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Masquerading microbial pathogens: Capsular polysaccharides mimic host-tissue molecules

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Summary

Bacterial pathogens bearing capsular polysaccharides identical to mammalian glycans benefit from an additional level of protection from host immune response.

The increasing prevalence of antibiotic resistant bacteria portends an impending post-antibiotic age, characterized by diminishing efficacy of common antibiotics and routine application of multifaceted, complementary therapeutic approaches to treat bacterial infections, particularly multidrug-resistant organisms. The first line of defense for most bacterial pathogens consists of a physical and immunological barrier known as the capsule, commonly composed of a viscous layer of carbohydrates that are covalently bound to the cell wall in Gram-positive bacteria or often to lipids of the outer membrane in many Gram-negative bacteria. Bacterial capsular polysaccharides are a diverse class of high molecular weight polysaccharides contributing to virulence of many human pathogens in the gut, respiratory tree, urinary tract, and other host tissues, by hiding cell-surface components that might otherwise elicit host immune response. This review highlights capsular polysaccharides that are structurally identical or similar to polysaccharides found in mammalian tissues, including polysialic acid and glycosaminoglycan capsules hyaluronan, heparosan, and chondroitin. Such non-immunogenic coatings render pathogens insensitive to certain immune responses, effectively increasing residence time in host tissues and enabling pathologically relevant population densities to be reached. Biosynthetic pathways and capsular involvement in immune system evasion are described providing a basis for potential therapies aimed at supplementing or replacing antibiotic treatment.

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Introduction

Bacterial capsular polysaccharides (CPSs) are major virulence factors that confer protective effects to their bearers against a wide range of environmental pressures, most notably against the immune system during infection of their animal hosts. Although capsules are often associated with descriptions of pathogenic bacteria due to the large proportion of encapsulated invasive pathogens, non-pathogenic and commensal bacteria also benefit from the ability to envelope themselves with a capsule (Hafez *et al.*, 2009; Dasgupta & Kasper, 2010). In Gram-negative bacteria, capsular polysaccharides are often attached to the outer membrane at their reducing end through covalently-linked lipids that are inserted into the lipid bilayer of the membrane. This provides a surface layer of water-saturated, high molecular weight polysaccharides that limit desiccation in the face of harsh environmental conditions, block infection by most bacteriophages, and thwart phagocytosis and other host immune responses by physically restricting access to cell surface antigens. These polysaccharide cloaks are likely rational targets for wide-spectrum therapeutic compounds aimed at replacing or supplementing antibiotic treatment of microbial infections, as removal of the capsule exposes bacteria to routine immune clearance pathways mediated frequently by activation of the complement system.

Historical perspective

The molecular compositions of CPSs vary extensively between organisms and even between strains within a single species, but, despite this diversity, some species from distinct orders have been shown to biosynthesize identical CPS structures (DeAngelis, 1999; DeAngelis & White, 2002). The existence of highly homologous biosynthetic machinery for production of identical polysaccharides between microbes suggests that capsular gene clusters have been acquired through horizontal gene transfer; conversely, non-homologous glycosyltransferases biosynthesize identical polysaccharides in disparate organisms (Vann *et al.*, 1981; Finne *et al.*, 1983; Korhonen *et al.*, 1985; Jann & Jann, 1998), an occurrence that has likely developed through functional convergent evolution (DeAngelis, 2002a, b) facilitated by inter-kingdom coevolution of prokaryotic pathogens with their eukaryotic hosts (Gagneux & Varki, 1999; Chen & Varki, 2010). Capsule structure diversity was originally investigated as part of a broad effort to classify bacterial strains based upon their interaction with human serum; that is, bacteria were serotyped through differentiation of their cell surface antigens (Lancefield, 1933). Serotyping is critical for understanding pathogenicity from medical, diagnostic, and immunological perspectives and has remained the dominant method for classifying strains of capsular bacteria. While studies in the early twentieth century demonstrated that polysaccharides present in the capsules of both Gram-negative and Gram-positive bacteria were antigenically distinct from cellular protein fractions (Heidelberger & Avery, 1923), research throughout the following decades further differentiated and classified

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these polysaccharidic antigens and ultimately implicated the eponymous CPSs in elevated serum resistance and inhibition of granulocytic phagocytosis (Peterson *et al.*, 1978; Horwitz & Silverstein, 1980).

Complementary serological and clinical studies during the latter half of the twentieth century identified a subset of streptococcal, staphylococcal, meningococcal, and *Escherichia coli* CPSs associated with increased virulence and widespread incidence in severe bacterial infections, provoking investigation of the relationship between CPS structure and immunogenicity (Robbins *et al.*, 1974; Kaijser *et al.*, 1977). Elucidation of the chemical structures of various K-antigens (an alternative name for the CPS of *E. coli*) and apparent demarcation based upon their physical properties prompted development of a K-antigen classification system (Orskov *et al.*, 1977) that was revised over time to incorporate genetic and biomolecular evidence (Jann & Jann, 1997), ultimately being supplanted by a more robust grouping scheme based on genetic, biochemical, and molecular criteria (Whitfield & Roberts, 1999). Serotyping systems for other species were developed in a similar manner, but the relative ease of Gram-negative CPS structural characterization and the genetic tractability of *E. coli* enabled more rapid development of the *E. coli* antigen classification scheme. Owing to their antigenicity in mammals, most CPS structures elicit T lymphocyte-independent immune responses that induce IgM antibody production but fail to stimulate T cell-dependent IgM-IgG switching, an important attribute to ensure long-lasting immunity (Weintraub, 2003; Avci & Kasper, 2010). However, purified CPSs from some of the most commonly isolated strains were determined to be non-immunogenic due to structural identity with human glycans (Edwards *et al.*, 1982; Johnson, 1991; Hérias *et al.*, 1997). Capsule-deficient mutants of these strains generally exhibit decreased virulence, persistence, and serum sensitivity (Pluschke *et al.*, 1983; Hérias *et al.*, 1997). As discussed in greater detail later, antibody generation proved difficult against purified mammal-like bacterial CPSs composed of hyaluronan (HA), heparosan, or certain congeners of unsulfated chondroitin or polysialic acid (PSA).

It should be noted here that there are reports of antibodies generated against these CPSs under unique circumstances (Frosch *et al.*, 1985; Jennings *et al.*, 1985; Kabat *et al.*, 1986; Kröncke *et al.*, 1990; Finke *et al.*, 1991; Troy 1992; Born *et al.*, 1996). However, careful consideration should be given to possible antigenic determinants for antibodies generated in such experiments and whether access to the epitopes would result in protective responses *in vivo*. If serum-accessible portions of these CPSs are identical to mammalian glycans, it seems unlikely that antibodies could be elicited against these “self” epitopes. In some cases, antibodies were raised in autoimmune animal hosts, where self-protection capacity was diminished due to immune dysregulation (Bitter-Suermann *et al.*, 1986). Immune response by healthy animal hosts requires other possible explanations to clarify this paradox:

- The CPS possesses an exposed antigenic determinant not found in the corresponding mammalian glycan, such as a deacetylated amino sugar or terminal unsaturated bond generated by a lyase (a class of enzymes acting to cleave acidic polysaccharides through an eliminase mechanism, in contrast to hydrolyzing glycosidases) (Linhardt *et al.*, 1986) or some other non-self chemical decoration.

- CPS purification exposes a non-mammalian antigenic determinant, like an anchoring moiety composed of a phospholipid or a monosaccharide or oligosaccharide linker constituent not biosynthesized in mammals.
- The antigenic determinant spans a self and non-self domain on the purified CPS, thereby cross-reacting with the self-domain.
- The antibody is not specific for the CPS, but is cross-reacting due to structural similarity to the true antigen.

In one example, a human IgM class antibody reactive against PSA was shown to be non-specific due to the antibody's reactivity with polynucleotides, a scenario where cross-reactivity was enabled possibly due to similar surface charge distributions in these two negatively charged biopolymers (Kabat *et al.*, 1986). An IgG antibody against PSA was also isolated in autoimmune NZB mice (Frosch *et al.*, 1985). Nevertheless, evidence supports the conclusion that non-immunogenic bacterial CPSs sharing structural identity with host glycans confer an additional protective advantage over immunogenic, non-host CPSs.

Due to their poor immunogenicity, mammal-like CPSs were originally considered non-typeable by traditional means. However, "typing" of some non-immunogenic CPSs was later achieved by screening strains against phages only capable of attachment and subsequent infection when a specific CPS is displayed on the bacterial surface, such as *E. coli* K1 and K5-specific phages (Roberts *et al.*, 1986; Scholl *et al.*, 2001). With increasing genetic characterization of CPS-producing strains, polymerase chain reaction and sequencing-based methods such as multi-locus sequence typing (MLST) can now be used for fast and accurate molecular typing (Townsend *et al.*, 2001; O'Hanlon *et al.*, 2004; Durso *et al.*, 2005; Kong *et al.*, 2005; Zhu *et al.*, 2012). Despite the utility of antibody-based serotyping, confounding variables at the immunological level make molecular diagnosis an attractive alternative.

Bacterial glycans

Bacteria produce an array of carbohydrates that are not limited to CPSs, and an understanding of these bacterial glycans is critical for appreciating the role of the CPS in the pathogenesis of infection. As depicted in Fig. 1, Gram-negative bacteria possess an external layer of long CPS chains that are covalently anchored by phospholipids to the outer leaflet of the outer membrane, an asymmetric lipid bilayer with an external layer composed primarily of lipid A (also known as endotoxin) and an internal phospholipid layer. Anchored to the outer membrane by lipid A, the lipopolysaccharide (LPS) serves as a hydrophilic barrier to natural hydrophobic antibiotics and is composed of three regions: (1) lipid A, a highly conserved region that possesses two phosphorylated, β-1,6 linked *N*-acetyl-D-glucosamine (GlcNAc) saccharide residues bearing variable numbers and lengths of fatty acyl chains; (2) the core oligosaccharide, which can be subdivided into the inner and outer cores. The inner core is covalently bound to lipid A and possesses a species-dependent or strain-dependent nonlinear oligosaccharide composed of 3-deoxy-D-mannooctulosonic acids (KDO), heptoses, and some non-glycan components such as pyrophosphoethanolamine (PPEtn). The outer core is linked to the terminal heptose of the inner core and possesses a more variable nonlinear structure of primarily hexose residues; (3) the last region of the LPS

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is known in *E. coli* as the O-antigen due to its distinct antigenicity from the K-antigen. The O-antigen is a repetitive glycan that varies in composition and length between species and strains, but it is typically masked from the environment by the K-antigen. Finally, an oligopeptide-cross-linked lattice of alternating β-1,4 linked GlcNAc and *N*-acetylmuramic acid (MurNAc) residues known as peptidoglycan is constrained within the periplasmic space between the outer and inner (cytoplasmic) membranes. In many species, peptidoglycan sugar moieties are further modified after installation (Vollmer, 2008). Water-soluble β-glucan polymers known as membrane-derived oligosaccharides (MDOs) are found near the inner membrane and are decorated with negatively charged ethanolamine, phosphoglycerol, and succinyl groups. These highly charged MDOs protect the inner cell membrane from low osmotic conditions (Esko *et al.*, 2009). Thus, glycans are critical components of the cell wall that contribute to structural integrity and interaction with the environment (Comstock & Kasper, 2006) (Fig. 2). As Gram-positive bacteria do not possess an outer cell membrane, the peptidoglycan layer is thicker compared to Gram-negative bacteria. Lipoteichoic acids anchor the inner layers of peptidoglycan to a glycolipid extending from cytoplasmic membrane, while wall-associated teichoic acids tether additional outer layers together through covalently linkages to MurNAc residues. CPSs biosynthesized by Gram-positive bacteria can be anchored to the inner membrane, to oligopeptide cross-linkers within peptidoglycan, or to GlcNAc residues in the peptidoglycan lattice (Hanson & Neely, 2012).

