Bacterial catabolism of nonulosonic (sialic) acid and fitness in the gut

Salvador Almagro-Moreno and E. Fidelma Boyd*

Department of Biological Sciences; University of Delaware; Newark, DE USA

The term nonulosonic acid or sialic acid encompasses a varied group of nine-carbon amino sugars widely distributed among mammals and higher metazoans. Among bacteria, the ability to synthesize sialic acid was first examined in a small number of human pathogenic species that deposit sialic acid on their outer surface. New phylogenomic data suggest that the ability to synthesize sialic acid and sialic acid-like compounds is not a novel bacterial innovation but a much more widespread ancient trait. In contrast, the genes required for the catabolism of sialic acid are found only among pathogenic and commensal bacterial species. This ability to utilize sialic acid as a carbon source is correlated with bacterial virulence, especially, in the sialic acid rich environment of the gut. In this article, we present the most recent findings in sialobiology with a focus on sialic acid catabolism.

Introduction

In recent years, the topic of sialic acid or nonulosonic acid occurrence and metabolism has gained increased attention from microbiologists. This family of nine-carbon amino sugars, the most abundant of which is *N*-acetylneuraminic acid (Neu5Ac), was once thought to be confined to higher metazoans and absent from prokaryotes. However, a growing number of pathogenic bacteria are found to encode genes involved in the metabolism of sialic acid.[1-3] Bacterial sialometabolism falls into three broad functional categories: synthesis and sialylation, scavenging, and catabolism, with each functional category conferring specific advantages to the microorganisms (**Fig. 1**).[2,3] The most widely studied of the three categories is the synthesis and sialylation (or deposition) on the bacterial surface of sialic acid and sialic acid-like compounds. To date, at least three different types of sialic acid have been identified that are produced by bacteria: neuraminic (Neu5Ac), pseudaminic (Pse5,7Ac) and/or legionaminic (Leg5,7Ac) acid.[1,2,4] *Campylobacter jejuni, Escherichia coli* K1, *Helicobacter pylori, Neisseria gonorrhoeae, N. meningitidis* and *Pasteurella multocida* can synthesize Neu5Ac.[4] Others, such as *Aeromonas caviae, Helicobacter pylori, Pseudomonas aeruginosa*, can synthesize Pse5,7Ac. *Legionella pneumophila, Clostridium botulinum* and *Vibrio parahaemolyticus* can synthesize Leg5,7Ac.[4] Some bacteria, such as *C. jejuni, Photobacterium profundum* and *V. vulnificus* can synthesize multiple types of sialic acid depending on the strain examined (Boyd EF, unpublished data).[4-6] Pathogens that synthesize sialic acids can coat or glycosylate three different structures on the bacterial cell surface, the flagellum, the capsule polysaccharide (CPS), or the lipopolysaccharide (LPS), masking them from the immune system of their host and changing host cell specificity.[1,2] The phylogenetic distribution of the genes involved in the synthesis of sialic acid among bacteria is widespread.[4]

