Mini-Review

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Sialic acid utilization by bacterial pathogens

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Sialic acid occupies the terminal position within glycan molecules on the surfaces of many vertebrate cells, where it functions in diverse cellular processes such as intercellular adhesion and cell signalling. Pathogenic bacteria have evolved to use this molecule beneficially in at least two different ways: they can coat themselves in sialic acid, providing resistance to components of the host's innate immune response, or they can use it as a nutrient. Sialic acid itself is either synthesized *de novo* by these bacteria or scavenged directly from the host. In this mini-review we will summarize recent findings relating to sialic acid transport, modification of sialic acid by O-acetylation, and the mechanisms of sialic acid-mediated complement resistance.

Introduction

The surfaces of many cells, both prokaryotic and eukaryotic, are decorated with glycoconjugates that play important roles in a wide range of biological processes, including cell–cell and small molecule–cell recognition. Sialic acid is a generic term to indicate a wide family of related nine-carbon sugar acids that feature prominently at terminal positions of many eukaryotic surface-exposed glycoconjugates, where they confer important properties upon the resulting cell surface. It is therefore perhaps not surprising that many pathogenic bacteria have also evolved to decorate their cell surfaces with sialic acid, which results in important phenotypes regarding their ability to resist the host's innate immune response and also their ability to interact specifically with different host-cell surfaces. The most abundant and best-studied sialic acid is *N*-acetylneuraminic acid (Neu5Ac, Fig. 1), although there are numerous naturally occurring variations including substitutions at carbon 5, or covalent modifications of the sugar's hydroxyls (for a more general overview of the diversity of sialic acid forms in nature see Angata & Varki, 2002). Most experimental work on bacterial sialic acid metabolism relates to Neu5Ac, therefore the term 'sialic acid' has been used in the literature to mean this particular chemical primarily. For the sake of simplicity we will not attempt to distinguish between these two terms; however, bacteria may encounter other sialic acid forms in their environment and process them through the same pathways as used for Neu5Ac metabolism. This review will summarize recent literature describing how pathogenic bacteria use sialic acid in their lifestyles and will draw primarily from a limited number of well-studied pathogens, including *Escherichia coli* K1, *Haemophilus influenzae*, *Pasteurella multocida*, *Neisseria* spp., *Campylobacter jejuni*

and *Streptococcus agalactiae*. These bacteria use sialic acid for a variety of different purposes that play important roles in their ability to colonize, persist and cause disease in mammalian species.

Although bacterial sialic acid metabolism is not restricted to bacterial pathogens, this topic will not be covered by this review, which will rather focus on the role of sialic acid in pathogenesis [for a complete review of bacterial sialic acid metabolism, readers are directed to the excellent article by Eric Vimr and colleagues (Vimr *et al.*, 2004)]. The mini-review will consider recent discoveries in three areas related to pathogenic bacteria and sialic acid, namely (i) how bacteria acquire sialic acid, either by scavenging from the environment or by *de novo* biosynthesis, (ii) how bacteria incorporate this compound into their cell-surface features, and (iii) how these sialic acid-bearing surfaces modify the interaction of the bacterium with its host.

Preparing the supply line – sialic acid acquisition and biosynthesis

Bacteria have two primary routes to obtain sialic acid: *de novo* biosynthesis or acquisition from the environment (Fig. 1) (Vimr & Lichtensteiger, 2002; Vimr *et al.*, 2004). The first route is used by a number of bacteria including *E. coli* K1, *Neisseria meningitidis* and *C. jejuni* (Vimr *et al.*, 2004). The metabolite UDP-GlcNAc is the precursor for sialic acid biosynthesis (Fig. 1; abbreviations for the names of metabolites are reported in the legend) and is produced by most cells, as it is used in cell wall biosynthesis; the combined actions of the NeuC and NeuB proteins convert this to Neu5Ac via ManNAc (Fig. 1).