In addition to LPS and CPS, many species of Gram-negative and Gram-positive bacteria also biosynthesize and secrete an assortment of high-molecular weight glycopolymers known as exopolysaccharides, which have long been considered determinants of biofilm physicochemical properties (Costerton *et al.*, 1987). Surprisingly, over the last decade exopolysaccharides from certain microbes have also been shown to inhibit biofilm formation by other microbial species (Valle *et al.*, 2006; Kim *et al.*, 2009; Nithya *et al.*, 2010; Bendaoud *et al.*, 2011; Jiang *et al.*, 2011). As an example of the diversity of bacterial glycans, the glycocalyx of a strain of *E. coli* can simultaneously possess six exopolysaccharides. In addition to biosynthesis of covalently bound O-antigen and K-antigen, a linear heteropolysaccharide known as enterobacterial common antigen (ECA) is produced by all members of the *Enterobacteriaceae* family and is also frequently bound to the outer membrane. ECA consists of a conserved $[\rightarrow 3)$-α-Fuc4NAc-$(1\rightarrow 4)$-β-ManNAcA-$(1\rightarrow 4)$-α-GlcNAc-$(1\rightarrow]_n$ repeating trisaccharide unit that can be bound by the reducing end of GlcNAc to the LPS core, anchored to the outer membrane by a phosphate bridge with diacylglycerophosphate, or secreted in a water-soluble, cyclized, and partially 6-*O*-acetylated (on GlcNAc) form with polymerization degree typically between 3 and 6 trisaccharide repeats (Erbel *et al.*, 2003; Fregolino *et al.*, 2012). Other exopolysaccharides, such as colanic acid (known as M-antigen), are also secreted into the environment by many *E. coli* strains. Although only some colanic acid (CA) remains loosely associated around the cell, especially when constrained within a biofilm during suboptimal growth, an *E. coli* strain has been isolated in which CA is ligated to the outer core of the LPS in place of the O-antigen (Meredith *et al.*, 2007). Another exopolysaccharide of *E. coli*, commonly secreted by many other bacteria as well, is the adhesin poly-β-1,6-GlcNAc, a homopolymer that encourages adherence and biofilm formation (Wang *et al.*, 2004, 2005). Finally, *E. coli* and other bacteria also biosynthesize and secrete an exopolysaccharide known as bacterial

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cellulose, or poly-β-1,4-glucan, as a component of the bacterial extracellular matrix (Zogaj *et al.*, 2001). It can be inferred that dynamic regulation of this suite of bacterial extracellular glycans allows sampling of many glycocalyx states to adapt to a wide range of environments (Meredith *et al.*, 2007).

While Gram-positive CPS structures are diverse and can be difficult to characterize, Gram-negative CPS structures are comparatively simple and have thus been more amenable to categorization (Whitfield, 2006). In spite of the depth and breadth of chemical and serological analysis of CPS structures, however, there remains a dearth of evidence regarding linkage of Gram-negative CPS to the cell. A very recent and fascinating report has resolved this longstanding enigma for a class of model *E. coli* and *Neisseria meningitidis* strains, demonstrating that capsules assembled through a common ATP-binding cassette (ABC) transporter pathway are biosynthesized on a nearly conserved lyso-phosphatidylglycerol anchoring moiety through an oligo-KDO linker, presumably guiding the translocation of such CPSs to the cell surface in a CPS- and organism-independent manner (Willis *et al.*, 2013). CPSs in this category are known as Group 2 K-antigens in *E. coli*, but all characterized *N. meningitidis* strains possess a homologous transport system described below. Despite the variety of bacterial glycans, CPSs comprised of non-immunogenic polysaccharides are of primary medical interest due to their conspicuous ability to evade the immune system. Those sharing identity with human polysaccharides have been cataloged in a number of pathogenic species, but the most well characterized CPS structures are found in *E. coli, N. meningitidis, Pasteurella multocida*, and *Streptococcus pyogenes*. It is interesting to compare *P. multocida, Avibacterium paragallinarum*, and *E. coli* since strains of all species have been found to produce chondroitin and heparosan, while certain strains of *P. multocida* also possess HA capsules. Although *P. multocida* and *A. paragallinarum* are predominantly animal pathogens rather than human pathogens, the ability of these microorganisms to produce identical non-immunogenic capsules to *E. coli* through similar yet distinct genetic and enzymatic processes warrants inclusion of the species in this review. Moreover, the diseases caused by these organisms in livestock pose economic threats and cause concern regarding the contribution of antibiotic-laden livestock feed to the spread of antibiotic resistance. Identical PSA capsules are also produced between different species, including meningitis-causing strains of *E. coli* and *N. meningitidis*, while HA is found in the capsules of strep throat and necrotizing fasciitis-causing *S. pyogenes* and *P. multocida*, the etiological agent of fowl cholera and many other mammalian and bird diseases. Bacteria containing these CPSs are compiled in Table 1 and are included in the discussion where relevant.

Molecular mimicry and coevolution

In evaluating the ability of host-like CPSs to evade the immune system, it is important to understand which host tissues contain similar molecules and how pathogens coevolved with humans to enable such mimicry. The first capsule type addressed in this review is known as polysialic acid or PSA, consisting of *N*-acetylneuraminic acid (Neu5Ac) monomers joined with various glycosidic linkages. PSA capsules produced by strains of K1 *E. coli, N. meningitides* serotype B, *Moraxella nonliquefaciens*, and *Mannheimia haemolytica* (previously *Pasteurella haemolytica*) A2 are characterized by α-2,8 glycosidic linkages

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(Adlam *et al.*, 1987; Devi *et al.*, 1991), while PSA produced by other microorganisms possess α-2,9 glycosidic linkages (*N. meningitidis* serotype C strains) or alternating α-2,8 and α-2,9 glycosidic linkages (K92 *E. coli* strains) (Glode *et al.*, 1977; Lifely *et al.*, 1986). In mammals PSA is an α-2,8-linked polysaccharide on neural cell adhesion molecule (NCAM), which is found on the surface of neurons, glial cells, and natural killer cells, a type of lymphocyte functioning as an integral part of the innate or non-specific immune response (Rutishauser, 2008; Chang *et al.*, 2009). Increased surface polysialylation leads to charge repulsion and is associated with decreased NCAM adhesion in animals (Rutishauser *et al.*, 1985) and resistance to phagocytosis by PSA capsular bacteria (King *et al.*, 2007). Structural identity of mammalian PSA with *E. coli* K1 and *N. meningitidis* type B CPSs—particularly embryonic NCAMs, which contain more than 50 α-2,8 Neu5Ac repeating units, compared to a much lower degree of polysialylation in adult NCAMs (Jann & Jann, 1998)—contributes to the neuroinvasiveness of these incredibly virulent neuropathogens (Robbins *et al.*, 1974; Sarff *et al.*, 1975). The PSA capsule is thought to enable traversal of the blood-brain barrier (Kim *et al.*, 2003), thus leading to high rates of morbidity and mortality in neonatal meningitis and serious neurological conditions in survivors of the disease (Kaper *et al.*, 2004).

The other three host-like CPSs discussed in this review are considered glycosaminoglycans (GAGs), or negatively charged, linear polysaccharides identical to the backbones of GAGs found in animals and composed of a repeating core disaccharide unit, comprised of an uronic acid residue linked to an amino sugar (Höök *et al.*, 1984). Although the monomeric sugar precursors constituting these core disaccharide units are conserved in GAGs, the disaccharide units in the animal GAGs heparan sulfate and chondroitin sulfate are not strictly repeating because they are variably sulfated and acetylated within a single chain. The dominant disaccharide unit in the polysaccharide and the distribution of specific disaccharide types, glycosidic linkage configurations, molecular weight, degree of sulfation and acetylation, and in some cases degree of epimerization define the class of GAG and contribute to heterogeneity within each class. GAGs exhibit their numerous biological activities by interacting with proteins including growth factors, chemokines, and adhesion molecules (Capila & Linhardt, 2002; Linhardt & Toida, 2004). The interactions between GAGs and pathogens can also represent the first line of contact between pathogen and host cell and are crucial to a pathogen's invasive potential (Kamhi *et al.*, 2013). Symbolic representations of bacterial CPS structures and related animal glycan structures are illustrated in Fig. 3.

In particular, the CPS produced by K4 *E. coli* and *P. multocida* type F strains are related to CS, a class of sulfated GAG characterized by a [→4) β-D-glucuronic acid (GlcA) (1→3) *N*-acetyl-β-D-galactosamine (GalNAc) (1→]$_n$ disaccharide repeat, where position and extent of sulfation are tissue and organism-dependent (Rodriguez *et al.*, 1988; Volpi, 2007; Volpi *et al.*, 2008). While K4 CPS GlcA residues are substituted with bisecting β-fructofuranose units between C2 of fructose and C3 of GlcA, the fructose is acid-labile, and K4 CPS is thought to exist as an unsubstituted backbone in certain low pH environments (Jann & Jann, 1990). Conversely, *P. multocida* type F CPS is identical to the unsulfated chondroitin precursor of animal CS (DeAngelis *et al.*, 2002). Considering the limited patterns of *O*-sulfo

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group substitution within the disaccharide-repeating unit of animal CS (Sugahara & Mikami, 2007), it is apparent that the order of the sulfonation reactions is important in CS biosynthesis and that *O*-sulfo groups in certain positions can preclude the activity of downstream sulfotransferases (Schiraldi *et al.*, 2010). CS occurs in animals as an *O*-linked glycan chain, covalently bound to serine residues of proteins, through a specific tetrasaccharide linkage, resulting in glycoconjugates known as proteoglycans (Esko, 2009). This class of GAG is found primarily in the extracellular matrix where one or more CS chain is attached to an array of core proteins affording proteoglycans with various structural and regulatory roles. Proteoglycans mediate a myriad of physiological interactions such as cellular recognition, communication, migration, adhesion, and proliferation (Thelin *et al.*, 2013).

The CPS produced by K5 *E. coli* strains such as Bi 8773-41 and the probiotic strain Nissle 1917 (Lodinová-Žaadniková *et al.*, 1992) and also by *P. multocida* type D strains is composed of heparosan (Vann *et al.*, 1981; DeAngelis & White, 2002). Heparosan, comprised of [→4) β-D-GlcA (1→4) α-D-GlcNAc (1→]$_n$ repeating disaccharide units, is identical to the mammalian precursor for heparin and heparan sulfate (Ly *et al.*, 2011) (Fig. 3). Heparan sulfate is typically found on the cell membrane and in the extracellular matrix as a component of proteoglycans (Gallagher, 1989). Heparin, a highly sulfated variant of heparan sulfate, is biosynthesized as an intracellular proteoglycan, serglycin (Li *et al.*, 2012). The GAG heparin is a widely used anticoagulant pharmaceutical (Capila & Linhardt, 2002). Although the natural function of heparin is not well understood, its release from the granules of mast cells is localized to damaged tissue and contributes to wound healing and defense against opportunistic infection (Zehnder & Galli, 1999). The non-template-driven biosynthesis of heparan sulfate and heparin affords a diverse range of disaccharide units from a modest number of biosynthetic enzymes (Fig. 3).

The GAG known as hyaluronan or HA is structurally identical in animals and in capsules of *S. pyogenes* groups A and C, *P. multocida* type A, and some other species of bacteria (DeAngelis, 1999). HA GAG consists of an unmodified [→4) β-D-GlcA (1→3) β-D-GlcNAc (1→]$_n$ disaccharide repeating unit in both animals and bacteria. In animals, this high molecular weight polysaccharide is the predominant GAG in the extracellular matrix and is found in high quantities in skin, connective tissues, cartilage, synovial fluid, and the vitreous humor of the eye (Höök *et al.*, 1984; Dougherty & van de Rijn, 1992).

The role of these host-like, or “self”, capsules enveloping the surfaces of invasive pathogens seems quite clear: molecular mimicry enables such pathogens to evade an immune system that has learned to ignore self molecules based on cell surface interactions. As glycans are ubiquitous on cell surfaces (Gallagher, 1989), they are likely the first molecules contacted by pathogens that utilize cellular adhesion during infection, so pathogens bearing these capsules have an evolutionary advantage. The question that remains is how humans and other animals have evolved to combat these camouflaged pathogens and how pathogens continually evolve to successfully colonize their animal hosts. In a series of papers, Ajit Varki argues that genetic evidence suggests pathogens—which evolve orders of magnitude faster than their hosts due to horizontal gene transfer, high mutation rates, fast growth, and short life spans—develop self-CPSs through convergent evolution, where the

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glycosyltransferase genes responsible for biosynthesizing the glycans in pathogen and host are not homologous in most cases (Gagneux & Varki, 1999). Considering that glycosyltransferases are highly conserved within the host and yet biosynthesize highly diverse glycan structures and distributions in different cell types and tissues, coupled with the combinatorial style of glycan interactions and the ability to maintain functional specificity when a participating glycan is modified, Varki also argues that sexual reproduction-enabled mutations in host glycosyltransferases and subsequent change in glycan profile allow these multicellular organisms to adapt to pathogenic pressure. Futhermore, Varki speculates that the coevolution of pathogens and their hosts has not only tailored the diversity of glycan structures and expression patterns, but that pathogenic pressures stemming from host-like capsules contribute significantly to speciation of multicellular organisms (Varki, 2006).

Immune response and clearance of encapsulated pathogens

Both evasion of complement-mediated killing and failure of being ingested by phagocytic cells enhance the virulence of the CPS. The polysaccharide capsules are effective physical barriers that protect the bacteria from being killed. The fact that bacteria capsules are commonly hydrophilic and negatively charged diminishes their removal through phagocytosis. The hydrophilic nature causes high-level hydration and reduces the surface tension at the phagocyte and bacterium interface (Kuberan & Linhardt, 2000). Additionally, the negatively charged polysaccharides on the bacterial surface repel the negatively charged surface of phagocytes, thus increasing the unfavorable interaction when phagocytosis or complement-mediated lysis occurs (Moxon & Kroll, 1990; Kuberan & Linhardt, 2000). According to van Oss and Gillman, the phagocytic cells such as polymorphonuclear leukocytes (PMNs), monocytes, and macrophages repel the encapsulated bacteria due to the net Lewis AB repulsion between the hydrophilic outer layers (Klainer & Geis, 1975; van Oss *et al.*, 1975), which reduce the surface tension between the phagocytic cell and the bacterium (Moxon & Kroll, 1990). For example, the cell surface of *Staphylococcus aureus* became less hydrophilic after removing the capsule and its phagocytic uptake was enhanced (van Oss *et al.*, 1975). A similar phenomenon was observed with the encapsulated strain of *Salmonella typhimurium*, which resists phagocytosis, but when unencapsulated it is readily phagocytized (Cunningham *et al.*, 1975). Non-effective contact can often lead to the failure of phagocytic engulfment. More intuitive is simple charge-charge repulsion between the negative charge of the CPS and the glycocalyx of the phagocytic cell. The more highly charged the CPS the more likely a bacterium is to avoid opsonophagocytosis (Moxon & Kroll, 1990). Poor phagocytosis of a ‘smooth surface’ may directly result from the physical surface properties instead of biological interaction of capsules with phagocytic signaling and complement-mediated molecules. Direct experimental testing of this hypothesis remains challenging.

