NCB accession # WP_005897390.
[n]NCBI protein database = NCB accession # WP_005897387.
[o]NCBI protein database = NCB accession # WP_005897384.
[p]NCBI protein database = NCB accession # WP_005897381.
[q]NCBI protein database = NCB accession # WP_032842809.
[r]NCBI protein database = NCB accession # WP_032842808.
[s]NCBI protein database = NCB accession # WP_005897376.
[t]NCBI protein database = NCB accession # WP_005897374.
[u]NCBI protein database = NCB accession # WP_032842709.
[v]NCBI protein database = NCB accession # WP_005897365.
[w]NCBI protein database = NCB accession # WP_005897360.

sequences from strain ATCC10953. We also used the well-characterized sialic acid biosynthetic enzymes from *E. coli* as BLAST seeds in this analysis. We note that some of these strains are designated only by strain names and not by subspecies in the database. However, we were able to assign their subspecies classification based on whole genome phylogeny (see Figure 5). These analyses revealed that two of four *polymorphum* strains (including ATCC10953) encoded all three required enzymatic steps for *de novo* sialic acid synthesis (encoded by NeuC, NeuB and NeuA). Another two strains (of six queried) within the subspecies *vincentii* encoded full pathways that were nearly identical to those found in the *polymorphum* strains (Figure 6). Several other strains appeared to encode one or more putative homologs of these enzymes, but their amino acid sequences shared very limited identity compared to the *F. nucleatum* ATCC10953 or *E. coli* UTI89 pathways. Our inability to find a complete pathway in some strains could theoretically be related to the degree of sequence coverage. However, there were a number of concrete examples of genomes with complete sequence coverage, but still no evident sialic acid pathway.

F. nucleatum sbsp. *polymorphum* ATCC10953 expresses high levels of Neu5Ac as determined by HPLC measurements and mass spectrometry

We tested our bioinformatic predictions by assessing sialic acid production in *F. nucleatum* strains ATCC25586, ATCC23726 and ATCC10953. *E. coli* UTI89 was included as a positive control, whereas *E. coli* Top10, which lacks a sialic acid biosynthesis pathway, was used to establish baseline levels of Neu5Ac in strains that do not express sialic acids but encode machinery for their uptake and catabolism. Compared to UTI89, the *polymorphum* strain ATCC10953 contained high levels of sialic acid, while *nucleatum* strains ATCC23726 and ATCC25983 contained very low levels (Figure 7A and B). This result strongly suggests that *F. nucleatum* subsp. *nucleatum* strains ATCC23726 and ATCC25586 do not express an operational sialic acid biosynthesis pathway under these conditions. This is consistent with our bioinformatic findings that these two strains do not encode a complete sialic acid biosynthesis pathway orthologous to the ATCC10953 strain.

In our final set of experiments, mass spectrometry was performed to provide conclusive evidence that *F. nucleatum* sbsp. *polymorphum* expresses Neu5Ac (*m/z* 308.1, Figure 7C and D). This is important because many other sialic acid-*like* molecules (e.g. legionaminic and pseudaminic acids) are also expressed by microorganisms and there are specific examples in the literature where they have been mistaken for Neu5Ac (Samuel et al. 2007; Lewis et al. 2009). Briefly, sialic acids were released from cultured bacterial cells by mild acid hydrolysis. We were unable to reproduce results of a previous study that reported sialic acid removal from various *F. nucleatum* strains and subspecies with commercially available sialidase enzymes. In our hands, treatment of *F. nucleatum* ATCC10953 with commercial sialidases from several bacterial species failed to release higher levels of sialic acid into the supernatant (determined biochemically) compared to the level of spontaneous hydrolysis observed in the parallel mock control (data not shown). Along these lines, we note a number of published examples in Gram-negative bacteria where Neu5Ac is linked internally within a polymer such that both the anomeric carbon and a side chain carbon link this

molecule within a chain of carbohydrates. Such internally linked sialic acid residues are not susceptible to typical sialidases, which are exo-glycosidases. Nevertheless, acetic acid hydrolysis was effective for removal of sialic acids and a low molecular weight fraction was subjected to LC-MS analysis without any derivatization. As expected, analysis of the *E. coli* K1 positive control yielded a peak of material at the expected mass of Neu5Ac (308.1) eluting at a retention time of 6.9–8.1 min (Figure 7C). As a negative control, material was isolated in parallel from *E. coli* TOP10, which does not synthesize sialic acid. This control gave the expected negative result (not shown). Analysis of *F. nucleatum* ATCC10953 confirmed that this strain produces Neu5Ac (*m/z* 308.1) (Figure 7D). However, experiments performed in parallel on ATCC25586 and ATCC23726 did not yield evidence of Neu5Ac production (not shown). These findings are consistent with the DMB-HPLC results and the bioinformatic analysis presented here.

