YjhS (NanS) Is Required for *Escherichia coli* To Grow on 9-O-Acetylated *N*-Acetylneuraminic Acid

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The nanATEK-yhcH, yjhATS, and yjhBC operons in *Escherichia coli* are coregulated by environmental *N*-acetylneuraminic acid, the most prevalent sialic acid in nature. Here we show that YjhS (NanS) is a probable 9-O-acetyl *N*-acetylneuraminic acid esterase required for *E. coli* to grow on this alternative sialic acid, which is commonly found in mammalian host mucosal sites.

The coregulated nanATEK-yhcH, yjhATS, and yjhBC operons involved in sialic acid catabolism in *Escherichia coli* are thought to be induced by the most common sialic acid, *N*-acetylneuraminic acid (Neu5Ac), through reversible inactivation of the NanR repressor encoded by nanR mapping immediately upstream of nanA (15, 27, 28; http://vetmed.illinois.edu/path/sialobiology/). Sialic acids are a family of over 40 naturally occurring 9-carbon keto sugar acids found mainly in metazoans of the deuterostome (starfish to human) developmental lineage and in some, mostly pathogenic, bacteria, where sialic acids expressed at the microbial cell surface inhibit host innate immunity (27). By contrast, most bacterial commensals and pathogens catabolize sialic acids as sole carbon and nitrogen sources, indicating exploitation of the sialic acid-rich host mucosal environment by a wide range of species (2, 27, 28). Interestingly, in vivo experimental evidence further indicates that sialic acid catabolism functions directly (nutrition) or indirectly (surface decoration and cell signaling) in host-microbe commensal and pathogenic interactions in organisms such as *E. coli*, *Haemophilus influenzae*, *Pasteurella multocida*, *Salmonella enterica* serovar Typhi, *Streptococcus pneumoniae*, *Vibrio vulnificus*, and *Vibrio cholerae* (1, 3, 5, 6, 10, 14, 23, 24, 26, 29). The animal species used for these studies include rodent models and natural hosts such as cattle and turkeys. The structural diversity of sialic acids at the terminal positions on glycoconjugates (glycoproteins and glycolipids) of mucosal surfaces of these hosts requires sialidases, acetyl esterases, and probably other enzymes that convert alternative or at least minor sialic acids to the more digestible Neu5Ac form (8, 9). We have previously demonstrated that *E. coli* has an epicurean propensity for metabolizing alternative sialic acids (30, 31). In the current communication, we show that YjhS is required for growth of *E. coli* on 9-O-acetyl-*N*-acetylneuraminic acid (Neu5,9Ac2).

Because most sialic acids are bound to other sugars, including other sialic acids, as part of the oligosaccharide chains on glycoconjugates, either microbial or endogenous (host) sialidases (NanH, or *N*-acetylneuraminidate hydrolases) are needed to release free sugar, which is then transported by NanT in *E. coli* (15, 16, 26, 31). Once internalized, sialic acid is cleaved by an *nanA*-encoded aldolase or lyase to yield the 6-carbon hexosamine, *N*-acetylmannosamine (ManNAc), and pyruvate, with the latter entering the tricarboxylic acid cycle or gluconeogenesis. ManNAc is converted to its 6-phosphate derivative by a specific kinase encoded by *nanK* and epimerized by NanE to yield *N*-acetylgulosamine 6-phosphate, which is converted to fructose 6-phosphate by products of the *nag* operon (15, 17, 18, 32). The functions of the coregulated *yjhS*, *yjhB*, *yjhC*, and *yhcH* gene products are unknown but are not required for growth on Neu5Ac (15). However, YjhA (NanC) is an outer membrane porin required for diffusion of Neu5Ac in the absence of the major porins (7), while YjhT (NanM) is a mutarotase that catalyzes the conversion of the alpha sialic acid isomer to the more thermodynamically stable beta form (21). Neither *nanC* nor *nanM* is required for growth on Neu5Ac (16), suggesting that *yjhS*, *yjhBC*, and *yhcH* are involved in reactions that convert alternative sialic acids to Neu5Ac (22, 23). YhcH was crystallized and has been suggested to be an isomerase or epimerase involved in processing *N*-glycolylneuraminic acid (Neu5Gc) (25), but deletion of *yhcH* did not affect growth on this sialic acid as a sole carbon source (16).

Computer-assisted analysis indicated that YjhB is a permease similar to NanT (16) whereas YjhC is a likely oxidoreductase or dehydrogenase. Orthologs of *yhcH, nanC, nanM*, and *yjhBC* are found in most bacterial species with intact Neu5Ac utilization systems, while *yjhS* is confined to *E. coli* and shigellae, either as part of the chromosomes in these strains or integrated with phages or phage remnants. However, a significant match (E value = 0.0007) was found between YjhS and AceA in *Rhodopirellula baltica*, where AceA is an acetyl xylan esterase (11), suggesting YjhS might be a sialate esterase. We propose that YjhS should be designated NanS to indicate its direct participation in utilization of an alternative sialic acid.