The interaction between the CPS of the bacterial surface and the host's complement system is also a key contributor to bacterial virulence. In the early stage of the immune response, the control and defense mechanism of the host are contingent on the classic and alternative complement pathways. The classic pathway is usually initiated by antigen-antibody binding. The C1 complement complex, which is a multi-molecular protease consisting of three

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subunits C1q, C1r and C1s, triggers the classical pathway of complement, first binding to the aggregated antibody molecule, then sequentially cleaving and activating the complement protein C4 and proenzyeme C2 to form a C3 convertase, C2bC4b (Jann & Jann, 1997). This process is regulated by C4-binding protein C4bp (Roberts, 1996) and is usually retarded during the encapsulated bacterial invasion. The C3 convertase then converts C3 to the activated C3b, which will be deposited on the bacterial cell surface. This process is controlled by factors B and H of the alternative pathway (Jann & Jann, 1997). The alternative pathway can be activated in the absence of antibody binding to the bacterial surface and therefore is very important in immunity to encapsulated or unencapsulated bacteria. In other words, the alternative pathway provides a way for the immune system to kill bacteria in the blood in the absence of specific antibodies. The alternative pathway utilizes the serum protein C3b, which is then activated by serum factor B, D and properdin (Moxon & Kroll, 1990), to form convertase C3bBb that amplifies the complement cascade for more C3 conversions and C3b deposition (Roberts, 1996). The activation of C3b results in a ligand targeting specific receptors on PMNs or macrophages. The binding of a C3b opsonized microbe to the complement receptor on PMNs or macrophages initiates phagocytosis and ultimately killing of the encapsulated bacteria. In addition, following C3b deposition, the sequential activations of C5 to C9 forms a membrane attack complex that directly leads to the lysis and death of some Gram-negative bacteria (Moxon & Kroll, 1990; Roberts, 1996).

This bacterial defense mechanism and the subsequent response of complement-mediated bacterial killing by the host can be blocked at numerous sites by CPSs avoiding serum-mediated killing and enhancing virulence. Some capsules protect the bacteria from being attacked by steric mechanisms. Bacteria such as pneumococci promote C3b deposit on the bacterial cell surface underneath the capsule, shielding it from recognition by the phagocytic cell (Winkelstein, 1981). Some bacterial capsules interrupt the binding of C3b to the bacterial surface by affecting regulatory proteins, such as factor B and H (Loos, 1985; Cross, 1990). Capsules that exert such a defense mechanism usually contain *N*-acetylneuraminic acid (Neu5Ac) since it contains a factor H binding site. The stimulation of H-C3b, correspondingly decreases the amplification convertase C3bBb, leading to failure of the complement cascade (Moxon & Kroll, 1990). Some capsules cannot bind to factor B, thus causing more H-C3b formation (Winkelstein, 1981). Strains such as *E. coli* K1, *E. coli* K92, *N. meningitides* types B and C and Group B *Streptococcus* polysaccharides have capsules that inhibit alternative complement activation by these mecahnisms (Stevens *et al.*, 1978; Wessels *et al.*, 1989).

The mimicry of the CPS structure to substances within the host serves as a virulence factor preventing bacteria phagocytosis. A CPS can mimic a similar structure found within the host representing "self", and therefore both avoid recognition as foreign and circumvent triggering the host immune response (Kuberan & Linhardt, 2000). The CPS K1 has the same poly-α-2,8-Neu5Ac (PSA) structure as carbohydrate portion of NCAM, required for organogenesis and neural cell growth (Finne, 1982; Kuberan & Linhardt, 2000). Similarly, the CPS K5 strain of *E. coli* shares the same structure as mammalian heparosan (Navia *et al.*, 1983). An X-ray diffraction study showed that the K4 capsule was poorly immunogenic

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due to its similar helix structure to CS. The removal of fructosyl linkage under low pH environment transforms K4 CPS into non-immunogenic chondroitin (Jann & Jann, 1997).

Capsular polysaccharide transport, genetics, biosynthesis, and role in immune system evasion

The chemical properties and immunogenicity of CPSs are dictated by variations in number, order, and diversity of monosaccharide constituents, anomeric centers (α- or β-), glycosidic linkage positions, absolute configuration (L or D), ring forms (pyranose or furanose), degree of chemical modification (*O*-acetylation, for example), and overall conformation (Mazmanian & Kasper, 2006). There is a wide range of capsule types among bacterial orders and even within a single species. For instance, strains belonging to one of the most well-studied CPS-producing species, *E. coli*, are known to biosynthesize approximately 80 CPS structures. The number of known capsule types increases dramatically when considering other genera, but capsules in other organisms are less well characterized due to limited biochemical studies and relative genetic recalcitrance. Nevertheless, studies in the model capsular species *E. coli* suggest that the capsule assembly pathways are comparatively limited in scope, where a diverse assortment of CPSs are assembled and translocated to the cell surface using identical strategies. Biochemical and genetic evidence in Gram-negative bacteria paints a picture of a veritable orchestra of catalytic enzymes, structural proteins, and transport proteins interacting in a transmembrane complex that spatially and temporally organizes biosynthesis and transport. The modularity of the cooperating sub-complexes allows distinct CPS biosynthetic enzymes, complexed at the inner membrane, to utilize identical transport systems for translocation of disparate CPS. Whitfield and coworkers recently showed an ABC-transporter dependent pathway common to some *E. coli* and *N. meningitidis* strains results in the biosynthesis of unique CPSs on a common anchor structure (Willis *et al.*, 2013). This apparently ensures successful CPS transport and outer membrane attachment. Similarly, another commonly conserved transport system, known as the Wzy-dependent pathway, shares the ability to assemble CPSs with relaxed specificity for CPS structure. Although a wide range of bacteria utilize the ATP-dependent and Wzy-dependent pathways for CPS assembly, the majority of experimental evidence has been acquired in *E. coli.* Homologous genes between species have been identified by sequence similarity in many cases rather than by functional characterization. Hence this section of the review will focus on *E. coli* as a model system and draw comparisons between related bacteria where relevant.

Transport pathways

In *E. coli*, CPS structures have been classified into four groups. Group 1 and 4 CPS structures (as well as colanic acid) are found in enteropathogenic (EPEC), enterotoxigenic (ETEC), and enterohemorrhagic *E. coli* (EHEC) strains and are assembled through what is known as the Wzy-dependent pathway. This pathway is distinct from the so-called ABC-transporter dependent pathway that is responsible for assembly and transport of Group 2 and 3 CPSs and that is described in detail later. While uronic acid sugars are common to Group 1 CPS repeat units, Group 4 CPS repeats are characterized by the presence of acetamido sugars. Despite this apparent structural distinction between Group 1 and 4 CPSs, both are

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polymerized and transported to the cell surface in a similar manner. In the Wzy-dependent system, serotype-specific repeating units are assembled from cytosolic sugar precursors and linked to undecaprenyl diphosphate by glycosyltransferases, unique to the specific type of CPS being synthesized, which are embedded in the cytosplasmic membrane. Individual undecaprenyl diphosphate-linked repeating units are then transferred across the inner membrane to the periplasm by a flippase, Wzx, which also passes the repeat unit to an integral membrane protein known as Wzy. Wzy processively catalyzes addition of these individual Group 1 and 4 CPS repeat units to the reducing end of the growing polysaccharide chain, which elongates in the periplasm without being released by Wzy until chain termination (Yi *et al.*, 2006). Wza, Wzb, and Wzc are responsible for control of chain length and export from the periplasmic face of the inner membrane to the cell surface. In Group 4 strains, longer polysaccharide chains can be incorporated into the LPS structure and effectively anchored by lipid A, although these K-antigens are classified as K_{LPS} to distinguish their unique attachment mechanism (Whitfield, 2006). In Group 1 strains, shorter polysaccharide chains can also form K_{LPS}, but longer chains are known to assemble capsules without covalent attachment to LPS. Although the outer-membrane protein Wzi had been implicated in attachment of Group 1 CPSs (specifically the K30 antigen) to the outer membrane (Rahn *et al.*, 2003), the exact mechanism was unknown until recently. A paradigm shift in understanding CPS attachment resulted from a study that concluded K30 CPS remained associated with the outer surface of the cell due to interactions with an outer-membrane lectin, Wzi, that captures secreted CPS and serves as a nucleation site for further CPS recruitment (Bushell *et al.*, 2013). Wzy-dependent capsules are also biosynthesized in *Klebsiella pneumoniae*, and much of the molecular insight for early steps in this pathway came from studies of *Salmonella enterica* O-antigen assembly. As CPSs in this class do not share identity with animal glycans, they elicit an immune response and are thus out of the scope of this review. The reader is directed to two excellent reviews compiling recent research in this area (Whitfield, 2006; Reid & Cuthbertson, 2012).

Group 2 and 3 *E. coli* CPSs are produced in strains commonly associated with extraintestinal infections (ExPEC), while all known *E. coli* CPS structures sharing identity with animal glycans belong to Group 2. It is also interesting to note that Group 2 and 3 *E. coli* CPSs share certain similar structure and assembly characteristics with strains of *N. meningitidis, P. multocida, Haemophilus influenzae*, and *Campylobacter jejuni* (Whitfield, 2006). In contrast to Group 1 and 4 CPSs, the repeat units of Group 2 and 3 CPSs exhibit extensive variation in structure. Similar to the Wzy-dependent transport system, the ABC-transporter dependent system expressed by Group 2 and 3 *E. coli* strains has relaxed specificity for CPS structure, successfully transporting very distinct structures across the cell wall. A striking difference compared to Wzy-dependent assembly is that Group 2 and 3 CPSs, assembled by ABC-transporter dependent pathways, are completely polymerized in the cytoplasm and then transported across the cell wall to the outside of the cell. CPSs of this class are elongated by processive, CPS-specific glycosyltransferases that are co-localized to the cytoplasmic surface of the inner membrane with other proteins belonging to the coordinated biosynthetic-transport complex. Details regarding polymerization initiation are not fully resolved, but CPSs from *N. meningitidis* group B, *E. coli* K1, and *E. coli* K5 strains (all Group 2 type capsules) were recently shown (Willis *et al.*, 2013) to be linked to a well-

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conserved lyso-phosphatidylglycerol (lyso-PG) terminus by a poly-β-KDO linker. It should be noted that slight variation in fatty acyl chain length and number of KDO repeats was measured within single cultures and between organisms. For instance, the single fatty acyl chain of lyso-PG in most cultures varied between saturated C16 (palmitoyl-PG) or monounsaturated C18 (oleoyl-PG), but one culture produced diacyl-PG with either two C16 chains (dipalmitoyl-PG), two C18 chains (dioleoyl-PG), or one C16 adjacent to a C18 chain (palmitoyl-oleoyl-PG). Furthermore, the number of KDO monomers exhibited inter-strain and intra-strain variation between 5 and 9 KDO repeats. This discovery suggests that the common glycolipid carrier is the anchor by which the ABC-transporter guides Group 2 CPSs from the cytoplasm to the outer membrane. However, the mechanism by which the glycolipid carrier is assembled and attached to the nascent polysaccharide remains undetermined. Comparatively little is known about assembly and transport of Group 3 CPS, but high sequence homology with Group 2 transport machinery suggests that the two groups share a common transport mechanism. Studies on Group 3 strains, none of which are known to produce animal-like glycans, are reviewed elsewhere (Barrett *et al.*, 2002).

Genetics

Genes involved in CPS biosynthesis and transport are typically organized within a so-called capsular gene cluster. Gene products participating in transport are generally more well conserved, while capsule biosynthetic enzymes are specific to capsule type, again suggesting an organization in which CPS transport proteins interface with CPS biosynthetic enzymes in a modular, interchangeable fashion. In fact, episomal expression of CPS biosynthetic enzymes from one Group 2 strain has been shown to lead to functional capsule "transplantation" in another acapsular Group 2 strain, where CPS is secreted by the common transport complex as expected (Zhang *et al.*, 2012). General characteristics of capsular gene clusters include distinct GC-content compared to the rest of the chromosomal DNA, lending additional evidence that these genes were acquired through horizontal transfer. Furthermore, CPS gene clusters are often encoded within regions of the genome known as genomic (also pathogenicity-associated) islands (Sun *et al.*, 2005; Wiles *et al.*, 2008), or segments of the genome prone to horizontal gene transfer that often encode virulence factors (Ostblom *et al.*, 2011). Since the pioneering experiment in which Silver and coworkers cloned and heterologously expressed the *E. coli* K1 capsule—the first study to clone an entire CPS gene cluster—Group 2 K-antigen assembly systems have become the prototype for genetic and biochemical characterization of ABC-transporter dependent pathways (Silver *et al.*, 1981).