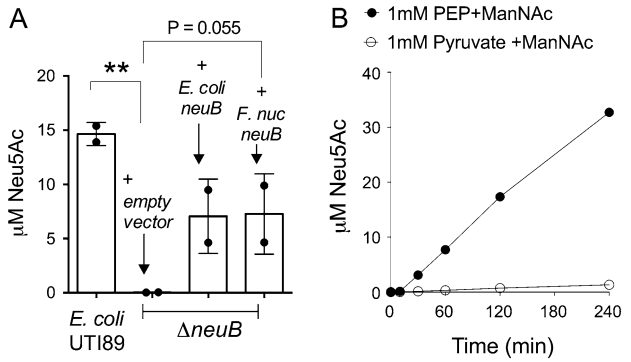

Fig. 2. The *F. nucleatum* subsp. *polymorphum* NeuB homolog encodes a functional Neu5Ac synthase. (**A**) The *F. nucleatum neuB* homolog was cloned and used in complementation studies to examine whether it could restore sialic acid biosynthesis to a sialic acid-expressing strain of *E. coli* in which *neuB* was deleted (Δ*neuB*). As expected, the *neuB* deletion rendered *E. coli* unable to produce sialic acid and plasmid-based expression of *E. coli neuB* was able to partially restore sialic acid synthesis. Expression of *F. nucleatum neuB* was also able to restore sialic acid production to the mutant at levels indistinguishable from complementation with the native *E. coli neuB*. The values shown are from two independent experiments (with this specific configuration of strains and plasmids). Similar results were obtained in several other experiments with different configurations of strains and plasmids. We note that the variability between the two experiments shown here is likely due to somewhat different time points of harvest in stationary phase. (**B**) *F. nucleatum* NeuB was expressed as a his-tagged protein and purified by nickel chromatography. Enzymatic activity was tested by incubating enzyme with its putative substrate, ManNAc, in the presence of PEP or pyruvate. Production of Neu5Ac was monitored over time by DMB-HPLC. Similar results were obtained using sonicated bacterial lysates, without purifying the enzyme.

Discussion

Here we provide the first biochemical and genetic evidence that some strains of *F. nucleatum* are capable of synthesizing sialic acids *de novo*. We provide definitive biochemical proof linking specific gene products with the synthesis of Neu5Ac from ManNAc as well as nucleotide activation of Neu5Ac. Mass spectrometry provided definitive proof that *F. nucleatum* synthesizes Neu5Ac as opposed to chemically similar di-N-acetylated molecules that are common among microorganisms and have been previously mistaken for Neu5Ac (Samuel et al. 2007; Lewis et al. 2009). The experiments also demonstrate that *F. nucleatum* sbsp. *polymorphum* strain ATCC10953 generates higher levels of this molecule than a pathogenic strain of *E. coli* (the encapsulated K1 strain UTI89) under the conditions tested. Our study contrasts with another recent paper that examined sialic acid metabolism among *F. nucleatum* subspecies and strains (Yoneda et al. 2014). The earlier study concluded that *de novo* sialic acid biosynthesis is widely conserved among the subspecies of *F. nucleatum*. By systematically querying *F. nucleatum* genomes using amino acid sequences from the ATCC10953 strain, we clarify that only 4 of the 31 sequenced *F. nucleatum* strains encode the complete pathway of *de novo* sialic acid synthesis. These findings are consistent with our rigorously controlled biochemical analyses of *F. nucleatum* sialic acid biosynthesis showing that strains ATCC23726 and ATCC25586, which do not encode a complete pathway, also do not contain sialic acids at levels above baseline. In summary, our experiments show that about 15% of *F. nucleatum* strains encode complete pathways for sialic acid synthesis and formally demonstrate the genes and enzymes involved. These findings are consistent with what we know about sialic acid biosynthesis in other taxa. For example, only certain strains of *E. coli* produce K or

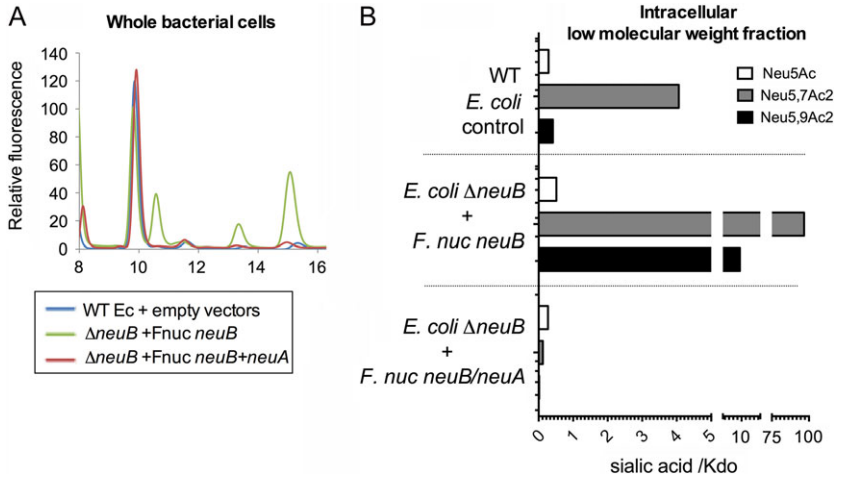

Fig. 3. HPLC analysis of sialic acids reveals higher levels of sialic acid O-acetylation upon complementation of *neuB* compared to WT *E. coli*. (**A**) DMB-HPLC analysis of whole bacterial cells, as described in the methods. (**B**) Intracellular sialic acids were isolated from bacteria and expressed relative to levels of the eight-carbon α-keto acid Kdo as described in the methods. This figure is available in black and white in print and in color at *Glycobiology* online.