The second source of sialic acid is the environment, which for the pathogens mentioned in this review is the mammalian host. Many pathogens secrete a sialidase that

Abbreviations: these are defined in the legend of Fig. 1.

Fig. 1. Overview of the major pathways for sialic acid utilization in bacterial pathogens. This figure summarizes the major pathways and mechanisms discussed in this review (see text for a description of each pathway) and is not meant to represent all known pathways for prokaryotic sialic acid utilization. Sialic acid is shown as Neu5Ac (inset), as most, if not all, experimental research has been carried out with this particular form, and as Neu5Ac is the most abundant form of sialic acid in nature. The site of interaction of factor H (fH) on the gonococcal cell surface is deliberately indicated in an ambiguous manner (black arrow) as it is still unclear whether the sialylated LPS is an actual binding site for fH. The monomeric O-acetylated Neu5Ac produced by *E. coli* NeuD is believed to enter the normal PSA biosynthetic pathway via NeuA/NeuS (Steenbergen *et al.*, 2006); hence the omission of the activated intermediate from the figure. The light-green discontinuous arrow represents the pathways (mostly not defined) by which the LPS is exposed on the outer membrane. The asterisk indicates O-acetylation of the disialylated LPS of *C. jejuni*; the reaction is catalysed by the product of the gene *orf11*, which is defined as a sialic acid O-acetyltransferase (SOAT) (Houliston *et al.*, 2006). The figure takes into account that the modified sialylated LPS of *C. jejuni* is exposed on the cell surface (Houliston *et al.*, 2006); however, as the role of LPS sialylation in *C. jejuni* is not clearly established, the interaction indicated between LPS and C3 must be taken to refer solely to *H. influenzae*. **Abbreviations:** IM, inner membrane; OM, outer membrane; Neu5Ac, N-acetylneuraminic or sialic acid; ManNAc, N-acetylmannosamine; GlcNAc, N-acetylglucosamine; GlcN, glucosamine; Fru, fructose; PSA, polysialic acid; PEP, phosphoenolpyruvate; Lst, *Neisseria* LPS sialyltransferase; NanC, *E. coli* Neu5Ac-specific porin; Kps, *E. coli* PSA capsule export system; SatABCD, *H. ducreyi* Neu5Ac ABC (ATP-binding cassette) transporter; SiaPQM, *H. influenzae*/*P. multocida* Neu5Ac TRAP (tripartite ATP-independent periplasmic) transporter; NanT, *E. coli* Neu5Ac MFS (major facilitator superfamily) transporter; SiaB and NeuA, respectively *H. influenzae* and *E. coli* CMP-Neu5Ac synthetases (NeuA also possesses deacetylase activity: Steenbergen *et al.*, 2006); Lic3A, Lic3B: *H. influenzae* sialyltransferases; SOAT, *C. jejuni* Neu5Ac O-acetyltransferase; NeuC, *E. coli* UDP-GlcNAc 2-epimerase; NeuB, *E. coli* Neu5Ac synthase; NeuS, *E. coli* polysialyltransferase; NeuO, *E. coli* PSA O-acetyltransferase; NeuD, *E. coli* Neu5Ac O-acetyltransferase; NanA, Neu5Ac aldolase; NanK, ManNAc kinase; NanE, ManNAc-6P epimerase; NagB, GlcNAc-6P deacetylase; NagA, GlcN-6P deaminase (the catabolic pathway is present in several bacteria: Vimr *et al.*, 2004).

releases sialic acid from a diverse range of host sialoglycoconjugates (Fig. 1) (Corfield, 1992); however, other sialic acid-utilizing bacteria, such as the respiratory pathogen *H. influenzae*, lack genes for a sialidase yet are reliant on host-derived sialic acid (Bouchet *et al.*, 2003). Presumably free sialic acid is made available to such pathogens by other, sialidase-expressing bacteria living in the same niche (Shakhnovich *et al.*, 2002), or, as hypothesized by Sohanpal *et al.* (2004, 2007), by host sialidases that are activated in the course of inflammation. The latter process is part of the normal recycling of sialic acid and there has been a recent suggestion that the host cells might use free sialic acid to help them cope with oxidative stress (Iijima *et al.*, 2007).