**Phenotypic characterization of nanS mutants.** Neu5,9Ac2 (lot no. 160309-75) and Neu4,5Ac2 (lot no. 58-5#9) were purchased from Applied Biotechnology, Austria. Fluorometric analysis of Neu5,9Ac2 by detection of 1,2-diamino-4,5-methylenedioxobenzene (DMB)-labeled sialyl derivatives after separation by reverse-phase chromatography (22) indicated contamination with 7% Neu5,8Ac2 and 3% Neu5Ac, while Neu4,5Ac2...
contained 34% unknown contaminants compared to the 10% reported by the manufacturer. The O-acetylated sialic acids were used as supplied for growth experiments by dissolving them to achieve a 0.1% final concentration in minimal salts medium followed by filter sterilization before immediate inoculation with the indicated bacterial strains. All chromatographic peak assignments were made on the basis of the specifications provided by the manufacturer and previous analyses of modified sialic acids as previously described (22). To determine whether nanS is required for E. coli K-12 growth on O-acetylated sialic acids, we constructed a deletion of nanS in strain BW30270 (E. coli Genetic Stock Collection) and tested its ability to utilize Neu5,9Ac2 as a sole carbon source. It and the other nanS mutants described below were constructed with forward (5'-CATATGCTGACAATCTTGGG
GCCTCATATTGATATGGGCGTCATAG-3') and reverse (5'-ATATGGCGTAATATCGGGCGTCATATGGTATTGTAGAAGACTGAGGCTGGACGTGCTCG-3') primers designed to amplify the kanamycin resistance cassette in pKD13 (pKD13 homologous regions are underlined) to yield in-frame deletions as previously described for construction of the Keio collection (4). All constructions were verified by diagnostic PCR analyses. Overnight cultures of the wild type and its isogenic nanS mutant derivative grown on glycerol as the carbon source were diluted 1:50 into fresh medium containing either glycerol or Neu5,9Ac2 as follows: BW30270 with glycerol (closed squares); nanS mutant with glycerol (closed triangles); BW30270 with Neu5,9Ac2 (open squares); nanS mutant with Neu5,9Ac2 (open triangles).

FIG. 1. nanS is required for E. coli to grow on Neu5,9Ac2 as the sole carbon source. Overnight cultures of BW30270 and its isogenic nanS mutant derivative grown on glycerol as the carbon source were diluted 1:50 into fresh medium containing either glycerol or Neu5,9Ac2 as follows: BW30270 with glycerol (closed squares); nanS mutant with glycerol (closed triangles); BW30270 with Neu5,9Ac2 (open squares); nanS mutant with Neu5,9Ac2 (open triangles).

FIG. 2. Chemical analysis of Neu4,5Ac2 in spent culture medium. (A) Neu4,5Ac2 at a 0.1% final concentration was incubated in minimal glycerol medium without cells for 7 h and then subjected to DMB analysis as described in the text. (B) Neu4,5Ac2 in the spent culture supernatant of wild type was grown for 7 h in the presence of glycerol. (C) Neu4,5Ac2 in the spent culture supernatant of the nanS mutant was grown in glycerol medium for 7 h. Note that the same profiles were obtained after comparable chemical analyses of 24-h cultures, indicating the stability of Neu4,5Ac2 and its resistance to metabolism by E. coli K-12. RF, relative fluorescence.
strains cocultured with glycerol and Neu4,5Ac₂ by chromatography of DMB derivatives. As shown in Fig. 2, the peak representing Neu4,5Ac₂ plus contaminants in medium alone was indistinguishable from that seen with the spent media of either the wild type or the mutant, indicating that Neu4,5Ac₂ is neither transported nor deacetylated by E. coli K-12. We conclude that nanS is required for growth on Neu5,9Ac₂, with the growth process most likely involving deacetylation prior to transport by NanT, and that failure to utilize Neu4,5Ac₂ is caused by an inability to remove the carbon-4 acetyl group of this sialic acid. The idea of a requirement for NanS as a probable exocyclic sialyl deacetylase is supported by the ability of a pGEM-T Easy clone (Promega, Madison, WI) harboring a full-length nanS PCR amplicon to restore growth of the nanS mutant on Neu5,9Ac₂ as a sole carbon source.

To demonstrate that the phenotype of the nanS mutant was not strain dependent and that the general Neu5Ac catabolic pathway was not affected by disruption of nanS, we determined the growth of the BW30270-derived nanS mutant compared to that of two independent nanS deletion mutants constructed from the E. coli K-12/K1 EV36 hybrid strain (31) and strain EV291, derived from clinical isolate RS218 (12); both strains produce Neu5Ac⁺ (22). As shown in Fig. 3A and B, all three wild-type strains and mutant derivatives grew similarly in the presence of the common sialic acids Neu5Ac and N-glycolyl-neuraminic acid (Neu5Gc), demonstrating that the central sialocatabolic system encoded by nanATEK genes is not affected by loss of NanS. As expected from the results shown in Fig. 1, growth on glycerol was unaffected by the loss of NanS. As expected from the results shown in Fig. 1, when Neu5,9Ac₂ was the carbon source (Fig. 3C), thus confirming the results represented in Fig. 1. We conclude that the growth defect of nanS mutants on Neu5,9Ac₂ results specifically from loss of NanS.