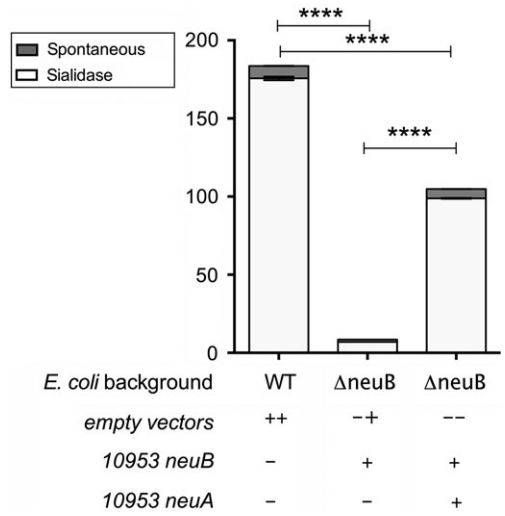

Fig. 4. *F. nucleatum* NeuA and NeuB together restored surface sialic acid expression to the *E. coli* Δ*neuB* strain. Levels of surface sialic acids were measured following their removal using *Arthrobacter urafacians* sialidase as described in the "Materials and methods" section. As a negative control, bacterial cells were incubated in parallel with buffer alone to illustrate the extent of spontaneous hydrolysis under otherwise identical conditions. Mean values with standard deviation of three technical replicates are provided for the spontaneous and enzyme catalyzed reactions. Similar results were obtained in multiple independent experiments. Differences between enzyme catalyzed release of sialic acids between the strains were evaluated using one way ANOVA with Tukey's multiple corrections test. ****$P < 0.0001$.

O-antigens that contain sialic acid. The same is true among several other taxa that are known to produce sialic acids or related molecules (Lewis et al. 2011; Lubin et al. 2015).

There are several interesting parallels between the *F. nucleatum* Neu5Ac biosynthesis pathway and the pathways encoded by the distantly related organisms GBS and *E. coli* K1. First, the NeuB and NeuC proteins in *F. nucleatum* have a closer phylogenetic relationship with orthologous enzymes encoded by *E. coli* K1 and GBS than to Neu5Ac pathways in several other human pathogens including *Neisseria meningitidis*, *Campylobacter jejuni* and *Haemophilus influenzae* (Lewis et al. 2009). In fact, we showed previously that the enzymatic route encoded by GBS, *E. coli* and *F. nucleatum* has a closer phylogenetic relationship with pathways that synthesize the related di-N-acetylated molecule legionaminic acid than to other convergently evolved bacterial pathways for Neu5Ac synthesis (Lewis et al. 2009). These findings suggest that this particular route of Neu5Ac synthesis may have evolved from a pathway that synthesized a related di-N-acetylated sialic acid-like molecule. Additional evidence supporting this interpretation comes from the observation that *F. nucleatum* encodes two additional gene products in common with GBS and *E. coli* that are unique from other sialic acid biosynthetic pathways. Specifically, *neuD* encodes a conserved O-acetyltransferase shown in GBS and *E. coli* K1 to participate in the biosynthesis of O-acetylated sialic acids prior to capsular assembly. In fact O-acetylation of Neu5Ac is found at the same carbon position where N-acetylation occurs in the related legionaminic acid pathway and uses a homologous enzyme to NeuD (Lewis et al. 2009). As described in the "Results" section, the NeuA CMP-sialic acid synthetase of *F. nucleatum* ATCC10953 contains a C-terminal O-acetylesterase domain that participates in the de-O-acetylation of intracellular sialic acids. This NeuA C-terminal esterase is also present in GBS and *E. coli* K1, but not in all pathways of bacterial *de novo* sialic acid synthesis.

As described above, machinery for the addition and removal of sialic acid O-acetylation appears to be present in *F. nucleatum* ATCC10953. The *F. nucleatum* sialic acid biosynthetic gene cluster encodes a NeuD homolog that shares 48% identity and 60% similarity in amino acid sequence with *E. coli* NeuD. However, HPLC analysis of *F. nucleatum* sialic acids *did not* reveal evidence that *F. nucleatum* synthesizes O-acetylated sialic acids. *F. nucleatum* also encodes a C-terminal NeuA O-acetylesterase domain, also found in GBS and *E. coli*, suggesting that like these organisms, *F. nucleatum* de-O-acetylates the transient pool of sialic acid molecules prior to assembly of sialylated surface structures. We note the relatively high level of intracellular 7-*O*-acetylated Neu5Ac in WT *E. coli* relative to unmodified Neu5Ac (Figure 3B). This is in contrast to intracellular sialic acids isolated from wild type (WT) GBS, which do not exhibit O-acetylation (Lewis et al. 2004). Notably, the level of surface O-acetylation in *E. coli* is determined not by the activity of NeuD, but rather by another enzyme (NeuO) that acts on the fully assembled polysaccharide (Steenbergen et al. 2006). Whereas *E. coli* uses the NeuA C-terminal esterase to de-O-acetylate monomeric sialic acids intracellularly prior to capsule assembly (Steenbergen et al. 2006), GBS de-O-acetylates only a portion, leaving some of the O-acetylation intact to be assembled into the capsular polysaccharide. For a summary of the different bacterial strategies for capsule sialic acid O-acetylation, see Figure 8 in Lewis et al. (2007). Together, these findings strongly support the interpretation that polymerization of the polysialic acid capsule in *E. coli* may be hampered by *O*-acetyl esters on the sialic acid side chain and therefore the *O*-acetyl groups need to be hydrolyzed by the NeuA O-acetylesterase prior to polymerization of sialic acids into the α2-8-linked capsule. Taken together, the findings that *F. nucleatum* ATCC10953 encodes both NeuD transferase and NeuA esterase, but does not produce measurable levels of *O*-acetylated sialic acids, suggests that the *de novo* pathway in *Fusobacterium* may behave more similarly to *E. coli* than to GBS.