Regardless of the mode of release of free monomeric sialic acid, bacteria must then capture this resource using specific transporters (Fig. 1). The one exception to this is *Neisseria gonorrhoeae*, which appears to have obviated the need for uptake by secreting the enzymes that sialylate its LPS and by using the activated form of sialic acid, CMP-Neu5Ac, from the host as a substrate (see next section). Our understanding of sialic acid transport in bacteria has until recently been quite poor and, at the molecular level, limited to the characterization of the NanT sialic acid transporter from *E. coli* K-12 (Vimr *et al.*, 2004). This is a classical secondary transporter of the major facilitator superfamily (MFS) and is essential for Neu5Ac uptake by *E. coli* (Fig. 1) (Vimr *et al.*, 2004). The route of sialic acid uptake in *H. influenzae* and *P. multocida* has recently been established; it is dependent on a tripartite ATP-independent periplasmic (TRAP) transporter, SiaPQM (Steenbergen *et al.*, 2005; Allen *et al.*, 2005; Severi *et al.*, 2005). This family of transporters are unusual as they are secondary transporters like NanT, but utilize an extracytoplasmic solute receptor (ESR) protein in the transport mechanism (Fig. 1). The ESR component of the *H. influenzae* sialic acid transporter, SiaP, binds sialic acid with high affinity and specificity (Severi *et al.*, 2005), and the structure of this protein revealed a fold not dissimilar to that seen in other ESR proteins from ATP-binding cassette (ABC) transporters (Muller *et al.*, 2006). These data suggest that SiaPQM functions as a high-affinity transporter *in vivo*, which would be consistent with the scavenging requirements of *H. influenzae*. The sialic acid transporter from *Haemophilus ducreyi* (SatABCD) has also been characterized; interestingly, it is not a TRAP transporter but rather is an ABC transporter and hence presumably of high affinity (Fig. 1) (Post *et al.*, 2005). Therefore it appears that bacteria have evolved multiple routes to capture sialic acid from the environment, supporting the importance of this process *in vivo*. This is certainly true for organisms such as *H. influenzae* and *H. ducreyi*, which are unable to synthesize sialic acid, and rely on their respective transporters for the provision of sialic acid to the LPS sialylation pathways (Severi *et al.*, 2005; Allen *et al.*, 2005; Post *et al.*, 2005). As we explain below, LPS sialylation is an important virulence factor for some pathogens, which again stresses the

importance of sialic acid uptake in the lifestyle of these bacteria.

There has also been a recent characterization of the Neu5Ac-inducible porin NanC (YjH4) from *E. coli* K-12, which is essential for growth on Neu5Ac when both the general OmpC and OmpF porins are not expressed (Fig. 1) (Condemine *et al.*, 2005). However, the occurrence of this porin is not widespread and the role of Neu5Ac-specific transport across the outer membrane of bacterial pathogens has not been established.