Neuraminidase or sialidase (NanH) is a glycohydrolase that cleaves bound sialic acid from cell surfaces. Neuraminidase is found in viruses, bacteria and vertebrates; however the occurrence among bacteria is limited to a handful of species.[7] In bacteria, apart from releasing sialic acid molecules from higher-order gangliosides found in mucous surfaces, NanH has been found to be involved in the unmasking of toxin receptors (*V. cholerae*) and biofilm
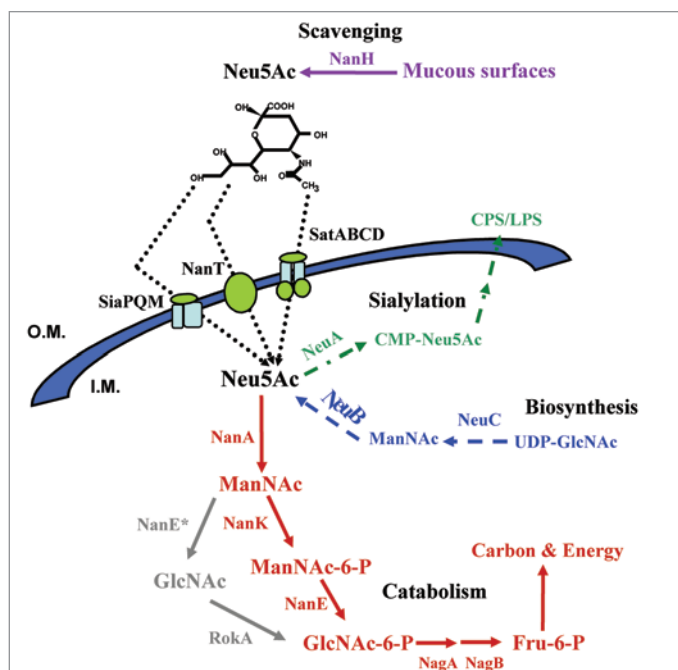
Figure 1. Schematic representation of the metabolism of sialic acid among Bacteria. The catabolic pathway of sialic acid in bacteria is highlighted in red, apart from that of *B. fragilis* which is highlighted in grey. The biosynthesis pathway is highlighted in blue. The sialylation pathway is highlighted in green. The scavenging of sialic acid by neuraminidase (NanH) is highlighted in purple. The three known transporters of sialic acid in bacteria are indicated. Abbreviations: NanH, Neuraminidase; Neu5Ac, *N*-acetylneuraminic acid, sialic acid; NanA, *N*-acetylneuraminic acid aldolase; ManNAc, *N*-acetylmannosamine; NanE*, *N*-acetylmannosamine epimerase; RokA, N-acetylglucosamine kinase; NanK, *N*-acetylmannosamine kinase; ManNAc-6-P, *N*-acetylmannosamine-6-phosphate; NanE, *N*-acetylmannosamine-6-P epimerase; GlcNAc-6-P, *N*-acetylglucosamine-6-phosphate; NagA, *N*-acetylglucosamine-6-phosphate deacteylase; GlcN-6-P, Glucosamine-6-phosphate; NagB, Glucosamine-6-phosphate deaminase; Fru-6-P, Fructose-6-phosphate; NeuA, CMP-*N*-acetylneuraminic acid synthetase; CMP-NeuAc, CMP-*N*-acetylneuraminic acid; NeuB, *N*-acetylneuraminic acid synthase; LPS, lipopolysaccharide; O.M. Outer membrane; I.M. inner membrane.

formation (*P. aeruginosa*).[8,9] The free sialic acid can then be taken up by a range of bacterial species that inhabit the human gut to either sialylate their surface or utilize them as a carbon, nitrogen and energy source.

The first evidence that bacteria could utilize sialic acid (Neu5Ac) as a carbon source was determined in *C. perfringens*.[10] Since then only a limited number of bacterial species, all of which have a close association with a host, have been found to utilize Neu5Ac as an energy source.[3,10-16] The distribution of the genes required for sialic acid degradation was recently shown to be confined to pathogenic and commensal bacteria.[3] Furthermore, current studies have found an intimate relationship between sialic acid catabolism and bacterial fitness in the gut, where the presence of sialic acids is widespread.[11,12,17,18]

Next, we will discuss these exciting new findings, framing them within the evolutionary and metabolic context of sialic acid catabolism.

General Catabolic Pathway of Sialic Acid in Bacteria

Five enzymes are required in order to catabolize *N*-acetylneuraminic acid (Neu5Ac), the most commonly found sialic acid (**Fig. 1**).[2] First, Neu5Ac lyase (NanA) breaks down Neu5Ac into *N*-acetylmannosamine (ManNAc) and phosphoenolpyruvate (PEP). ManNAc kinase (NanK) adds a phosphate group to carbon six of ManNAc generating *N*-acetylmannosamine-6-phosphate (ManNAc-6-P). ManNAc-6-P epimerase (NanE) converts ManNAc-6-P into *N*-acetylglucosamine-6-P (GlcNAc-6-P). In bacteria, the genes for the first

three enzymes (NanA, NanK and NanE) are usually found clustered together forming what is denominated as the Nan cluster.[3] Finally, GlcNAc-6-P deacetylase (NagA) and glucosamine-6-P deaminase (NagB) converts GlcNAc-6-P into fructose-6-P (Fru-6-P), which is a substrate in the glucolytic pathway (**Fig. 1**). The genes encoding NagA and NagB vary in their locations among the different genomes that encode the Nan cluster.