In recent years, several studies of periodontal disease in pregnancy have found that women with this condition have increased risks of intrauterine infections and preterm birth (Offenbacher et al. 1996; Hill 1998; Jeffcoat, Geurs, Reddy, Cliver et al. 2001; Jeffcoat, Geurs, Reddy, Goldenberg et al. 2001; Khader and Ta'ani 2005; Nabet et al. 2010; Riche et al. 2002; Pizzo et al. 2005). Interestingly, both live bacteria and bacterial products such as lipopolysaccharide can be found in the bloodstream of persons with periodontal disease following eating, routine oral hygiene and other periodontal procedures (Geerts et al. 2002; Maestre et al. 2008; Olsen 2008). Attempts to prevent preterm birth by providing pregnant women with oral intervention (scaling and root planning) have met with mixed results (Lopez et al. 2002; Jeffcoat et al. 2003; Tarannum and Faizuddin 2007; Macones et al. 2010; Weidlich et al. 2013). However, successful reductions in preterm birth among women with periodontal disease may depend on whether therapy was effective in improving periodontal health (Jeffcoat et al. 2011). *F. nucleatum* has gained attention recently as a cause of intrauterine infections during pregnancy that may originate from the mouth. However, mechanisms whereby oral bacteria evade immune processes in the bloodstream to reach the intrauterine environment have remained elusive. Sialic acids are known to play important roles in bacterial survival during bacterial blood-borne pathogenesis. Moreover, it may not be coincidental that GBS, *E. coli* K1 and *F. nucleatum* (although distantly related), share a tropism for infection during pregnancy and infancy. We suggest that some strains of *F. nucleatum* may mimic sialic acids to ensure survival upon exposure to blood, which is likely a common event for bacteria that live in gingival crevices. Unfortunately, not all

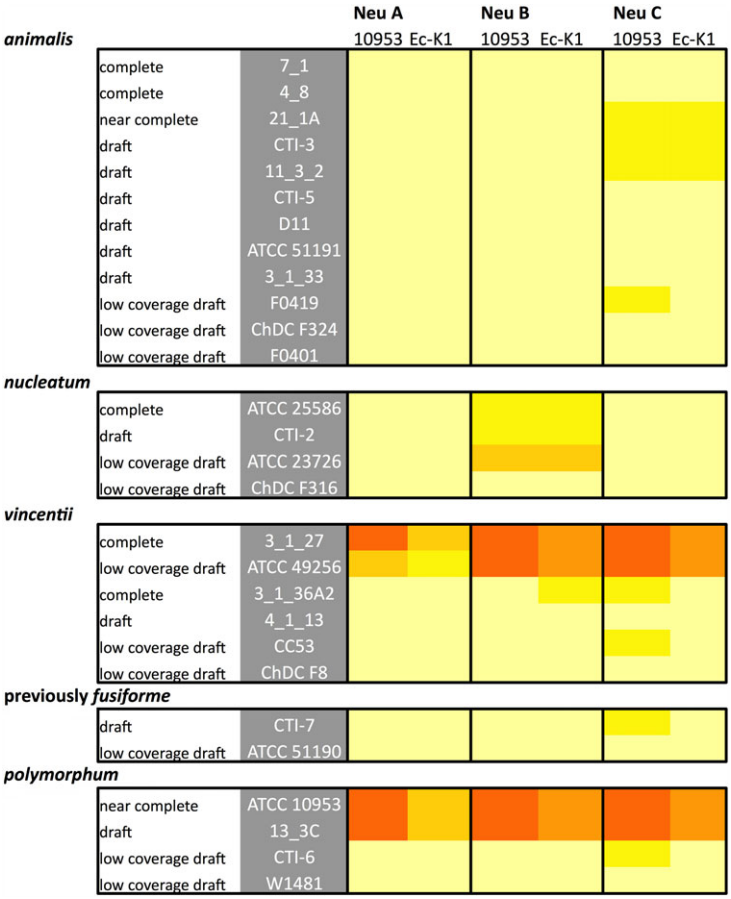

Fig. 5. De novo sialic acid biosynthesis is strain specific in *F. nucleatum,* not widely conserved. Heatmap showing percent identity of NeuA, NeuB and NeuC homologs in sequenced *F. nucleatum* strains compared to the prototype strain ATCC10953 and to the well-characterized *E. coli* pathway (Ec-K1). Darker shades refer to higher percent identity. This figure is available in black and white in print and in colour at *Glycobiology* online.

strains of *F. nucleatum* are amenable to genetic approaches (including ATCC10953), a limitation that prevents further characterization of the potential pathogenic role of sialic acids, at least until additional tools become available.

Materials and methods

Bacterial strains and culture conditions

WT strains of *E. coli* [TOP10, Invitrogen and MG1655 and UTI89 (provided by Scott Hultgren)] were grown in Lysogeny broth (LB) at 37°C unless otherwise noted. For plasmid maintenance, ampicillin was used at 100 μg/mL and chloramphenicol at 20 μg/mL. *F. nucleatum* strains ATCC25586, ATCC23726 and ATCC10953 were obtained from ATCC. All *Fusobacterium* strains were grown in Columbia broth at 37°C in an anaerobic chamber. See Table III for a complete list of strains.