In addition to 'preparing the supply line' for making sialic acid-modified cell surfaces, some bacteria, such as *H. influenzae* and *E. coli*, can also utilize the transported sialic acid as a carbon and nitrogen source (Vimr *et al.*, 2004; Severi *et al.*, 2005). The pathway by which this occurs has been well characterized and uses the *N*-acetylneuraminate aldolase NanA that cleaves Neu5Ac to ManNAc and pyruvate (Fig. 1). ManNAc is then ultimately converted to fructose 6-phosphate and ammonia, via the action of the NanK, NanE, NagB and NagA proteins, where it can enter central metabolism (Fig. 1) (Vimr *et al.*, 2004). How these bacteria maintain the balance between the catabolic and anabolic pathways is not clearly defined, nor is it clear how sialic acid catabolism contributes to virulence. There is evidence that in *H. influenzae* sialic acid catabolism competes with the LPS sialylation pathway for sialic acid that has been transported into the cell (Fig. 1), as demonstrated by the LPS hyper-sialylation phenotype and increased fitness in an animal model of a *nanA* mutant (Vimr *et al.*, 2000). In *E. coli* K1 catabolism has the potential to compete with the polysialic acid (PSA) synthesis pathway (Fig. 1); however, whereas the *neu* genes are expressed constitutively the *nan* genes are induced by Neu5Ac (Vimr *et al.*, 2004), and the hypothesis of tight coupling of Neu5Ac synthesis and activation by NeuB/NeuA might explain the lack of a futile cycle of Neu5Ac synthesis and degradation, and implies that the catabolic operon is expressed only when exogenous sialic acid is made available (Vimr *et al.*, 2004). Interestingly Chang *et al.* (2004) have shown that the *nanAT* genes are required for *E. coli* colonization of the mouse colon, where sialic acid-rich mucin appears to be an important nutrient source *in vivo*. However, studies on the contribution of sialic acid catabolism to virulence using animal models must be interpreted with caution as the distribution and nature of sialic acid may be significantly different in the human host, which affects any possible extrapolation.

Deploying sialic acid onto the battlefield – synthesis of sialic acid-containing cell-surface features

Once obtained (by either *de novo* biosynthesis or acquisition from the host), sialic acid can be incorporated into cell-surface macromolecules that modulate the pathogen's interaction with the host (see next section).

The obligatory first step in this process is the conversion of sialic acid into the activated form CMP-Neu5Ac (catalysed by CMP-sialic acid synthetases), which is then added to appropriate acceptors by linkage-specific sialyltransferases (Fig. 1). As mentioned above, *N. gonorrhoeae* relies instead on an outer-membrane-associated sialyltransferase to scavenge CMP-Neu5Ac directly from the secretions of the host (Fig. 1) (Shell *et al.*, 2002).

A significant part of our knowledge about the incorporation of sialic acid into bacterial macromolecules has come from the analyses of the processes for synthesis of the PSA capsules of *E. coli* and *N. meningitidis*. In *E. coli*, NeuA activates Neu5Ac prior to its incorporation into the K1 and K92 capsules while the *N. meningitidis* orthologue carries out the analogous function relating to both capsule and LPS synthesis (Fig. 1). In *E. coli*, NeuS functions as the main polysialyltransferase adding Neu5Ac to oligosialic acid receptors to form the PSA capsule, which is then exported through the Kps system (Fig. 1) (Vimr *et al.*, 2004). An initial step in PSA synthesis is thought to include the addition of Neu5Ac by the NeuE protein to some as-yet-unidentified initiator molecule, presumed to be a lipid (Vimr *et al.*, 2004). Once synthesized, sialic acid residues in the PSA capsule of both *N. meningitidis* and *E. coli* can be modified by *O*-acetylation (Claus *et al.*, 2004; Steenbergen *et al.*, 2006; Deszo *et al.*, 2005), an emerging and exciting new feature of sialic acid metabolism also seen in unencapsulated organisms (see below). In *E. coli* the *O*-acetyltransferases NeuO and NeuD modify PSA and monomeric Neu5Ac, respectively, the latter of which can be deacetylated by NeuA acting as a bifunctional enzyme (Fig. 1) (Steenbergen *et al.*, 2006; Deszo *et al.*, 2005; Bergfeld *et al.*, 2007). These new discoveries highlight the huge potential diversity of capsule structures that may be presented on the cell surface by varying the patterns of acetylated sialic acid (King *et al.*, 2007). The only Gram-positive bacterium reported to produce a sialic acid-containing capsule is *S. agalactiae*, which possesses a sialyltransferase (CpsK) that adds a terminal α -2,3-linked Neu5Ac to galactose within the capsule's oligosaccharide repeat (Chaffin *et al.*, 2005). Again, recent work has shown that this Neu5Ac residue can be modified by *O*-acetylation (Lewis *et al.*, 2006).