The gene cluster encoding biosynthesis and transport of Group 2 capsules, including the capsule types of primary interest in this review, is depicted in Fig. 4a. Regions 1 (*kpsFEDUCS*) and 3 (*kpsMT*) genes are conserved in Group 2 capsular *E. coli* and encode the enzymes and transport proteins responsible for initiation of chain elongation and translocation to the cell surface, while Region 2 genes encode the glycosyltransferases and other enzymes responsible for biosynthesis of the K-antigen-specific CPS. In comparison to Groups 1, 3, and 4, expression of Group 2 CPS is subject to thermoregulation. Promoters upstream of Region 1 and Region 3 are sufficient for transcription of all genes within the CPS cluster at temperatures near the optimum of 37°C, but no Region 1 or 3 transcripts are detectable at temperatures below 20°C (Cieslewicz & Vimr, 1996; Simpson *et al.*, 1996;

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Stevens *et al.*, 1997; Whitfield & Roberts, 1999; Rowe *et al.*, 2000; Xue *et al.*, 2009). Specifically, *kpsMT* of Region 3 encodes the ABC transporter responsible for translocation of the fully synthesized CPS across the inner membrane (Reizer *et al.*, 1992). The transporter consists of multiple protein products, where two units of KpsM function as the inner membrane spanning domain and two units of KpsT serve as the nucleotide-binding domain (Pavelka *et al.*, 1994; Pigeon & Silver, 1994; Steenbergen & Vimr, 2008). Region 3 is organized into a single transcriptional unit such that the start codon of *kpsT* overlaps the stop codon of *kpsM* by two base-pairs, and it has been suggested that the two proteins are translationally coupled to facilitate their interaction at the inner membrane (Smith *et al.*, 1990; Pavelka *et al.*, 1991). Upon binding of ATP, KpsT undergoes a conformational change that is conveyed to KpsM, which then experiences a change in conformation to enable transport of CPS using the energy gained from ATP hydrolysis (Bliss *et al.*, 1996).

Region 1 encodes genes implicated in biosynthesis of the poly-KDO linker, as well as in translocation initiation and transport through the periplasm. The genes *kpsED* encode two proteins that receive the CPS from KpsMT and transport it to the outer membrane (Wunder *et al.*, 1994; Rosenow *et al.*, 1995). Functional deletions of *kpsED* lead to accumulation of CPS in the periplasm, which supports the role of KpsED in CPS translocation (Silver *et al.*, 1988; Bronner *et al.*, 1993; Pazzani *et al.*, 1993). KpsE has been described as an adaptor protein that spans the periplasm to guide CPS from the ABC-transporter toward KpsD, a channel allowing CPS passage through the outer membrane (Rosenow *et al.*, 1995). The gene *kpsF* encodes D-arabinose 5-phosphate isomerase, a homotetramer (Meredith & Woodard, 2006) that interconverts D-ribulose 5-phosphate and D-arabinose 5-phosphate with higher turnover toward D-arabinose 5-phosphate. The adjacent gene *kpsU* encodes CMP-KDO synthetase, a dimer (Jelakovic *et al.*, 1996) that converts D-arabinose 5-phosphate provided by KpsF to nucleotide-activated CMP-KDO (Rosenow *et al.*, 1995). The absolute roles of the cytosolic proteins encoded by *kpsC* and *kpsS* are not entirely elucidated. Group 2 strains with deletions of *kpsC* (*kpsC*) or *kpsS* (*kpsS*) accumulate high molecular weight polysaccharide intracellularly, implicating these two proteins in control of polymer length as well as in translocation initiation (Larue *et al.*, 2011; Willis *et al.*, 2013). The accumulating cytosolic polysaccharide inside *kpsC* and *kpsS* K1 strains was recently found to be non-lipidated (Willis *et al.*, 2013) despite conflicting past reports (Frosch & Müller, 1993; Tzeng *et al.*, 2005), suggesting that either KpsC or KpsS might catalyze the transfer of CPS to the glycolipid anchor. Given that most genes required for CPS biosynthesis and transport are typically located within the CPS biosynthetic gene cluster, it is also possible that either KpsC or KpsS is a β-KDO-polymerase catalyzing the biosynthesis of the poly-β-KDO-linker (Willis *et al.*, 2013). Further studies are required to determine the roles played by KpsC and KpsS in biosynthesis and translocation initiation.

Common to Region 2 are genes encoding the glycosyltransferases required for assembly of the K-antigen-specific CPS structure. Glycosyltransferases in Group 2 *E. coli* are processive and catalyze the addition of high-energy nucleotide-activated sugar monomers to the nonreducing end of the growing CPS. It should be noted that identical CPSs in disparate bacteria, such as *P. multocida*, are biosynthesized by non-processive enzymes (DeAngelis *et al.*, 2003). Studies have shown that Group 2 glycosyltransferases colocalize to the

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cytoplasmic side of the inner membrane with other proteins from the CPS gene cluster and that the proteins form a hierarchical transenvelope hetero-oligomeric complex to efficiently couple CPS assembly and transport (Rigg *et al.*, 1998). Also often encoded in Region 2 with the glycosyltransferases are enzymes that biosynthesize CPS precursors but that do not actively participate in chain elongation (Cimini *et al.*, 2012). In certain instances, enzymes within the CPS biosynthetic gene cluster are predicted to duplicate the function of enzymes encoded elsewhere in the chromosome (Muñoz *et al.*, 1998). However, sequence divergence between the copies suggests that there could be an advantage conferred by the extra copy. Spatial co-localization of such duplicated enzymes with the CPS biosynthetic complex might ensure higher local concentrations of CPS precursors. Finally, CPS clusters possess genes with unknown functions that do not appear necessary for CPS production (Krahulec *et al.*, 2005), while other encoded proteins lacking detectable catalytic activity have been shown to associate with the biosynthetic complex and increase biosynthetic productivity, possibly by lending structural integrity to the biosynthetic complex or by fostering protein-protein interactions (Hodson *et al.*, 2000).

CPS biosynthesis in *N. meningitidis* is not as well characterized as in the more genetically tractable microbe, *E. coli.* However, the genomes of representative strains from all known serogroups have been sequenced, and an ABC-transporter dependent capsule assembly pathway with homology to Group 2 and 3 *E. coli* strains is conserved among all *N. meningitidis* capsular strains (Harrison *et al.*, 2013). Unique to each serogroup, of course, are CPS biosynthetic enzymes for serotype-specific polysaccharide production. Despite the homology of many CPS transport genes between *N. meningitidis* and *E. coli*, the two distinct CPS loci exhibit limited synteny. Six regions known as A-D, D′, and E exist within the CPS gene locus of *N. meningitidis*, occurring in the order D-A-C-E-D′-B (Fig. 4b). CPS-specific biosynthetic genes are encoded within Region A, and CPS transport proteins are encoded within Regions C and B. Protein sequence alignments have been used to identify CPS transport proteins in *N. meningitidis*, and a new gene nomenclature has recently been proposed to ensure consistent descriptions between strains, where the names of all *N. meningitidis* CPS transport genes begin with "ctr" to denote capsule transport (Harrison *et al.*, 2013). The four genes encoding the transmembrane complex *ctrA, ctrB, ctrC*, and *ctrD* are adjacent to each other within Region C of *N. meningitidis*, which contrasts the organization of the *E. coli* homologs *kpsD, kpsE, kpsM*, and *kpsT*, respectively. It seems intuitive that the proteins required for CPS translocation across the cell wall, including the ABC transporter proteins (KpsM/CtrC and KpsT/CtrD), the periplasm spanning adaptor protein (KpsE/CtrA), and the outer membrane protein (KpsD/CtrB), would be encoded within a single operon as in *N. meningitidis*. However, it is likely that ancient genomic rearrangements have led to the separation of *kpsED* and *kpsMT* between two independent transcripts in *E. coli. N. meningitidis* genes *ctrE* (formerly *lipA*) and *ctrF* (formerly *lipB*) are encoded in Region B and are homologs of *E. coli* genes *kpsC* and *kpsS*, respectively. Despite the recent demonstration that *kpsC* and *kpsS E. coli* K1 strains accumulate non-lipidated CPS (Willis *et al.*, 2013), mutations in *N. meningitidis* genes *ctrE* and *ctrF* have been shown to lead to intracellular accumulation of lipidated CPS (Tzeng *et al.*, 2005). This disparity could represent a slight variation between species in an otherwise highly similar transport system, where lipidation and translocation initiation events are decoupled in *N. meningitidis*

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but intertwined in *E. coli.* Further research differentiating between these steps and assigning biochemical functions to CtrE and CtrF will help resolve this discrepancy. Region D and D′ encode duplicates of genes required for biosynthesis of *N. meningitidis* LPS (Hammerschmidt *et al.*, 1994), while the function of Region E is unknown.

P. multocida also harbors a CPS gene locus that shares significant homology with Group 2 *E. coli* and *N. meningitidis* CPS gene loci (Fig. 4c). The topology of *P. multocida* type A, D, and F gene clusters more closely resembles that of *E. coli*, where a central CPS-specific region is flanked by two regions, Region 1 and 3, coding for translocation proteins. Specifically, Region 1 encodes the transport genes *hexA, hexB, hexC*, and *hexD* that are homologous to *E. coli kpsT, kpsM, kpsE*, and *kpsD*, respectively, while Region 3 encodes *phyA* and *phyB*, homologs to *kpsC* and *kpsS* genes predicted to lipidate CPS and initiate translocation (Chung *et al.*, 1998). The genes in both regions are highly conserved among members of all five serogroups (A, B, D, E, and F), but *P. multocida* type B and E CPS loci exhibit slight rearrangements in gene order (Boyce *et al.*, 2010). Region 3 gene *lipA* (homologous to *phyA*) is instead located between Regions 1 and 2, whereas *lipB* (homologous to *phyB*) maintains synteny with other serogroups due to its preserved location on the opposite side of Region 2 (Boyce *et al.*, 2000a). For serogroups A and B, Region 1 genes *hexA-D* are known as *cexA-D*. CPS-specific biosynthesis in all serogroups is guided by the variable enzymes encoded in Region 2, including the synthases responsible for CPS polymerization. Experimental validation of the putative functions of most *P. multocida* Region 1 and 3 proteins is lacking, but a *hexA* mutant strain was acapsular, supporting the role of HexA in CPS transport through the cell wall (Chung *et al.*, 2001).

Biosynthesis

Several model *E. coli* strains possessing K1, K4, and K5 capsules have been sequenced, and their amino/nucleotide sugar metabolism is well conserved with only slight genetic and metabolic differences (Chen *et al.*, 2006; Shuting Lu *et al.*, 2011; Cress *et al.*, 2013a, b, c). The conserved biosynthetic steps for these *E. coli* CPSs are representative of many bacteria and are shown alongside major competing metabolic pathways in Fig. 5, with CPS intermediates boxed in black. Two activated UDP-sugar intermediates are required for biosynthesis of the GAG-like CPSs, while only one of these two precursors is required for biosynthesis of PSA, a non-GAG CPS. The cytosolic reactions constituting these two intermediate pathways act as sinks on upper glycolysis and can be considered as two distinct modules, represented by the two branches in Fig. 5.

In the first module, glucose-6-phosphate (G6P) is converted to UDP-glucose by sequential action of two enzymes, while UDP-glucose is further converted to UDP-GlcA (the immediate GAG precursor) in GAG-producing K4 and K5 strains. Phosphoglucomutase (encoded by *pgm*) isomerizes G6P to glucose-1-phosphate (G1P), which is then converted by the UTP:G1P uridylyltransferase *galU* to UDP-glucose through transfer of a uridylyl group from UTP, releasing a pyrophosphate (PP_i). The second module consists of four enzymes catalyzing the formation of UDP-*N*-acetylglucosamine (UDP-GlcNAc) from fructose-6-phosphate (F6P). F6P-amidotransferase encoded by *glmS* transfers an amine group from glutamine to F6P to form glucosamine-6-phosphate, which is further isomerized

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to glucosamine-1-phosphate by phosphoglucosamine mutase (*glmM*). An acetyl group is then transferred to glucosamine-1-phosphate to form *N*-acetylglucosamine-1-phosphate, a reaction catalyzed by glucosamine-1-phosphate *N*-acetyltransferase (*glmU*). The gene *glmU* encodes a bifunctional enzyme that subsequently transfers an uridylyl group from UTP to *N*-acetylglucosamine-1-phosphate, forming UDP-GlcNAc and releasing PP_i. Specific biosynthesis of CPS from these intermediates and transport out of the cell in these model strains will be described in greater detail within. It is important to note here that these CPSs have been found in other species (Table 1), and it is expected that the rapid increase in microbial genome sequencing projects will continue to reveal disparate bacteria sharing related capsular gene loci. Since HA capsules are not known to exist in *E. coli*, the genetic and biosynthetic description will be presented later.