Deletion of neuB in *E. coli* UTI89

Restriction enzymes and Phusion DNA polymerase were obtained from New England Biolabs. The *E. coli* UTI89 Δ*neuB* strain (LSR8) was generated using the Red recombinase system (Datsenko and Wanner 2000). Briefly, the kanamycin resistance cassette from pKD13 was amplified with the primers UTI89 neuB KO F and UTI89 neuB KO R (Table II), which contain 3′ homology arms targeting the kan cassette combined with 5′ homology arms targeting the flanking regions of *E. coli neuB*. The purified product of this reaction was transformed into competent UTI89 harboring the temperature-sensitive helper plasmid pKM208. Specifically, cells were grown at 30°C to an OD^{600} of 0.3 and the Red recombinase was induced for 30 min with 1 mM IPTG (Gold Bio, Olivette, MO). The culture was heat shocked in a 42°C water bath for 15 min to promote transformant survival, and incubated on ice for 10 min. Cells were then pelleted by centrifugation at 12,000 × *g*, washed three times in 1 mL ice cold 20% glycerol with 1 mM MOPS, and then resuspended in 75 μL of the same solution. One microgram of the purified PCR product was mixed with this cell suspension and the bacteria were electroporated at 20 kV/cm in an Eppendorf 2510 electroporator. Cells were recovered for 1.5 h at 37°C in SOC medium and transformants were selected on LB agar containing kanamycin at 20 μg/mL. Following confirmation of the *neuB* deletion by PCR, the kanamycin resistance cassette was excised using the helper plasmid pCP20. The *E. coli* MG1655 Δ*nanA* strain (LSR4) was generated using the same protocol as above with the primers nanA KO F and nanA KO R.

Plasmid construction

The *neuB* genes from *E. coli* UTI89 and *F. nucleatum* ATCC10953 (see Table I) were amplified using the primer sets UTI89 neuB F Nco and UTI89 neuB his R Bam, and 10953 neuB F Nco and 10953 neuB his R Bam, respectively (see primer sequences in Table II and

gene accession numbers in Table I). PCR products were desalted, digested with NcoI and BamHI, and cloned into the NcoI and BamHI sites of pTrc99A, yielding pLR11 (*F. nucleatum neuB-his* in pTrc99A), and pLR14 (*E. coli neuB-his* in pTrc99A). The *F. nucleatum* ATCC10953 *neuA* gene was similarly amplified with the primers 10953 neuA F Nco and 10953 neuA R Bam and digested with NcoI and BamHI. The PCR product was then cloned into the NcoI and BamHI sites of the pBAD33-derivative pLR92 (Robinson et al. 2006), resulting in pLR15.

Complementation of the *E. coli* Δ*neuB* mutant

pTrc99A (vector control), pLR11 (*F. nucleatum neuB*) and pLR14 (*E. coli neuB*) were transformed into the UTI89 Δ*neuB* mutant LSR8. The strains were grown to stationary phase at 37°C in Circle Grow media supplemented with ampicillin. Bacteria from 2 mL of each culture were pelleted, washed twice in PBS and resuspended in 1 mL PBS. Suspensions were acid hydrolyzed prior to sialic acid analysis by DMB-HPLC.

To counteract the polar effect of the *neuB* deletion on downstream *neuA* expression in LSR8, pLR15 (harboring *F. nucleatum neuA* under the control of the *araBAD* promoter) was transformed into LSR8 containing pLR11 (*F. nucleatum neuB-his* in pTrc99A). Bacteria were grown as above, with the addition of chloramphenicol at 20 μg/mL and arabinose at 100 μg/mL.

Release and HPLC analysis of sialic acids

Sialic acids were released by resuspending washed bacteria in 2 N acetic acid and incubating at 80°C for 3 h. Insoluble debris were pelleted, and the supernatant was derivatized with DMB (Sigma, St. Louis, MO) as we have previously described (Lewis et al. 2006, 2007, 2009). Standards such as Neu5Ac and sialic acids isolated from bovine submaxillary mucin were derivatized in parallel to monitor retention times of known sialic acids. Kdo (3-deoxy-D-manno-octulosonic acid) is a monosaccharide synthesized by most Gram-negative bacteria and incorporated into the core region of lipopolysaccharide. As an eight-carbon backbone α-keto acid, Kdo is derivatized alongside any nonulosonic α-keto acids. Thus, in some experiments, Kdo served as a convenient internal standard for normalization of data from the *E. coli* system. We also considered using the molecule Kdo as an internal control for potential normalization of the data comparing sialic acid levels between strains and species of *Fusobacterium*. However, we were surprised to find that many strains of *Fusobacteria* had very low or undetectable levels of Kdo. Thus, we measured protein content of the original cell pellet using the BCA assay (Pierce, Rockford, IL) to normalize and compare between samples. Derivatized samples were injected into a Waters HPLC equipped with a reverse-phase C18 column (Tosoh Bioscience, Tokyo, Japan) and Waters fluorescence detector set to excite at 373 nm and detect emission at 448 nm. Peak integrations were used to quantitate sialic acid