Recently, a surprising exception was found in the catabolic pathway of sialic acid in the gut commensal *Bacteriodes fragilis*.[13] Brigham and colleagues identified a novel epimerase that had no requirement for a phosphorylated substrate. They demonstrate that, in *B. fragilis*, Neu5Ac is broken down into ManNAc by an aldolase (NanA) and an epimerase (NanE) converts it directly into GlcNAc, then a Rok kinase adds a phosphate group to GlcNAc converting it into GlcNAc-6-P.[13] Thus far, this variant pathway appears to be unique to the genus *Bacteroides*.

The role of a novel mutarotase (NanM) encoded within the sialic acid catabolism cluster was recently discovered in *E. coli*.[19] Neu5Ac, when associated with the sialoglycoconjugates, is commonly found in its α-anomer form; however microbes utilize the β-anomer.[19] Severi and colleagues found that *E. coli* secreted an extracellular mutarotase (NanM) that converts α-Neu5Ac into β-Neu5Ac thus allowing the organism to utilize it as a carbon source.[19]

Distribution and Evolution of Nan Cluster

Recently, we demonstrated that the distribution of the genes involved in the catabolism of sialic acid is exclusively confined to pathogenic and commensal bacteria (**Fig. 2**).[3] Among the bacteria shown to encode the Nan cluster are several pathogenic species frequently associated with colonization of the human gut, *V. cholerae*, *Yersinia enterocolitica*, *C. perfringens*, *Salmonella enterica*, pathogenic strains of *E. coli*, and *Shigella boydii*. In addition, many of the most abundant commensal species of the human gut encode the Nan cluster, *B. fragilis*, *Parabacteroides distasonis*, *Faecalibacterium prausnitzii*, *Ruminococcus*

gnavus, *Lactobacillus plantarum*, *L. sakei* and *L. salivarius* (**Table 1**).[3]

The genes within the Nan cluster show independent evolutionary histories, with the NanA phylogenetic tree separate from that of NanE and NanK trees. Several horizontal gene transfer (HGT) events are noted in the phylogenetic trees for all three proteins (**Fig. 2**).[3] Most significantly, NanA shows several putative HGT events between Eukaryotes and Prokaryotes. For instance, *Trichomonas vaginalis* clusters firmly within members of the Pasteurellaceae, such as *Haemophilus influenzae*.[3,20] Strikingly, all members of the *Bacteroides*, *Yersinia* and *Vibrio* branch closely with members from the kingdom Eukarya (**Fig. 2**).[3,20]

Sialic Acid Catabolism and Bacterial Pathogenesis

Several pathogenic and commensal species have been found to be able to utilize sialic acid as a carbon source: *C. perfringens*, *E. coli*, *P. multocida*, *H. influenzae*, *B. fragilis*, *V. vulnificus*, *V. cholerae*, *Y. enterocolitica* and *S. enterica* serovar Typhimurium.[3,10,13-16] Indeed, only in recent years has the in vivo advantage of sialic acid catabolism by bacteria begun to be elucidated. In 2004, Chang et al. found that mice infected with a commensal strain of *E. coli* with *nanA* knocked out, thus unable to utilize sialic acid as carbon source, shed less colony forming units (CFUs) in the faeces than its wild-type parent strain.[18] Their findings suggested for the first time that sialic acid is an important source of carbon and energy for gut dwellers.[18] Interestingly, another study by the same group examined the carbon nutrition of a pathogenic *E. coli* strain, EDL933, and found no difference between *E. coli* EDL933 wild-type and a *nanA* mutant strain in the number of CFU recovered in the faeces of the infected mice.[17] The authors suggested that these findings, together with others related to carbon nutrition, indicated an interesting system of carbon preferences between commensal and pathogenic strains of the same organism.[17]

We demonstrated that the catabolism of sialic acid plays a significant role in colonization of the gut by *V. cholerae* pathogenic