Polysialic acid

PSA is not a GAG, but like GAGs it is an acidic, linear polysaccharide found in vertebrate tissues. PSA is composed of repeating sialic acid (Neu5Ac) monomers, where the glycosidic linkage configuration is organism-dependent and commonly found as either α-2,8 or α-2,9 linkages or a combination of the two. In *E. coli* K1, *N. meningitidis* serogroup B, *M. nonliquefaciens*, and *M. haemolytica* A2, PSA possesses the mammalian-like [→8) Neu5Ac $(2\rightarrow]_n$ structure seen in Fig. 3. Other strains possess immunogenic PSA capsules due to the non-animal glycosidic linkages. For instance, sialic acid units in *E. coli* K92 CPS are α-2,9 linked, and in *N. meningitidis* serogroup C are alternating α-2,8 and α-2,9 linked.

Polysialic acid biosynthesis

E. coli strains expressing K1 CPS share Region 1 and 3 of the CPS gene cluster with other Group 2 capsular bacteria (Fig. 4a) (Roberts, 1996). Region 2 consists of 6 genes specific for biosynthesis of K1 PSA. As depicted in Fig. 6, the first committed step of PSA biosynthesis is catalyzed by UDP-GlcNAc 2-epimerase (encoded by *neuC*), which epimerizes UDP-GlcNAc to *N*-acetylmannosamine (ManNAc) (Vann *et al.*, 2004). NeuNAc synthase (*neuB*) then catalyzes the condensation of ManNAc and phosphoenolpyruvate (PEP) to NeuNAc and inorganic phosphate (P_i), where three carbons from PEP extend the monosaccharide from six to nine carbons (Annunziato *et al.*, 1995). CMP-NeuNAc cytidyltransferase (*neuA*) utilizes a single molecule of CTP to activate NeuNAc with the transfer of CMP, thereby releasing pyrophosphate (PP_i) (Silver *et al.*, 1988). The processive sialic acid polymerase, polysialyltransferase, encoded by *neuS* sequentially adds NeuNAc to the nonreducing end of the nascent PSA chain (Silver *et al.*, 1988). Although it has been suggested that *neuD* encodes a Neu5Ac *O*-acetyltransferase due to the presence of a hexapeptide repeat motif characteristic of an acyltransferase superfamily (Vimr & Steenbergen, 2006), this possibility is unlikely since K1 CPS is not *O*-acetylated in many strains encoding *neuD*. Another study demonstrated heterodimerization between NeuD and NeuB, suggesting that NeuD plays a stabilization role during chain elongation (Daines & Silver, 2000). The exact function of NeuE is unknown, and further efforts will be required to understand its role in polymerization initiation (Reid & Cuthbertson, 2012).

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Many K1 CPS strains have been found to possess PSA that has been *O*-acetylated at the C7 or C9 hydroxyl group (Orskov *et al.*, 1979). These are chemical modifications that increase resistance to desiccation and reduce biofilm formation, while coincidentally increasing immunogenicity (Mordhorst *et al.*, 2009). However, the *O*-acetylation is a dynamic phenotype that varies within a population and appears to be controlled by a stochastic "on-off" switching mechanism at the genetic level (King *et al.*, 2007). It was determined that this phase variation (also known as form variation) only occurs in K1 strains that have been lysogenized by a K1-specific lambdoid bacteriophage and thus possess a chromosomal accretion element known as CUS-3, a remnant of the infection. The *neuO* gene, encoding the K1 *O*-acetyltransferase responsible for the chemical modification of PSA, is encoded within CUS-3. The 5′ end of *neuO* is subject to slip strand DNA mispairing in which a 7 nucleotide repeat sequence is gained or lost at the 5′ end, leading to a frameshift and corrupted translation. By this mechanism, individuals in a CUS-3-harboring K1 population randomly partition between acetylation-on and acetylation-off variants, a phenomenon that presumably confers a population-level evolutionary advantage where the likelihood of persistence increases in adverse environmental pressures (Deszo *et al.*, 2005). Incredibly, the *O*-acetyltransferase catalytic efficiency has also been shown to increase linearly with the number of tandem, in-frame repeats that manifest as tandem heptapeptide repeats and form a disordered N-terminal domain (Schulz *et al.*, 2011). The function of the disordered region remains unknown. PSA purified from K1 strains lacking the CUS-3 region was invariably lacking *O*-acetylation (Deszo *et al.*, 2005).

N. meningitidis serogroup B CPS is identical to K1 CPS, although the genes encoding the biosynthetic enzymes are organized differently and share only 30-40% amino acid sequence identity. *N. meningitidis* serogroup B Region A encodes *cssABC* (formerly *synABC* or *siaABC*), *csb* (formerly *synD* or *siaD*), and *ctrG* (formerly *NMB0065*) (Fig. 4b). The *cssA* gene encodes an UDP-GlcNAc 2-epimerase that shares 32% identity with NeuC from K1 (Murkin *et al.*, 2004), while *cssB* codes for a CMP-Neu5Ac cytidylyltransferase with 34% identity to K1 NeuA (Edwards & Frosch, 1992; Ganguli *et al.*, 1994). The *cssC* gene shares 37% identity with its K1 homolog NeuB, a Neu5Ac synthase (Vimr *et al.*, 1989; Ganguli *et al.*, 1994). The *csb* gene codes for a polysialyltransferase with 33% identity to K1 NeuS (Frosch *et al.*, 1991). Finally, the gene products of *ctrG* and K1 *NeuE* share 27% identity. The role of CtrG in polymerization initiation is not entirely elucidated, but is appears to play a similar role as NeuE in coupling CPS biosynthesis with traversal through the cell wall (Hobb *et al.*, 2010). Thus, the pathways biosynthesizing polysialic acid in these two Gram-negative organisms are metabolically and functionally identical. Furthermore, protein homology demonstrates high functional conservation of the ATP-dependent transport pathways. Biosynthesis of α-2,8-PSA capsules in other species, including *M. haemolytica* A2 (Adlam *et al.*, 1987) and *M. nonliquefaciens* (Devi *et al.*, 1991), has not been studied in depth.

Polysialic acid in evasion of immune system

Compared to other CPSs, K1 *E. coli* strains are particularly non-immunogenic due to the fact that the structure of CPS mimics the substance in the host (Brooks *et al.*, 1980). The chemical structure of K1 polysaccharide is identical to the PSA on the embryonic form of

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NCAM, which is related to the organization of the neural tissue (Finne *et al.*, 1983). Therefore, they are relatively more virulent because the immune response towards K1 is usually non-existent due to the mistaken recognition of the encapsulated bacteria as “self”, letting them pass protective barriers. Interestingly, this only applies to a certain host age range. The K1 organism's carriage rates are 22-42% among infant and children without sex differentiation while the highest among women aged 16-31 years old (Sarff *et al.*, 1975). Some studies also indicate that K1 strains are poor activators for initiating the alternative complement pathway of immune response (Bortolussi *et al.*, 1979). The anti-complementary effect is due to PSA's increasing the binding of inhibitor B1H to C3b, preventing formation of C3 convertase and blocking activation of the complement cascade (Harber *et al.*, 1986; Leying *et al.*, 1990). The failure to accumulate C3b on the cell surface effectively prevents phagocytosis and, thus, enhances the virulence of *E. coli* K1 strain.

E. coli K1 strains frequently cause infections of the urinary tract (Johnson, 1991), which according to Wiles, is one of the most common sites associated with human disease (Wiles *et al.*, 2008). Moreover, K1 is also mainly responsible for causing acute pyelonephritis (Kaijser, 1973) since they can be easily found among the bacterial strains isolated from clinical specimens during acute pyelonephritis (Hanson *et al.*, 1977). In addition, the K1 antigen is also found on strains of the extraintestinal pathogenic *E. coli* (ExPEC) (Wiles *et al.*, 2008). K1 *E. coli* are generally thought to be the second most common cause of human neonatal meningitis (next to group B streptococci) and approximately 80% of American and European strains implicated in the disease have PSA capsules (Orskov & Orskov, 1992). Some studies have also suggested that bacterial survival within brain microvascular endothelial cells was enhanced by K1 CPS (Pluschke *et al.*, 1983; Kim *et al.*, 2003; Scholl *et al.*, 2005).

PSA is also the CPS of two serogroups of *N. meningitidis*, a common causative agent of meningitis in children and adults. Early work on the CPS of *N. meningitidis* serogroup B found that mutants deficient in capsule production lost all pathogenicity in mice (Masson *et al.*, 1982). Several studies have demonstrated that the CPS aids in the resistance of the bacterial cells to the innate immune system (Jarvis & Vedros, 1987; Spinosa *et al.*, 2007). Similar to other CPSs, the PSA capsule of *N. meningitidis* has been found to hinder adhesion and invasion (Spinosa *et al.*, 2007), and it is unable to activate the complement pathway (Jarvis & Vedros, 1987). This is most likely a result of the capsule masking immunogenic adhesins and invasins on the surface of the bacterial cell. However, it has also been shown that the CPS is vital for the survival of the bacterium in the bloodstream (Jarvis & Vedros, 1987) and important for survival inside human cells (Spinosa *et al.*, 2007). In the bloodstream, the CPS allows *N. meningitidis* to evade uptake and degradation by macrophages (Jarvis & Vedros, 1987), while intracellularly the encapsulated bacterial cells are resistant to antimicrobial peptides, which act by binding bacterial membranes and increasing their permeability (Spinosa *et al.*, 2007).

Several studies have shown the ability to successfully produce antibodies protective against *N. meningitidis* serogroup B by immunization with a vaccine containing *N*-propionyl and de-*N*-acetylated sialic acid derivatives (Pon *et al.*, 1997; Granoff *et al.*, 1998; Moe *et al.*, 2009). Importantly, the antibodies were shown to be unreactive to human PSA. More specifically,

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vaccines containing de-*N*-acetylated sialic acid derivatives were shown to possess the ability to protect against *N. meningitidis* in multiple ways, including complement-dependent bactericidal activity and passive protection in infant mice (Moe *et al.*, 2009). Because the vaccines show protection against *N. meningitidis* serogroup B, but not purified human PSA, it is possible that some amount of de-*N*-acetylated sialic acid is present in the CPS of *N. meningitidis* serogroup B. It has been proposed that de-*N*-acetylated sialic acid elicits a T-cell dependent immune response due to its zwitterionic nature, characterized by the presence of both positively-charged free amino groups at de-*N*-acetylated positions and negatively charged carboxyl groups along the polymer backbone (Moe *et al.*, 2009). In contrast to the more common negatively charged or neutral CPSs, zwitterionic CPSs are known to be T-dependent antigens, which are bound, processed, and presented by major histocompatibility complex class II (MHCII) to stimulate helper T-cells through what is known as the MHCII endocytic pathway (Cobb *et al.*, 2004; Surana & Kasper, 2012). These remarkable findings suggest that enzymatic de-*N*-acetylation of other acidic CPSs such as heparosan, chondroitin, and hyaluronan could represent a strategy for eliciting natural immune response to pathogenic infection.

Chondroitin

CPS produced by strains of K4 *E. coli* and *P. multocida* type F is structurally related to the GAG CS, which is involved in a range of molecular interactions in humans as previously described. As loss of CS from cartilage in humans leads to osteoarthritis, nutritional supplementation is a common treatment strategy, making CS a pharmaceutically and nutraceutically valuable product (Wildi *et al.*, 2011). CS is currently harvested from animal sources (cow, pig, shark, fish, and bird cartilage) (Huskisson, 2008), but there is a growing interest in moving to sustainable microbial production platforms to minimize contamination and to control product consistency. One such production strategy involves harvesting a CS precursor from cultures of *E. coli* strains biosynthesizing a K4 capsule (Zanfardino *et al.*, 2010; Restaino *et al.*, 2011, 2012; Schiraldi *et al.*, 2011, 2012; Trilli *et al.*, 2012). This biotechnological relevance has provoked increased interest in improving biosynthesis of K4 CPS by manipulating *E. coli* metabolism (Restaino *et al.*, 2012; Cimini *et al.*, 2013; Cress *et al.*, 2013b; Wu *et al.*, 2013). K4 CPS is similar in structure to unsulfated CS, with the exception of an acid-labile, bisecting β-fructofuranose attached to C3 of the GlcA residue (Fig. 3). Alternatively, the *P. multocida* type F CPS is a linear polysaccharide identical to unsulfated chondroitin (DeAngelis *et al.*, 2002). Commercial interest in this GAG has increased knowledge regarding its biosynthesis in microbes.