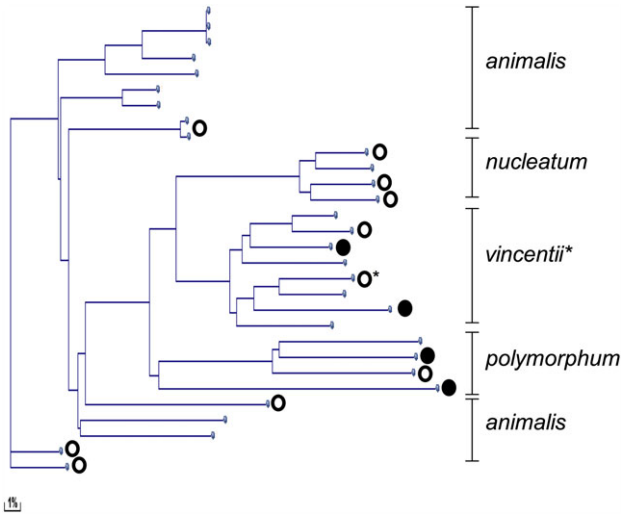

Fig. 6. Whole genome *F. nucleatum* phylogenetic tree overlaid with information about the presence and completeness of sialic acid biosynthesis pathways. Dendrogram of *F. nucleatum* genomes based on genomic blast, presenting distribution of *F. nucleatum* strains with complete (closed circles) and incomplete (open circles) sialic acid biosynthetic pathway. The minimum threshold defining whether strains of *F. nucleatum* had a "complete" pathway was defined as the presence of homologs of NeuA, NeuB and NeuC (see Figure 5). *The subspecies *vincentii* now includes what was previously known as *fusiforme*. This figure is available in black and white in print and in colour at *Glycobiology* online.

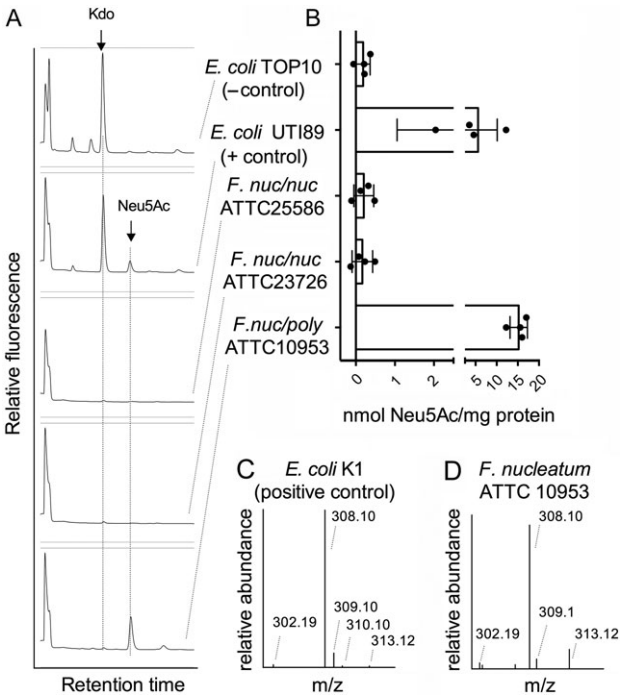

Fig. 7. *F. nucleatum* sbsp. *polymorphum* ATCC10953 expresses high levels of Neu5Ac. (**A**) Bacterial pellets were subjected to mild acetic acid hydrolysis to lyse bacteria and release any sialic acids. The low molecular weight supernatant was then subjected to derivatization with DMB, which fluorescently labels molecules containing an α-keto acid, including Neu5Ac, the most common sialic acid found in nature and Kdo, an eight-carbon backbone carbohydrate found in the core structure of lipopolysaccharides. *E. coli* strains known to synthesize sialic acids (K1 strain UTI89) or laboratory strain that does not synthesize sialic acids (TOP10) were used as positive and negative controls, respectively. (**B**) The BCA method was used to estimate protein content in the bacterial lysates, values that were used to normalize and compare between strains. Bars indicate the mean of four independent measurements from each strain performed on different days. Error bars are standard deviation. (**C**, **D**) Electrospray ionization mass spectrometry in negative ion mode was used in conjunction with liquid chomatography to isolate peaks at retention times matching parallel standards for Neu5Ac and determine molecular masses. Abundances of major ions between *m/z* 300 and 316 are shown. The expected mass is 308.1 for Neu5Ac.

Table II. Primers used in this study

Primer name	Sequence
UTI89 neuB KO F	GCTGGGACTCCAACAAGATTAATTAGGGGGAATGAATGAGGTGTAGGCTGGAGCTGCTTC
UTI89 neuB KO R	GCAATAATTTTTGTTCTCATTATTCCCCCTGATTTTTGAAATTCCGGGGATCCGTCGACC
UTI89 neuB F Nco	AAAACCATGGCAAGTAATATATATATCGTTGCTGAAATTGG
UTI89 neuB his R Bam	TTTTGGATCCTTAGTGGTGGTGGTGGTGGTGTTCCCCCTGATTTTTGAATTC
nanA KO F	ATAAAGGTATATCGTTTATCAGACAAGCATCACTTCAGAGGTGTAGGCTGGAGCTGCTTC
nanA KO R	TTCCCCTCACCCGGTAGGGGCGAGCGAGGGGAAACAACTCATTCCGGGGATCCGTCGACC
10953 neuB F Nco	AAAACCATGGCACAAAAAAAAGTTTTTATAGTTGCAGAAATAGG
10953 neuB his R Bam	AAAAGGATCCTTAGTGGTGGTGGTGGTGGTGTTCTCCTTGCATTTCAAAATTAGAATC
10953 neuA F Nco	AAAACCATGGCAAAAAAAAATTGCTATAATTCCAGCTAGATC
10953 neuA R Bam	TTTTGGATCCATATTCGGCTCTTGAACC