Table 1. Bacteria that encode NanA and are able to colonize the human intestine

Pathogens	Commensals
Clostridium perfringens	*Akkermansia muciniphila*
C. butyricum	*Anaerotruncus colihominis*
C. difficile	*Bacteroides caccae*
Citrobacter koserii	*B. fragilis*
E. coli	*B. ovatus*
Enterobacter sakazakii	*B. stercoris*
Salmonella enterica	*B. uniformis*
S. typhimurium	*B. vulgatus*
Shigella boydii	*Clostridium leptum*
Vibrio cholerae	*C. bolteae*
V. vulnificus	*C. scindens*
Yersinia enterocolitica	*Dorea longicatena*
Y. bercovieri	*D. formicigenerans*
Y. mollaretii	*Faecalibacterium prausnitzii*
	Lactobacillus sakei
	L. plantarum
	L. salivarius
	Parabacteroides distasonis
	P. merdae
	Ruminococcus gnavus
	Victivallis vadensis

isolates.[11] *V. cholerae* is the causative agent of the deadly diarrheal disease cholera, which is endemic on the Indian subcontinent, Africa, and South America. Conservative estimates indicate that there are over a million cases of cholera worldwide per year. In *V. cholerae*, the Nan genes are encoded within a 57 kb pathogenicity island, named Vibrio Pathogenicity Island-2, which is confined to pathogenic isolates of the species.[21] Neuraminidase, the enzyme that cleaves sialic acid from higher-order gangliosides in the gut, is encoded adjacent to the Nan cluster on VPI-2. Also associated with the region are homologues of genes that encode a TRAP transporter that was shown in *H. influenzae* to be highly efficient in the uptake of sialic acid into the bacterial cell.[14] Two putative mutarotases are also clustered with these genes. Interestingly, this entire region, which encompasses a 10 kb section of VPI-2, is also present in *V. vulnificus* on chromosome 2. *V. vulnificus* is a pathogen of humans but is associated with septicemia and wound infections, which, in susceptible individuals, can have up to a 75% mortality rate. On the other hand,

V. vulnificus is only occasionally associated with gastroenteritis in humans.

V. cholerae is unique among Vibrio species in its sialometabolism capacity, as it is the only species that encodes both neuraminidase and the Nan cluster. *V. vulnificus* encodes both the Nan cluster and the Neu cluster, required for de novo sialic acid synthesis, and can sialylate its surface (Boyd EF, unpublished data). No isolates of *V. cholerae* from the 15 sequenced genomes have been identified that can synthesize sialic acid or sialylate their surface. Among sequenced *V. parahaemolyticus* strains, an important cause of seafood borne gastroenteritis, all contain the genes for the synthesis of sialic acid but not the catabolism.

In *V. cholerae*, we determined the role of the Nan cluster in vitro and in vivo. We demonstrated that a knockout strain for *nanA* had a significant decrease in CFUs in the early stages of colonization when compared to the wild-type *V. cholerae* strain.[11] This finding prompted us to investigate whether that deficiency in early infection would have a fitness cost for the mutant strain in competition with

Figure 2. Phylogenetic tree based on *N*-acetylneuraminic acid aldolase (NanA) sequences in the database. Commensal organisms are highlighted in blue. Pathogenic organisms are highlighted in orange. The tree was constructed with 121 bacterial NanA sequences using the Neighbor-Joining method with the bootstrap values obtained after 10,000 generations as implemented in MEGA4.[38] The black triangle represents the collapsed branch of NanA from Eukaryotes.

the wild-type. We performed competition assays using the infant mouse model, and demonstrated a significant decrease in the fitness of the *nanA* mutant when compared to the wild-type strain of *V. cholerae*.[11] These data indicate that the ability to utilize an abundant carbon and nitrogen source in the human gut is important in in vivo survival.

Similarly, it was shown for *V. vulnificus* that a *nanA* mutant displayed diminished ability in its colonization of the gut.[12] Interestingly, Jeong et al. found some novel virulence features associated with sialic acid catabolism.[12] For instance, the *V. vulnificus nanA* mutant strain exhibited lower levels of cytotoxicity, reduced virulence in the mouse, less growth and adherence to cell lines, and less intestinal colonization than the wild-type parent strain.[12] A possible reason for this wider array of phenotypical effects displayed by the *V. vulnificus nanA* mutant might be due to a crosstalk or interplay between synthesis of sialic acid and sialic acid-like compounds, sialylation of the bacterial surface, and uptake and catabolism of sialic acid from the external environment. Visibly, further studies remain to be done in order to decipher the precise and wider role of sialometabolism in vivo. Our most recent data in this area indicates that *V. vulnificus* can synthesize multiple forms of sialic acid and that this ability is prevalent among clinical strains whereas environmental *V. vulnificus* strains produce generally only one type of sialic acid (Boyd EF, unpublished data).