Chondroitin biosynthesis

E. coli strains expressing the K4 capsule share Regions 1 and 3 of the Group 2 CPS biosynthetic cluster, but the biosynthetic enzymes unique to K4 CPS are encoded by Region 2 and presumably form a biosynthetic complex at the cytosolic side of the inner cell membrane (Fig. 4a). Biosynthesis of K4 CPS precursors can be segmented into two distinct modules drawing from upper glycolysis through the intracellular pool of UDP-glucose and UDP-GlcNAc (Fig. 7). In the first module, UDP-glucose is converted to UDP-GlcA by UDP-glucose dehydrogenase (UGDH) encoded by *kfoF* and associated with the K4 CPS

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biosynthetic enzyme complex (Ninomiya *et al.*, 2002). Although it is not uncommon for multiple copies of UDP-glucose dehydrogenase to exist in *E. coli* genomes, the existence of two other copies in the genome of the model K4 strain U1-41 (Cress *et al.*, 2013b) suggests that *kfoF* has evolved to perform a distinct physiological role. Since UDP-glucose is a key metabolite in many pathways, it is plausible that the association of the *kfoF*-encoded copy of UDP-glucose dehydrogenase with the capsular biosynthetic enzyme complex serves to spatially constrain the chemical reaction—conversion of UDP-glucose to UDP-GlcA near the K4 CPS glycosyltransferase on the inner cell membrane would increase the local concentration of UDP-GlcA and effectively channel valuable UDP-glucose toward production of K4 CPS without significant loss to other cellular reactions. In the second module, UDP-GlcNAc 4-epimerase encoded by *kfoA* catalyzes the formation of UDP-GalNAc (Ninomiya *et al.*, 2002). The two activated sugar precursors from each module are sequentially added to the nonreducing end of the growing polysaccharide chain by chondroitin polymerase (encoded by *kfoC*), a bifunctional glycosyltransferase catalyzing the transfer of both GlcA and GalNAc and release of two molecules of UDP per disaccharide extension (Ninomiya *et al.*, 2002). The crystal structure of KfoC has been obtained in the presence of UDP-activated precursors, indicating the existence of two active sites for addition of UDP-GalNAc by the N-terminal domain and UDP-GlcA by the C-terminal domain (Osawa *et al.*, 2009).

Several studies have suggested that K4 chondroitin is fructosylated subsequent to polymerization of the backbone (Lidholt & Fjelstad, 1997), but the enzyme responsible for fructosylation has remained unreported until recently. Initial searches for the fructosyltransferase focused on proteins in Region 2 of the K4 biosynthetic gene cluster that were predicted to possess glycosyltransferase motifs. One such enzyme encoded by *kfoG* possessed a putative glycosyltransferase domain; however, disruption/deletion of the gene did not prevent fructosylation (Krahulec *et al.*, 2005). A series of recent patents purport that deletion of *kfoE* results in production of unfructosylated chondroitin, suggesting that *kfoE* encodes a fructosyltransferase (Trilli *et al.*, 2012). The enzyme has not been characterized in its purified form, however, and biochemical characterization will be required to assign fructosyltransferase activity to *kfoE*. Several genes in the K4 CPS cluster, including *kfoB, kfoD*, and *kfoG*, have unknown function. However, *kfoB* shares 38% identity with a gene in *E. coli* K5 (*kfiB*) that is believed to be structurally important in K5 CPS biosynthesis. This might suggest a stabilizing role for KfoB that is critical for assembling or colocalizing biosynthetic enzymes near the transenvelope CPS assembly complex, but further work will be required to determine the precise functions of these proteins.

The unfructosylated, unsulfated chondroitin backbone constituting the *P. multocida* type F CPS is biosynthesized by genes in Region 2 of the *P. multocida* CPS locus. The region consists of four genes, *fcbB, fcbC, fcbD*, and *fcbE*, only one of which has been characterized. The chondroitin synthase known as PmCS is encoded by *fcbD* and is homologous to *E.coli* K4 *kfoC* (DeAngelis *et al.*, 2002). PmCS is also a bifunctional polymerase possessing two distinct glycosyltransferase domains that sequentially add GlcA and GalNAc residues to the growing polysaccharide chain (DeAngelis & Padgett-McCue, 2000). Similar to KfoC, the N-terminal domain of PmCS possesses GalNAc-transferase activity, and the C-terminus

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possesses GlcA-transferase activity (Osawa *et al.*, 2009; Otto *et al.*, 2012). While deletion of *kfoE* in *E. coli* K4 has been reported to abrogate chondroitin fructosylation, it would be interesting to determine if heterologous expression of *kfoE* in *P. multocida* type F complements expression of fructosylated CPS. *P. multocida* genes *fcbB, fcbC*, and *fcbE* are homlogous to *E. coli* K4 genes *kfoG, kfoF*, and *kfoB*, respectively, but the biological functions have not been studied (Townsend *et al.*, 2001).

Chondroitin in evasion of immune system

The K4 antigen is not implicated in human disease as frequently as K1 and K5 antigens, but K4 strains have been associated with human and animal infection. K4 *E. coli* strain U1-41 is a uropathogen (Rodriguez *et al.*, 1988), and the K4 capsule has been found on strains causing diarrhea in humans (Orskov *et al.*, 1985). EHEC K4 strains have also been isolated in calves (Moxley & Francis, 1986; Stordeur *et al.*, 2000). The unfructosylated chondroitin backbone is identical to the mammalian CS precursor, but unlike the K1 and K5 antigens, the K4 antigen host-related bacterial polymer is substituted with an additional sugar residue not present in the mature, sulfated GAG. The β-linked fructose on K4 CPS is an antigenic determinant that imparts immunogenicity to the *E. coli* K4 capsule and is responsible for a conformational change resulting in significantly higher viscosity compared to defructosylated K4 CPS (Rodriguez *et al.*, 1988). Therefore, K4 CPS may be easily recognized by anti-K4 antibodies and induce a complement dependent immune response. However, the fructosyl group is labile and can be easily removed in mild acidic conditions. The conversion of K4 antigen to a non-fructosylated chondroitin results in the non-immunogenicity of K4 CPS (Rodriguez *et al.*, 1988; Jann & Jann, 1997). Thus, it has been suggested that such lability enables dynamic physiological conditions in the host, such as low pH in certain host tissues, cells, or compartments (Jann & Jann, 1997), to modulate the presence of this immunodominant residue on the K4 capsule. A K4 strain also might benefit outside the host from a more viscous capsule and inside the host from a less viscous, non-immunogenic capsule.

The chondroitin CPS of *P. multocida* type F is non-immunogenic since it lacks the fructose residue present in *E. coli* K4 CPS and is thus identical to the animal precursor to CS. It has been shown that treatment of *P. multocida* type F with chondroitinase, a lyase that is known to cleave unsulfated chondroitin, results in increased phagocytosis by swine neutrophils (Rimler *et al.*, 1995), presumably through elimination of the capsule.

Heparosan

Pharmaceutical grade heparin has traditionally been derived from porcine or bovine mucosal tissues, but a contamination crisis leading to several hundred deaths worldwide and causing severe allergic reactions in many patients prompted exploration of microbial heparosan production followed by enzymatic conversion to bioengineered heparin with purity and biological activity meeting United States Pharmacopeia (USP) standards (Wang *et al.*, 2011). *E. coli* K5 strains have been shown to serve as a viable production platform due to the similarity between K5 CPS and the mammalian precursor for heparin (Wang *et al.*, 2010, 2011; Ly *et al.*, 2011). Recent work has also demonstrated the ability of an engineered

heparosan-producing *E. coli* BL21 strain to naturally secrete the polysaccharide (Zhang *et al.*, 2012), presumably utilizing transport proteins encoded by Region 1 and 3 genes from its endogenous Group 2 capsular export system (Andreishcheva & Vann, 2006). This work demonstrated the feasibility of producing heparosan in well-characterized production strains, albeit at much lower concentrations than wild-type K5 strains. Efforts in this area have contributed to the understanding of heparosan biosynthesis.

Heparosan biosynthesis

Similar to K4 CPS, the biosynthesis of K5 CPS can be partitioned into two modules each producing the requisite activated sugar precursor for heparosan polymerization. The monomeric constituents of K5 CPS are GlcA and GlcNAc, which draw carbon from G6P and F6P in the form of two activated sugar precursors, UDP-GlcA and UDP-GlcNAc, respectively. UDP-GlcA biosynthesis in K5 is identical to that in K4 with the exception of the UDP-glucose dehydrogenase associated with the capsular biosynthetic complex. Conversion of UDP-glucose to UDP-GlcA in the first module is catalyzed by an UDP-glucose dehydrogenase encoded by *kfiD* from Region 2 in the K5 capsule gene cluster (Fig. 4a) (Sieberth *et al.*, 1995). The *kfiD* gene encodes the third copy of UDP-glucose dehydrogenase in the genome of the model strain Bi 8337-41 (Cress *et al.*, 2013c) and the probiotic strain Nissle 1917 (Cress *et al.*, 2013a), and biochemical studies provide evidence for its association with the K5 CPS biosynthetic complex also comprised of proteins encoded by *kfiA, kfiB*, and *kfiC* (Rigg *et al.*, 1998). While *kfiB* is thought to perform a membrane-localization and complex-stabilizing or scaffolding role rather than a catalytic one (Hodson *et al.*, 2000; Zhang *et al.*, 2012), *kfiA* and *kfiC* are glycosyltransferases that alternatively elongate the heparosan chain at the nonreducing end (Petit *et al.*, 1995). The final reaction of the first module is catalyzed by *kfiC*-encoded UDP-GlcA glucuronosyltransferase, which attaches a GlcA residue to the nascent K5 polysaccharide and releases UDP. The second module in K5 CPS biosynthesis encompasses the reactions converting F6P to UDP-GlcNAc as shown in Fig. 8, and the final reaction involves transfer of GlcNAc from UDP-GlcNAc to the growing polysaccharide by *kfiA*-encoded UDP-GlcNAc *N*-acetylglucosaminyltransferase, a step that frees an additional UDP.

Relatively little is known about most of the proteins encoded in Region 2 of *P. multocida* type D (*dcbB, dcbC, dcbE*). An exception is the well-studied heparosan synthase PmHS1 encoded by *dcbF*, also known as *hssA* (Kane *et al.*, 2006). This bifunctional synthase possesses two fused glycosyltransferase domains, where a segment of the N-terminal β-1,4-glucuronosyltransferase domain is homologous to *E. coli* KfiC, and a section of the C-terminal α-1,3-*N*-acetylglucosaminyltransferase domain is homologous to KfiA (Kane *et al.*, 2006; Otto *et al.*, 2012). Drawing analogy with other CPS biosynthetic gene clusters, the putative UDP-glucose dehydrogenase encoded by *dcbC* likely supplies UDP-GlcA to PmHS1 for chain elongation. Finally *dcbE* encodes a homolog of *E. coli kfiB*, and *dcbB* is a putative glycosyltransferase sharing homology with *kfoG*. Surprisingly another heparosan synthase (PmHS2) with similar glycosyltransferase domain organization and ~70% identity to PmHS1 is located outside of the CPS gene locus in the GAG-producing *P. multocida* type A, D, and F strains (DeAngelis & White, 2004). The biological role of PmHS2 has not been characterized *in vivo*, but it has been speculated that PmHS2 and the HA and chondroitin

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synthases in type A and F strains, respectively, might be differentially regulated by environmental conditions to allow variation in the type of GAG displayed on the cell surface (DeAngelis & White, 2004). Owing to their broad substrate specificities compared to PmHS1, purified PmHS2 and KfiA have been used to synthesize novel unnatural polysaccharides from analogs of native UDP-sugar donors, and PmHS2 has also been used to prepare heparosan for the chemoenzymatic production of heparin (Liu *et al.*, 2010; Li *et al.*, 2013).

Heparosan in evasion of immune system

The structure of *E. coli* K5 CPS is identical to that of heparosan, which is the first polymeric intermediate during the biosynthesis of heparin in the host (Navia *et al.*, 1983). In addition, the mature heparin chain also contains some unsulfated and unepimerized [→4) β-D-GlcA (1→4) α-D-GlcNAc (1→]$_n$ repeats identical to the K5 disaccharide (Vann *et al.*, 1981). Thus, the mimicry of the K5 CPS structure makes it non-immunogenic. Since traditional CPS typing relied on antigenicity, the capsule of *E. coli* K5 was originally classified as K-non-typeable (Jann & Jann, 1987). In part due to the difficulty of identifying the K5 antigen and the frequency or extent in different infections, more effective methods of typing were developed, including one by Gupta *et al.* that utilized a specific phage to type K5 in various infections (Kaijser & Jodal, 1984).

The capsules of *E. coli* K5 strains are most commonly found in urinary tract infections (UTI) (Sandberg *et al.*, 1988) such as pyelonephritis, cystitis, and asymptomatic bacteriuria. Although K5 CPS has been found to be a common *E. coli* capsule antigen in UTI infections, it is also prevalent in strains causing sepsis (Kaijser & Jodal, 1984). In one of the studies using specific K5 phage typing, K5 was found in the 17.1% strains in the case of sepsis followed by 12.4% strains in the case of asymptomatic bacteriuria (Kaijser & Jodal, 1984). Moreover, the K5 capsule has also been shown to promote the persistence of *E. coli* in the rat large intestinal microflora (Hérias *et al.*, 1997). The same study indicated that the enhanced intestinal colonization of K5 encapsulated strains allowed higher cell densities to be reached and secondarily led to increased translocation through the intestinal mucosa to mesenteric lymph nodes. Translocation is thought to be a normal physiological process regulating immunity to gut bacteria (Wells *et al.*, 1988), but it can also reach the blood stream causing sepsis and meningitis (Lambert-Zechovsky *et al.*, 1992). In the tissues, the capsule might further protect the organisms from phagocytosis and complement-mediated killing (Hérias *et al.*, 1997).