content by referencing a standard curve of pure sialic acid (Neu5Ac) purchased from Sigma derivatized in parallel. Relative retention times of sialic acids and O-acetylated derivatives have been reported previously for Neu5Ac, Neu5,7Ac2 and Neu5,9Ac2 in this system (Manzi et al. 1990).

Expression and isolation of *F. nucleatum NeuB*

The pLR11 plasmid encoding his-tagged *F. nucleatum* NeuB-his was transformed into the *E. coli* Δ*nanA* strain LSR4, which lacks the ability to metabolize sialic acids. LSR4 pLR11 was grown at 37°C shaking to an OD 600 of 1.0 in 350 mL Circle Grow broth, and *neuB-his* expression was induced with 200 μM IPTG. Cells were grown overnight at room temperature. The next day, bacteria were pelleted, washed once in 30 mL PBS and resuspended in 10 mL lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0). The bacteria were then sonicated on ice with 100 one-second pulses at 35% amplitude with a model 505 Sonic Dismembrator (Fisher Scientific, Waltham, MA). Unbroken bacteria and cellular debris were pelleted at 20,000 × *g* for 10 min, and the clarified lysate was mixed with 600 μL His-Select Nickel Affinity Gel (Sigma). The lysate was rotated at 4°C for 90 min before being applied to a 5 mL disposable polypropylene column (Thermo Scientific, Waltham, MA). The beads were washed with three column volumes of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0), and protein eluted with ten 150 μL fractions of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). Expression and purification were confirmed by running each fraction on an Any kD precast polyacrylamide gel (Bio-Rad, Hercules, CA) and staining with Biosafe Coomassie Blue (Bio-Rad). Chemicals were obtained from Sigma.

Biochemical assays of sialic acid synthesis by *F. nucleatum NeuB*

For sialic acid synthesis experiments, 20 μL NeuB-his solution was mixed with 20 μL 10 mM ManNAc (Sigma) and 20 μL 10 mM pyruvate (Sigma) or 10 mM PEP (Sigma) in 140 μL PBS. Reaction mixes were incubated at 37°C and at each time point and samples were removed for analysis by DMB-HPLC.

Separation of intracellular from surface-associated sialic acids

Strains used for these experiments were: UTI89 (WT) pTrc99A pBAD33 (WT strain containing empty vector controls), LSR8 pLR11 pBAD33 (UTI89 Δ*neuB* containing *F. nucleatum neuB-his* in pTrc99A and empty vector control) and LSR8 pLR11 pLR15 (UTI89 Δ*neuB* containing *F. nucleatum neuB-his* in pTrc99A and *F. nucleatum neuA-his* in pBAD33-derivative pLR92). Strains were grown overnight shaking at 37°C in 1 mL LB supplemented with 100 μg/mL ampicillin and 20 μg/mL chloramphenicol. The next morning, each culture was diluted to 10^{-3} in 30 mL fresh LB containing ampicillin, chloramphenicol and 100 μg/mL arabinose. Following 4 h of growth in a shaking incubator at 37°C, bacteria were pelleted, washed three times with 1 mL of 100 mM sodium acetate pH 5.5, and resuspended in 400 μL 100 mM sodium acetate pH 5.5. Then 5 μL of each whole cell suspension was taken for acid hydrolysis and HPLC. The remaining suspensions were sonicated at 28% amplitude for 40 one-second pulses and centrifuged at 20,000 × *g* for 10 min. Three-hundred microliter of each supernatant was applied to 10 kDa cut-off columns and spun at 11,000 × *g* for 10 min. Flow through was taken for HPLC.

Release of bound extracellular sialic acid

The *E. coli* strains from the above experiment were grown under identical conditions. Bacteria from 9 mL of each culture were pelleted, washed twice in 100 mM sodium acetate pH 5.5 and resuspended in 1 mL of the same buffer. Three-hundred microliter of each suspension was then treated with 50 mU of *Arthrobacter ureafaciens* sialidase (EY Labs, San Mateo, CA) or mock treated in buffer alone (100 mM sodium acetate pH 5.5) overnight at 37°C. The next morning, bacterial cells were removed by centrifugation at 20,000 × *g* and supernatants taken for DMB-HPLC for measurement of released Neu5Ac.

Mild base treatment to eliminate *O*-acetyl esters

O-Acetyl groups were chemically removed from sialic acids by treating samples with 100 mM NaOH for 30 min at 37°C. Following deacetylation, the pH was neutralized with glacial acetic acid.

Liquid chromatography mass spectrometry

Mass spectrometry was conducted at the Glycotechnology Core Resource at the University of California San Diego. Briefly, washed bacterial pellets were hydrolyzed using 2 M acetic acid at 80°C for 3 h, followed by speed vac removal of acid. To ensure complete removal of acid, the sample was dissolved in 50% isopropanol and re-evaporated. Dried sample was dissolved in water and passed over a 5 K molecular weight cut-off filter, the flow through was collected and lyophilized. This was dissolved in water for LC-MS. To be clear, samples were not subjected to DMB derivatization.