The expression pattern of the Nan cluster in vivo has also been determined.[22,23] A highly significant increased expression of the Nan genes in *V. cholerae* was found in the rabbit ileal loop model of infection, and Alteri and co-workers found that uropathogenic *E. coli* strain CFT073 had increased levels of expression of the Nan cluster when growing in human urine.[22,23] It has been suggested that due to their very limited distribution, sialic acids might not only be used as a carbon source but also may act as a signaling molecule that indicates pathogen entry into its host. A recent study of *Streptococcus pneumoniae*, a major cause of bacteremia, pneumonia and otitis media, demonstrated that only *N*-acetylneuraminic acid enhanced

pneumococcal biofilm formation in vitro, and, in a murine model, intranasal inoculation of sialic acid significantly increased pneumococcal counts in the nasopharynx.[24] Trappetti and colleagues also correlated this phenotype with the presence of neuraminidase in these strains.

Taken together, all these findings undeniably highlight the significance of sialic acid catabolism and bacterial virulence, and more broadly, the role of bacterial carbohydrate availability and host nutrients in host-microbial interactions.

Scavenging Sialic Acid: Neuraminidase

An interesting relationship is found between the production of neuraminidase and the catabolism of sialic acid. In some bacterial pathogens, such as *S. pneumoniae* or *Pseudomonas aeruginosa*, that colonize the heavily sialylated upper respiratory tract, the presence and function of neuraminidase has been well documented.[9,25] In both organisms neuraminidase plays an essential role in biofilm formation, and therefore, in colonization of the lungs.[9,25] In *P. aeruginosa* neuraminidase also unmasks the receptors of the type-IV pilus, a major virulence-associated adhesion.[26,27] In addition, in *H. influenzae* the presence of sialic acid is required for the successful production and stability of biofilms.[28]

Surprisingly, the role that neuraminidase might play in biofilm formation and adherence of intestinal bacteria has not been studied, even though species such as *S. enterica* serovar Typhimurium, *V. cholerae*, *C. perfringens* and *B. fragilis* are known to encode at least one neuraminidase.[7,29-32] In the case of *V. cholerae*, it is well established that neuraminidase removes two molecules of sialic acid from the trisialogangliosides found in the intestinal mucus, subsequently unmasking the receptors of the cholera toxin, the GM1 gangliosides.[8,30,33] These released molecules of sialic acid can be utilized as a carbon source, thereby closing the cycle of *V. cholerae*'s sialometabolism. In *B. fragilis* it was shown by Godoy et al. that neuraminidase was required for efficient growth on CHO cells and on the rat granuloma pouch, possibly by providing an alternative source of carbon once glucose was depleted.[31]

Future Directions

The human gut contains one of the most complex and densely populated microbial ecosystems on the planet.[34] In an environment like this, competition for scarce resources among gut inhabitants is fierce, particularly in the case of pathogenic organisms attempting to colonize and multiply in a hostile new niche, where they are encountering numerous adverse conditions and competitors.[35,36] One of the main limiting factors is the immediate availability of nutrients.[18] It is fundamental for pathogenic bacteria, in order to survive and establish an infection within the human gut, to outcompete the current residents in this quest for limited resources. One way many bacterial species have overcome this bottleneck is through the utilization of alternative carbon sources other than highly utilized glucose.[37] Here we have presented some of the latest findings in sialic acid catabolism and pathogenesis in bacteria.[3,11,12,18] The future perspectives on this emerging field are enticing since the relationship between sialic acid catabolism and fitness in the gut has been studied only in a handful of the organisms encoding the Nan cluster (**Table 1**). Indeed the analysis of carbohydrate availability and utilization by pathogenic bacteria promises not only a greater understanding of host-pathogen interactions but also points to new prevention and treatment strategies by demonstrating the novel roles a host compound such as sialic acid plays in infectious disease.

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