Sialylation of the LPS is also catalysed by linkage-specific sialyltransferases. Both *N. meningitidis* and *N. gonorrhoeae* sialylate their LPS through the outer-membrane α -2,3 sialyltransferase Lst (Fig. 1), although the enzyme is expressed at a higher level in the gonococcus (Packiam *et al.*, 2006). LPS sialylation is also a feature of several pathogenic members of the *Pasteurellaceae*, including *H. ducreyi*, *H. influenzae*, *Haemophilus somnus* and *P. multocida*. *H. influenzae* has the greatest number of characterized LPS sialyltransferases, with up to four being present in any given strain (Fox *et al.*, 2006). The main sialyltransferase is Lic3A (Fig. 1), which adds α -2,3-Neu5Ac (Hood *et al.*, 2001), and has been shown to be absolutely required for bacterial survival in the middle ear in an

animal model of otitis media (Bouchet *et al.*, 2003). Amongst the other sialyltransferases, the Lic3A-homologue Lic3B is a bifunctional enzyme that can add mono- or disialic acid to the LPS acceptor (Fig. 1) (Fox *et al.*, 2006). *C. jejuni* also possesses mono- or bifunctional LPS sialyltransferases transferring either α -2,3-Neu5Ac or disialic acid (Gilbert *et al.*, 2000). It is of note that the terminal sialic acid residue in the disialylated LPS can also be modified by an *O*-acetyltransferase (Fig. 1) (Houliston *et al.*, 2006). Sialic acid, also in its *O*-acetylated form, can be included as part of the oligosaccharides that are repeated to form the O-antigen of LPS in *E. coli* (Ali *et al.*, 2006), although the enzymes required for this process are not well studied.

While sialylated LPS and PSA capsules have obvious benefits to bacteria in particular host compartments, for example in evading the immune response as we describe below, their presence may not always be desirable when the organism is within other compartments either inside or outside the host. Some pathogens, such as *Neisseria* spp., *H. influenzae* and *C. jejuni*, regulate expression of cell-surface features by stochastic genetic mechanisms relying on rapid and reversible ON to OFF switching of the expression of key biosynthetic genes, the so-called 'phase variation' (van der Woude & Baumler, 2004). Phase variation also modulates *O*-acetylation of the PSA capsule of *E. coli* K1 (Deszo *et al.*, 2005; Bergfeld *et al.*, 2007). Such variable expression makes it likely that any population of bacteria will be expressing alternative glycoforms and that any one expression pattern might confer a fitness advantage to the relevant bacteria under any given conditions.

Contact with the enemy – alteration of host responses by sialic acid-coated bacteria

As sialoglycoconjugates can be predominant components of mammalian cell surfaces, the incorporation of sialic acid into bacterial cell-surface features (be those sialic acid-containing capsules or sialylated LPS) is hypothesized to allow bacterial pathogens to disguise themselves as host cells and thus circumvent and/or counteract the host's immune responses through a strategy of 'molecular mimicry' (Harvey *et al.*, 2001).

The PSA capsule of *N. meningitidis* serogroup B and *E. coli* K1 is poorly immunogenic and this is thought to be a consequence of its being structurally identical to the PSA chains of the mammalian neuronal cell adhesion molecule, NCAM (Vimr & Lichtensteiger, 2002). The exact mechanisms by which the PSA capsule helps evade the host immune response are not clear, but in meningococci this capsule is required for resistance against the killing effect of human serum, possibly by hindering the insertion of the complement membrane-attack complex (MAC) in the bacterial membrane (Vimr & Lichtensteiger, 2002). The PSA capsule of *E. coli* K1 does not confer serum resistance,

but it inhibits opsonization and phagocytosis *in vivo* (Fig. 1) (Vimr & Lichtensteiger, 2002). The sialylated capsule of *S. agalactiae* has similar inhibitory effects on phagocytosis and acts by impairing C3 deposition on the cell surface, thus preventing activation of the complement alternative pathway (Marques *et al.*, 1992).