Table III. Strains and plasmids used in this study

Strain/plasmid name	Brief description	Reference/ source
WT strains		
ATCC10953	*F. nucleatum* sbsp. *polymorphum*	American Type Culture Collection
ATCC25586	*F. nucleatum* sbsp. *nucleatum*	American Type Culture Collection
ATCC23726	*F. nucleatum* sbsp. *nucleatum*	American Type Culture Collection
TOP10	*E. coli* laboratory strain (does not synthesize sialic acid)	Invitrogen
UTI89	Uropathogenic *E. coli* strain, K1 sialic acid capsule	Anderson et al. (2010)
Plasmids		
pKD13	Source of kanamycin resistance cassette for use in Red Recombinase system	Datsenko and Wanner (2000)
pKM208	Temperature-sensitive helper plasmid for Red Recombinase system	Datsenko and Wanner (2000)
pCP20	Helper plasmid for excision of antibiotic resistance cassettes	Datsenko and Wanner (2000)
pTrc99A	Expression vector	Amann and Brosius (1985)
pBAD33	Expression vector	Guzman et al. (1995)
pLR92	pBAD33 with RBS and start codon from pTrc99A	Robinson et al. (2006)
pLR11	*F. nucleatum neuB-his* in pTrc99A	This study
pLR14	*E. coli neuB-his* in pTrc99A	This study
pLR15	*F. nucleatum neuA-his* in pLR92	This study
Other strains used in the study		
UTI89 pTrc99A pBAD33	UTI89 empty vector control	This study
LSR8	UTI89 Δ*neuB*	This study
LSR8 pLR11 pBAD33	UTI89 Δ*neuB* expressing *neuB-his* (pLR11) + empty vector (pBAD33)	This study
LSR8 pLR11 pLR15	UTI89 Δ*neuB* expressing *neuB-his* (pLR11) and *neuA-his* (pLR15)	This study
LSR4	MG1655 Δ*nanA*	This study
LSR4 pLR11	MG1655 Δ*nanA* expressing *neuB-his* (pLR11)	This study

Instrument specifications were as follows: The mass spectral analysis was done on LTQ-Orbitrap Discovery (Thermo Scientific) attached with online Ultimate 3000 liquid chromatography unit (Dionex, Sunnyvale, CA). Separation of sialic acid was done using Phalanx C18 column (Higgins Analytical, Mountain View, CA) using ion-pairing reagents. The column specification is as follows, 150 mm × 1.0 mm and 5 µm particle size. A gradient run of two solvent systems with ion-pairing reagents are Solvent A: 5 mM dibutyl amine + 8 mM acetic acid and Solvent B: 5 mM dibutyl amine + 8 mM acetic acid in 70% methanol. The entire liquid chromatography run was performed at 50 µL/min flow rate with buffer gradient of 0–100% solvent B at 20 min span. The mass spectrum was acquired using FT-Orbitrap in negative mode with resolution set at 30 K. The ion-spray voltage set at 3.5 and capillary temp was 150°C.

Bioinformatic analysis

To evaluate the presence of putative enzymes involved in the biosynthesis of sialic acids in *Fusobacterium*, we conducted a bioinformatics analysis using protein blast (blastp ver. 2.2.31+) program from the BLAST suite (Altschul et al. 1997). Amino acid sequences of NeuC (*N*-acetylglucosamine epimerase), NeuB (sialic acid synthase) and NeuA (CMP-sialic acid synthetase/O-acetylesterase) from *F. nucleatum* subsp. *polymorphum* ATCC10953 and *E. coli* UTI89 were used as query to nonredundant protein sequences database for *F. nucleatum* (taxid:851). Any hits (as of 15 March 2015) with an *E*-value less than or equal to zero were considered significant.

Whole genome phylogeny

To examine the distribution of sialic acid biosynthesis enzymes among *F. nuclteatum* strains, we overlaid a dendrogam based on whole genomes of sequenced *F. nucleatum* strains (available from NCBI genome database, http://www.ncbi.nlm.nih.gov/genome/180) with information from the bioinformatic analysis described above. The phylogenetic tree shows evolutionary relationships between various *F. nucleatum* strains using *F. nucleatum* subsp. *nucleatum* ATCC25586 as a reference genome. For this purpose, our definition of whether strains of *F. nucleatum* had a "complete" pathway was defined as the presence of homologs of all three enzymes (NeuA, NeuB and NeuC) in any given subspecies.

Author contributions:

A.L.L. conceived and coordinated the study, participated in experimental design, analysis and interpretation of data, made the figures, and wrote the paper. L.S.R. participated in experimental design, performed experiments and data analysis, constructed all the tables, and participated in writing the paper. K.A. performed the bioinformatic analyses of *F. nucleatum* genomes, participated in interpretation of the bioinformatic data, and participated in writing the paper. W.G.L. coordinated the study, and participated in experimental design, analysis and interpretation of data, and writing the paper.

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database were used for bioinformatic analyses presented in this paper (http://www.ncbi.nlm.nih.gov/genome/180).

Conflict of interest statement

The authors declare that they have no conflicts of interest with the contents of this article.

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