The heparosan CPS of the animal pathogen *P. multocida* type D has also been demonstrated to be an important virulence factor for the organism. Acapsular variants of toxigenic *P. multocida* type D lose their virulence in a murine model, and lesions caused by acapsular strains are less severe than their encapsulated counterpart (Jacques *et al.*, 1993).

Hyaluronan

The unsulfated GAG known as HA exists in a wide-range of mammalian tissues and as the primary constituent of the CPS of several microbes, including the well-studied bacteria *S. pyogenes* type A and C and *P. multocida* type A (other species possessing HA capsules are

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listed in Table 1). Although the monosaccharide constituents of this acidic polysaccharide are identical to heparosan, the glycosidic linkages are distinct and lead to a slightly different repeating disaccharide structure. HA is noted as one of the most hygroscopic molecules to exist in nature, and it is estimated that hydrated HA contains a 1000-fold mass of water compared to its own weight (Laurent & Fraser, 1992). The high biocompatibility of HA stems from its natural presence in many mammalian tissues, and it is thus utilized in cosmetic and medical applications (Cimini *et al.*, 2012), including tissue engineering (Allison & Grande-Allen, 2006). HA has also long been known as a regulator of cancer progression, and a high-molecular-mass HA unique to naked mole rats was recently shown to mediate resistance to cancer, an unusual but famous property contributing to the species' extraordinary longevity (Tian *et al.*, 2013). Thus, commercial HA production is appealing for its use in a wide range of applications. Industrial preparation of HA was originally achieved by extraction of HA from animal sources such as rooster combs, but there is interest in moving towards more sustainable production practices such as microbial fermentation (Boeriu *et al.*, 2013). Since the existence of a bacterial HA synthase was first documented in pathogenic group A *S. pyogenes* (DeAngelis *et al.*, 1993a, b), HA production has been evaluated in nonpathogenic *Streptococci* and recombinant strains (Yu & Stephanopoulos, 2008; Yu *et al.*, 2008; Liu *et al.*, 2011), and now microbial HA has been fully commercialized (DeAngelis, 2012).

Hyaluronan biosynthesis

Microbial production of HA has primarily been studied in streptococcal species and *P. multocida* type A. Although not all enzymes catalyzing biosynthetic steps toward HA have been definitively proven, biosynthetic models have been proposed based on known CPS genes in other organisms (Liu *et al.*, 2011; Cimini *et al.*, 2012; Boeriu *et al.*, 2013). HA and heparosan share the same precursors (UDP-GlcA and UDP-GlcNAc), so the metabolic models for these CPSs are likely analogous. The similarity of early biosynthetic steps for nucleotide activated sugar precursors across a wide range of bacteria also supports the assertion that biosynthetic steps are conserved between heparosan and HA strains. Since UDP-glucose and UDP-GlcNAc are important building blocks for many microbial glycans and other glycoconjugates, it would be surprising to see significant inter-species variation in the anabolism of these critical components. The obvious distinction between heparosan and HA biosynthetic pathways are the glycosyltransferases that polymerize the distinct CPSs. HA synthases from *Streptococci* and *P. multocida* are bifunctional enzymes that alternatively add GlcA and GlcNAc to the nascent polymer (DeAngelis *et al.*, 1993b; Kumari & Weigel, 1997; DeAngelis *et al.*, 1998; Ward *et al.*, 2001). As opposed to heparosan synthases that form alternating α- and β-linkages between monosaccharides, however, HA synthases only form β-linkages. A striking feature of HA synthases from different species is that they polymerize in opposite directions; Class I HA synthases of *Streptococci* catalyze monosaccharide addition at the reducing end of the chain, while Class II HA synthases in *P. multocida* elongate the chain by addition to the non-reducing end (DeAngelis, 2012).

As discussed in previous sections, Regions 1 and 3 of the *P. multocida* CPS gene cluster are presumably responsible for CPS transport through an ABC-transporter dependent pathway.

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Genes *hyaB, hyaC, hyaD*, and *hyaE* in Region 2 of the *P. multocida* type A CPS locus are involved in HA biosynthesis (Chung *et al.*, 1998). Putative functions have been ascribed to *P. multocida hyaB, hyaC*, and *hyaE* based upon their homology to *kfoG, kfiD/kfoF*, and *kfiB/kfoB*, respectively, where HyaC is an UDP-glucose dehydrogenase providing UDP-GlcA to HA synthase, and HyaE could serve as a scaffold or structural component. In fact, a *P. multocida* type A *hyaE* mutant was shown to be acapsular, suggesting that HyaE is critical for proper translocation of the HA CPS (Crouch *et al.*, 2012). In another study, an acapsular *P. multocida* type A mutant was generated by deletion of *hyaB*, which implicates HyaB as another key protein in CPS translocation (Steen *et al.*, 2010). The *hyaD* gene encodes the HA synthase PmHAS, an enzyme surprisingly sharing high identity with the chondroitin synthase PmCS from *P. multocida* type F (DeAngelis *et al.*, 1998). Like most other GAG synthases, PmHAS possesses two independent glycosyltransferase domains that functions in a non-processive manner. Specifically, the N-terminus of PmHAS possesses a GlcNAc-transferase domain, while the C-terminus possesses a GlcA-transferase domain (Jing & DeAngelis, 2000; DeAngelis *et al.*, 2003).

Details regarding HA assembly in Gram-positive *Streptococci* are lacking as well, but the HA biosynthetic operon has been cloned from four species, including *Streptococcus pyogenes, Streptococcus uberis, Streptococcus equisimilis*, and *Streptococcus zooepidemicus* subsp. *equi.* This operon possesses *hasA* (HA synthase) and *hasB* (UDP-glucose dehydrogenase; homologous to *hyaC* in *P. multocida* type A) in all four species (Crater & van de Rijn, 1995). In all but *S. uberis, hasC* (UTP-glucose-1-P uridylyltransferase) is encoded downstream of *hasB.* In *S. uberis, hasC* is located elsewhere in the genome (Ward *et al.*, 2001). The CPS operon in *S. equisimilis* and *S. zooepidemicus* also possesses *hasD*, a gene encoding the bifunctional glucosamine-1-P *N*-acetyltransferase/UDP-GlcNAc-1-P uridylyltransferase with homology to *glmU* from Gram-negative bacteria. Finally, *S. zooepidemicus* subsp. *equi* has an additional gene in the operon known as *hasE*, which encodes phophoglucoisomerase (Widner *et al.*, 2005). Phosphoglucoisomerase is essential for many normal cellular processes, but with respect to CPS biogenesis, it could be important for balancing the intracellular abundance of the two UDP-sugar precursors to HA (Chen *et al.*, 2009; Prasad *et al.*, 2010).

Genes encoded in the CPS operons of different species might lead to more subtle variations in HA biogenesis through the expression of dedicated enzymes that increase the availability of UDP-sugar precursors to meet the demands of CPS production. It is noteworthy that the presence of such supplementary enzymes is inconsistent between species producing identical CPS, as exemplified by the extra copy of HasD in the CPS locus of *S. equisimilis* and *S. zooepidemicus* compared to *S. uberis* and *S. pyogenes* (Blank *et al.*, 2008). A possible explanation is that dedicated biosynthetic enzymes might be required in the metabolic background of one species to ensure sufficient intermediate metabolite availability, where, in a different species, sufficient precursor concentration exists without these dedicated enzymes. It cannot be discounted that the genetic difference is random; however, maintenance of function of these duplicate, CPS-dedicated enzymes suggests that they confer an evolutionary advantage.

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Hyaluronan in evasion of immune system

P. multocida is an animal pathogen, mainly in avian, bovine, and swine hosts, but can be transmitted to humans through animal bites, particularly from cats and dogs. Multiple studies have been performed that demonstrate the prevalence of the differing serotypes, based on the different CPSs utilized, of *P. multocida* in healthy and diseased organisms. In one study, 289 strains that were isolated from a variety of both healthy and ill animals (including bovine, small ruminants, buffalo, swine, rabbits, dogs, cats, and poultry) were serotyped based on PCR detection of the capsular biosynthesis genes *capA, B, D, E*, and *F*. The study found that the HA capsule of type A *P. multocida* was the most common amongst the isolates, followed by type D (Ewers *et al.*, 2006). A similar study investigated the capsules found in porcine pneumonia and atrophic rhinitis samples, and likewise found that A was the most common, followed by D (Davies, 2003). A 2012 study used multiplex PCR to determine the capsular genotype of isolates from 121 animals in Malaysia, and found that the capsular genotype was specific for infections in different hosts, with capsular type A predominantly found in avian, rabbit, and porcine samples, capsular type B found mostly in cattle and buffalo samples, capsular type D found mainly in goat samples, and type F capsules found only in a cattle sample (Mohamad *et al.*, 2012). While the organism is most often associated with disease in chickens and pigs, isolates have more recently been studied from chimpanzees and humans. In a study of isolates from the lungs of wild chimpanzees affected by an outbreak of respiratory disease in 2004, researchers found that all of the isolates had the biosynthesis genes required for the production of a HA capsule (Köndgen *et al.*, 2011). Another study, this time on 143 isolates of *Pasteurella* from humans, again found that type A was the most common serotype identified, and it was predominantly isolated from respiratory tissue, while isolates identified as type B, D, and F were found more commonly than type A in soft tissue infections such as bite wounds (Donnio *et al.*, 2004).

The capsule of *P. multocida* serotype A is composed of the GAG HA. Several studies have shown that this capsule plays an important role in both virulence and evasion of the host immune system. Studies on the capsule's role in virulence of *P. multocida* have shown that the HA capsule enhances virulence, and that inhibiting the production of the capsule attenuates the pathogenicity. For instance, one group showed that PBA930, a mutant *P. multocida* type A strain deficient in capsule export, was attenuated in mice when compared to the wild type strain, and that lethality was restored to wild type levels when the strain was complemented with a plasmid encoding the genes responsible for export (Chung *et al.*, 2001). Another such study showed that invasion of acapsular *P. multocida* mutant cells into chicken embryo fibroblast cells was decreased 12 to 16-fold when compared to the wild type of the same strain (Al-Haj Ali *et al.*, 2004). The same study also showed that encapsulated strains were more adhesive to the chicken embryo fibroblasts than the acapsular mutants. Interestingly, treatment with hyaluronidase did not affect invasion or adhesion, while trypsin and periodic acid treatments both inhibited invasion and adhesion only in encapsulated strains, indicating that HA may be playing a role in masking the receptors responsible for adhesion and invasion by these cells. Moreover, another study showed that the thickness of the capsule seems to also play a role in the virulence of *P. multocida* infections in chickens,

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with increasing capsule thickness correlating positively with virulence, with thinly-capsulated strains being attenuated even at high doses (Borrathybay *et al.*, 2003a).

Several studies have attempted to understand how the HA capsule of *P*. multocida type A aids the bacteria in evading the immune system of its host. Studies have shown that this capsule aids in evading multiple faucets of the immune system, including phagocytic uptake, phagocytic killing, and the complement system. One such study found that a mutant strain of *P. multocida* lacking the genes required for capsular export was sensitive to killing in 90% chicken serum, while encapsulated strains and complemented mutant strains were not (Chung *et al.*, 2001). Interestingly, the group had previously determined that the capsule of serotype B *P. multocida*, which has a non-GAG capsule, composed of mannose, galactose, and arabinose, did not infer resistance to complement in chicken serum (Boyce *et al.*, 2000b). This seems to indicate an important role in capsules composed of GAGs to infer resistance to complement in serum.

In addition to resistance against complement activity in serum, studies have also indicated that the HA capsule plays a role in making the bacteria resistant to phagocytosis by macrophages. A 2004 study showed that immunization of mice with crude capsular extract (serotype A) did not induce the production of antibodies against HA, but did induce antibodies against a 39 kDa protein that is only present in encapsulated strains of *P. multocida.* The study also used immunoelectron microscopy to show that the 39 kDa protein was localized at the capsule, and that mice that were immunized with the antibodies against this protein were protected from at least 2 serotype A strains of *P. multocida* (Al-haj Ali *et al.*, 2004). This illustrates that one function of the capsule in promoting virulence is to mask surface antigens on the bacterial cell from the host immune system. Another study examined the capsule's role in virulence by comparing phagocytic uptake of *P. multocida* cells with and without capsules and found that encapsulated strains were resistant to phagocytosis, and that this resistance was lowered when the capsules were removed enzymatically with hyaluronidase (Poermadjaja & Frost, 2000). Another study found that encapsulated strains of *P. multocida* type A were less hydrophobic than acapsular strains, which could be responsible for inhibiting interactions between the cells and hydrophobic components on the outer surface of macrophages, and also found that treatment with hyaluronidase or mechanical shearing of the bacterial cells significantly reduced the surface charge of the cells, which could also play a role in interactions with macrophages or other immune system components (Watt *et al.*, 2003). Together, the studies on the influence of the HA capsule of *P. multocida* serotype A indicate that capsule enhances virulence by evading the host immune system in a number of ways.