LPS sialylation also inhibits the complement alternative pathway in both *N. gonorrhoeae* and in non-typable *H. influenzae* (NTHi), although these two pathogens use different mechanisms to this effect (Figueira *et al.*, 2007; Ram *et al.*, 1998). Gonococcal sialylated LPS increases the binding to the bacterial cell surface of factor H (fH), an anti-activator of the complement alternative pathway (Fig. 1) (Ram *et al.*, 1998). This strategy mimics the effect that sialylation has on some eukaryotic cell membranes, which self-protect from C3 attack by recruiting fH, and results in higher serum resistance (Ram *et al.*, 1998). To date it is not known whether the sialylated LPS is an actual binding site for fH (Fig. 1) (Madico *et al.*, 2007). Interestingly, the positive effect of LPS sialylation on fH binding requires the presence of the gonococcal porin PorB (Fig. 1), suggesting that sialylated LPS and PorB might constitute a composite epitope, or that sialylation might cause a conformational change in the LPS that unmasks novel sites in PorB (Madico *et al.*, 2007). In NTHi, LPS sialylation inhibits deposition of C3 without entailing fH binding (Fig. 1) (Figueira *et al.*, 2007). Restoration of virulence to an avirulent, sialic acid-free mutant of NTHi inoculated into complement-depleted chinchillas demonstrated a role for the complement in eliminating unsialylated *H. influenzae in vivo* (Figueira *et al.*, 2007). Circumstantial evidence suggests that one target of C3 on unsialylated cell surfaces might be LPS, raising the possibility that sialylation acts by masking those sites (Figueira *et al.*, 2007).

There is evidence that the sialylated bacterial surfaces of *C. jejuni*, *N. meningitidis* and *S. agalactiae* (LPS for the former two species and capsule for the latter) can interact with sialic acid-specific lectins of the Siglec family, which are normally expressed on the surface of cell types of the immune system (Avril *et al.*, 2006; Carlin *et al.*, 2007; Jones *et al.*, 2003). This has led to the hypothesis of direct cellular interactions between these cell types and the bacterial cells, which may represent yet another way by which bacterial cell-surface sialylation might modulate the host's immune response (Avril *et al.*, 2006; Carlin *et al.*, 2007; Jones *et al.*, 2003).

Concluding remarks

We have attempted in this short review to introduce some of the interesting new research that is elucidating how bacteria build sialic acid-containing structures and the roles of these in pathogenesis. In an interesting variation on this theme, the group of Ian Blomfield (Sohanpal *et al.*, 2007, 2004) has demonstrated a different way by which sialic acid may modify bacterial cell surfaces. In uropathogenic strains of *E.*

coli, free Neu5Ac inhibits the phase-variable expression of type 1 fimbriae, which are known virulence factors mediating adherence to and invasion into epithelial cells (Sohanpal *et al.*, 2004). This is achieved by antagonizing the activator effect of key *N*-acetylsugar-responsive transcription factors (NanR and NagC) on the *fimB* gene, which is in turn a regulator of the expression of the *fim* operon (Sohanpal *et al.*, 2004). As sialic acid is produced by the host in the course of inflammation, it is possible that a sialidase-negative bacterium such as *E. coli* recognizes free sialic acid as an indicator of inflammation, and downregulates expression of adhesins in response (Sohanpal *et al.*, 2004, 2007). These recent discoveries along with new work on sialic acid transport, sialic acid acetylation and the mechanisms of serum resistance continue to further our understanding of sialic acid in biology and specifically of how it is exploited by pathogenic bacteria for a variety of different purposes *in vivo*.

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