Because S. enterica serovar Typhimurium has an intact nan-ATEK system and was previously shown to grow on Neu5Ac (13) but lacks an ortholog of NanS, we tested whether the growth of the BW30270-derived nanS mutant compared to that of two independent nanS deletion mutants constructed from the E. coli K-12/K1 EV36 hybrid strain (31) and strain EV291, derived from clinical isolate RS218 (12); both strains produce Neu5Ac⁺ (22). As shown in Fig. 3A and B, all three wild-type strains and mutant derivatives grew similarly in the presence of the common sialic acids Neu5Ac and N-glycolyl-neuraminic acid (Neu5Gc), demonstrating that the central sialocatabolic system encoded by nanATEK genes is not affected by loss of NanS. As expected from the results shown in Fig. 1, growth on glycerol was unaffected by the loss of NanS. As expected from the results shown in Fig. 1, when Neu5,9Ac₂ was the carbon source (Fig. 3C), thus confirming the results represented in Fig. 1. We conclude that the growth defect of nanS mutants on Neu5,9Ac₂ results specifically from loss of NanS.

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to convert Neu4,5Ac2 to Neu5Ac. However, in addition to the potentially large amounts of Neu4,5Ac2 reported in studies of the mouse intestine, the level of Neu5,9Ac2 in this tissue was reportedly low compared to levels in the human intestine (18). If these differences are real instead of just methodological artifacts, our current results indicate that the mouse would not be a relevant model for investigating enteric colonization of the human intestine in terms of sialometabolism. Despite these

FIG. 3. Growth of the wild type and the yjhS and nanA mutants on different sialic acids. In panels A to D, the indicated wild-type strains (open boxes) and their isogenic yjhS deletion derivatives (shaded boxes) were diluted 50-fold from stationary-phase cells grown in M63 medium plus glycerol (0.4%) into 0.1 ml of M63 medium containing 0.1% of the indicated sialic acids or glycerol in 96-well plates. (A) Growth on Neu5Ac. (B) Growth on Neu5Gc. (C) Growth on Neu5,9Ac2. (D) Growth on glycerol. The plate was incubated without shaking at 37°C for 16 h, and \( A_{600} \) values were recorded with a microplate reader. Data represent the means of three independent experiments ± standard deviations. (E) S. enterica serovar Typhimurium wild-type strain 14028, nanA strain LT2 derivative M2, and E. coli nanA mutant EV78 were diluted 60-fold into 0.5 ml of M63 medium containing 0.4% glycerol, 0.1% Neu5Ac, or 0.1% Neu5,9Ac2. Cultures were grown at 37°C with vigorous aeration; the \( A_{600} \) values were recorded after 7 h.
FIG. 4. Utilization of Neu5Ac or Neu5,9Ac₂ by BW30270 and its yjhS deletion derivative growing simultaneously in the presence of glycerol. BW30270 or its yjhS derivative was diluted 60-fold into 0.5 ml of M63 medium plus glycerol containing either 0.1% Neu5Ac or Neu5,9Ac₂ and incubated at 37°C with vigorous aeration for 7 h unless indicated otherwise. (A) Neu5,9Ac₂ incubated without cells. (B) Neu5Ac incubated without cells. (C) Wild type grown with Neu5Ac. (D) Mutant grown with Neu5Ac. Note that the minor peak eluting at about 10 min as shown in these panels and in the succeeding panel is likely to represent a trace amount of 2-deoxy-α-manno-octulosonic acid derived from sloughed lipopolysaccharide during the incubation that was not removed by the centrifugation step used to produce the spent culture media for the analyses (22). (E) Wild type grown with Neu5,9Ac₂. (F) Mutant grown with Neu5,9Ac₂. (G) Neu5,9Ac₂ incubated without cells for 17 h. (H) Mutant grown with Neu5,9Ac₂ for 17 h.
potential discrepancies, the metabolism of host-derived sialic acids is likely to involve a complex competitive as well as synergistic interplay between different bacterial species and the host, suggesting that a simplified model of mucusinolysis is urgently needed for progress in this research area. Because E. coli lacks nanH, our results further imply that this species and other sialidase-negative bacteria efficiently scavenge free sialic acids released by host sialidases or sialidase-positive bacteria. We conclude that further analysis of sialometabolism in E. coli and other bacteria should lead to a more complete understanding of host-microbe colonization and disease potential and might suggest new therapeutic targets, as we have already posited (30). Future studies will focus on determining the relative contributions of different sialic acids to colonization and diseases caused by mucosal pathogens and on understanding the functions of ychH and yjhBC.

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REFERENCES