S. pyogenes, also commonly known as Group A *Streptococcus* (GAS), is the causative agent of human streptococcal pharyngitis, rheumatic fever, and soft tissue infections including necrotizing fascitis. The capsule of *S. pyogenes* is also composed of HA. Many studies have been performed on GAS in order to elicit the importance and role of the HA capsule in virulence. As in *P. multocida*, the capsule of GAS has been shown to enhance virulence. An interesting 2004 study showed that incidence of mucoid (encapsulated) GAS isolates correlated temporally with incidence of rheumatic fever (Veasy *et al.*, 2004). One group used mutations in the CsrRS regulation system to show that increased capsule production

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correlated with increased virulence (Engleberg *et al.*, 2001). Another study on GAS looked to determine the extent to which the HA capsule contributed to virulence compared to another important virulence factor in GAS, the M protein. Similar to the capsule of *P. multocida* type A, this study found that the capsule of GAS played an important role in resistance to phagocytosis in serum (Fillit *et al.*, 1986). Another study showed similar results, finding that encapsulated GAS was able to grow in human blood, while acapsular GAS was not, and that encapsulated GAS was resistant to phagocytosis, while acapsular was not. The same study found that the loss of the HA capsule resulted in a 100-fold loss of virulence in mice (Wessels *et al.*, 1991).

In contrast to the immune-resistance roles in *P. multocida*, the HA capsule in GAS has been implicated in additional roles in virulence. One study using transmission electron microscopy found that the binding of the GAS capsule to skin epithelial CD-44 receptors induced cytoskeletal rearrangements that allowed the bacterial cells to invade (Cywes & Wessels, 2001). However, an earlier study found that the HA capsule of GAS did not correlate with invasion into skin cells, but did find that the non-encapsulated GAS was significantly less virulent when it invaded skin cells, causing fewer and less severe lesions (Schrager *et al.*, 1996). Another study, aiming to look at the role of both the HA capsule and the M protein in a baboon model, found that acapsular GAS persisted half as long in the throat as encapsulated GAS, and also indicated a role for the capsule in enhancing microbial resistance to antibody-mediated phagocytic killing (Ashbaugh *et al.*, 2000). The studies on the importance of the HA capsule in the virulence of Group A *Streptococcus* have shown that its role is very similar to that of the HA capsule of *P. multocida.* Its primary role seems to be to protect the bacterial cell from host immune response, but it also has additional roles in invasion of host cells.

Concluding remarks and future perspectives

Owing to the diversity and abundance of distinct microbial virulence factors and their multifunctional, synergistic properties, strictly decoupling the role of CPSs in pathogenicity from other contributing factors is a daunting and risky task. The wealth of research devoted to bacterial capsules has nevertheless expanded our understanding of the mechanisms utilized by pathogens to persist in host tissues and cavities without provoking severe immune responses. In light of the concerning trend of pathogenic strains evolving and acquiring drug-resistance, however, the scientific community should endeavor to identify vulnerabilities in this first line of defense for capsular pathogens and prioritize studies aimed at reducing virulence through capsule manipulation or interference with capsular biosynthesis. Given the nature of this review, we believe that a degree of speculation about possible studies and therapeutic strategies is warranted in this perspectives section.

Understanding virulence, pathogenicity, and protective roles of capsules

Further studies will help to better understand the role of the capsule in the pathogenicity of bacteria, as exemplified by the potential directions outlined in this section. For instance, the presence or absence of the capsule clearly impacts bacterial virulence, but little is known about the effect of intermediate degrees of encapsulation. Although CPS structure is likely

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the dominant virulence determinant, the mass of CPS per cell, CPS chain length, capsule density, and capsule thickness might be important contributing factors to persistence against host immune systems. A thick capsule of high-molecular weight, non-immunogenic polysaccharide might be expected to serve as a more efficacious shield of cell surface components than a tenuous coating composed of an identical polysaccharide of shorter chain length. Indeed, it should be noted that strains of the same capsular type will express different quantities or molecular weight of CPS (Hickey *et al.*, 2013), and it is clear that within many pathogenic bacterial species, the more capsule expressed the more virulent they are (Lee *et al.*, 1991; Luong & Lee, 2002). Since there are other genetic differences between such wild-type strains, however, specifically implicating quantitative capsule expression as a virulence factor has not been straightforward. Although studying the impact of variable microbial capsule "coverage" in animal infection models is a difficult prospect, the relationship between degree of coverage and pathogenicity is worth investigating because it could support or obviate the design of drugs that facilitate clearance of encapsulated pathogens through partial or complete removal of non-immunogenic CPSs. If even minimal capsule coverage is sufficient to inhibit immune response, then a more appropriate drug design strategy might target early stages of CPS biosynthesis to preclude capsule formation entirely.

The increasing prevalence of synthetic biology tools could help resolve some intriguing questions about immune response with respect to varying levels of encapsulation. Genetic mutations in past CPS virulence studies have consistently been static, where the presence or absence of capsule is set prior to inoculation by deleting or heterologously expressing capsule biosynthesis genes. One can envision engineering virulent wild-type strains with dynamic transcriptional regulatory circuits capable of modulating CPS production in response to environmental cues (Khalil & Collins, 2010; Chang *et al.*, 2012a), or even in an inducer-free, oscillatory manner that takes advantage of mutually repressible genes (Elowitz & Leibler, 2000; Danino *et al.*, 2010). Similarly, common metabolic engineering strategies such as overexpressing capsule biosynthesis genes or downregulating and deleting genes in competing metabolic pathways could be implemented to create strains producing different quantities of CPS (Yu & Stephanopoulos, 2008; Yu *et al.*, 2008; Zhang *et al.*, 2012). After inoculating model animals with these engineered strains, it is conceivable that the effect on immunogenicity of transient variation in CPS coverage could be monitored to understand critical coverage levels and to measure rates of capsule assembly and degradation *in vivo.* In any such studies, it will likely be important to consider that differential capsule production might not correlate with capsule coverage. For instance, if it can be assumed that total mass of CPS attached to the outside of a bacterium is intrinsically limited by surface area of the outer leaflet or number of available attachment sites, then where would excess CPS localize after this saturation point is reached? In strains engineered to overexpress capsular biosynthetic machinery, it might be expected that excess CPS would accumulate extracellularly without leading to a corresponding augmentation of capsule thickness or density and without affecting immunogenicity. Alternatively, excess CPS might remain loosely associated with the cell through noncovalent interactions with the capsule, a scenario that could lead to capsule thickening and attenuated host immune response. Related questions that remain to be fully resolved are the following: can bacteria sense change in the

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quantity of CPS attached to the outer membrane, and can such information actuate a change in CPS production levels? Alterations in bacterial metabolism upon capsule perturbation could also be examined using metabolomic, genomic, and proteomic studies to elucidate native capsule regulatory mechanisms and other unknown players in capsule assembly.

In contrast to modulating CPS production *in vivo*, an alternative method for studying the relationship between capsule coverage and immunogenicity would be to subject capsular bacteria to CPS-specific lyases or glycosidases that would remove the capsule post-colonization. For instance, since carbohydrate-cleaving enzymes have been shown to degrade bacterial capsules *in vitro* (Rimler *et al.*, 1995), it is conceivable that an infected animal could be treated with these purified enzymes to strip the invading pathogen of its capsule. Alternatively, pathogenic microbes could be engineered to secrete lyases or glycosidases to degrade their own CPS *in situ*, effectively exposing cell surface antigenic determinants to the host immune system on command from an external signal. In a related example, the wild-type *E. coli* K5 strain naturally produces and secretes a heparosan lyase that depolymerizes its own capsule (Legoux *et al.*, 1996). Engineering the secretion of enzymes that are not naturally secreted can be challenging, but recent successes in this area suggest that diverse types of recombinant proteins can be engineered for secretion in Gram-negative bacteria like *E. coli* or Gram-positive bacteria like *Bacillus subtilis* or other bacilli by fusing secretion peptide tags to one end of the protein. Interested readers are directed to comprehensive reviews on the subject (Simonen & Palva, 1993; Tjalsma *et al.*, 2004; Mergulhao *et al.*, 2005). Prior to such studies, however, an additional question that should be addressed is whether treatment with a lyase alters the immunogenicity of CPS. The lyase from K5-specific coliphage creates an unnatural double bond in the external-facing GlcA residue through a β-elimination mechanism (Hänfling *et al.*, 1996), which could presumably serve as an antigenic "flag" on the outer surface of the glycocalyx; in contrast, treatment of K1 capsule with a sialidase (the cognate polysialic acid glycosidase, or carbohydrate hydrolyzing enzyme) would preserve the natural polysaccharide terminus without introduction of an unsaturated bond. Thus, it is important to consider unintended immunogenic consequences of the class of depolymerizing enzyme utilized. To decouple the role of CPS structure from other virulence factors, similar inducible circuits could be designed to switch from expression of wild-type CPS machinery to expression of biosynthetic enzymes for production of a different CPS structure, a scenario in which the wild-type capsule would be progressively supplanted by a capsule with dissimilar physical properties and antigens. In this manner, the confounding variables stemming from different virulence factors between strains could be minimized when studying immune response against distinct CPS structures.

Another valuable approach would be to develop genome-scale metabolic reconstructions of capsular bacteria and study, *in silico*, environmental or media conditions leading to differential CPS production level. Constraints-based metabolic modeling techniques such as flux balance analysis (Orth *et al.*, 2010) and metabolite essentiality analysis (MEA) (Kim *et al.*, 2007) excel at identifying non-intuitive strategies for modulating bacterial metabolism toward a specific phenotype and have already been used to study pathogenic bacteria and predict validated antibiotics (Shen *et al.*, 2010). By individually constraining production of

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all metabolites to zero, one can probe the capacity of a metabolic network to sustain a cellular phenotype (such as maintaining biomass production/flux, the objective function of the optimization algorithm) when certain compounds are removed from metabolism. Metabolites that are found to be essential for the phenotype of interest can be derivatized to compete with the natural metabolite and hinder phenotype manifestation. In this manner, a novel antibiotic was rationally designed and shown to out-perform an existing therapeutic for *Vibrio vulnificus* infection (Kim *et al.*, 2011). MEA could be applied in a similar manner to genome-scale models of capsular pathogens while using CPS production as the objective function. As minimal subnetworks required to sustain the capsule are discovered in simulations, gene deletions could be used to validate predictions *in vivo*. Analogs of essential CPS metabolites could then be screened for CPS inhibition.

Although countless experiments could be contemplated, the implication is clear: there is still much to learn about the role of capsules in microbial pathogenesis, and creative strategies capitalizing on new genetic and computational tools should be devised to probe the limits of the immune system in detecting and clearing exposed pathogens and to predict molecules capable of interfering with capsule biosynthesis and transport. Exploration of the gap between outright killing of pathogenic bacteria and simply exposing them to the immune system could lead to development of therapeutic strategies for treating infection by multi-drug resistant strains, and it could also minimize society's contribution to the alarming spread of antibiotic resistance.

Potential therapeutic approaches

Therapeutic strategies that replace or supplement antibiotic treatment by capitalizing on capsule susceptibility will likely depend upon the type of CPS expressed by the invading pathogen, thus development of rapid and inexpensive diagnostic tools to guide treatment options will be a critical component of any therapeutic strategy. Serological characterization of surface antigens is hindered by the inability to generate, with high specificity, antibodies against important CPS structures that are identical to human glycans. Although MLST analyses and PCR based techniques guided by conserved capsule flanking regions are the current gold standard for molecular typing, these methods are limited due to the requirement of equipment that is not ubiquitous. Furthermore, molecular typing could lead to false positives due to expectations of CPS production that might not manifest due to the presence of mutations or additional CPS modifying enzymes located outside of the assayed gene loci. Rapid CPS diagnostics should be cheap, deployable to any location in the world, and should exhibit very high specificity for the target molecule to limit false positives.

One technology that satisfies these constraints is known as a molecular beacon, or a nucleic acid aptamer that has been engineered to emit a fluorometric or colorimetric signal upon binding of target molecules (Raj & van Oudenaarden, 2009). Aptamers are nucleic acid oligomers that have been selected from large libraries of random sequences due to their affinity and selectivity for a target molecule (Voigt, 2006). Highly specific aptamer affinity to a target molecule is easily attained by performing negative selection screens against structurally related molecules to remove cross-reacting oligonucleotides from the candidate aptamer pool. Aptamers with dissociation constants in the low nanomolar range have been

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generated against carbohydrates (Sun *et al.*, 2010), and even recently against the PSA from the capsule of *E. coli* K1 (Cho *et al.*, 2013). Target binding is associated with conformation change in the oligonucleotide; hence molecular beacons can be readily designed with covalently-bound fluorophore and quencher molecules that remain in close proximity in the unbound state to mask fluorescence but separate enough to unveil the fluorescent moiety when bound to the target (Tombelli *et al.*, 2005). Addition of molecular beacon to a sample containing the cognate CPS would enable fluorescence but would require a high intensity light source for excitation and detection. Alternatively, aptamer sequences have been fused with ribozymes to, upon binding of target molecule, actuate production of a colored compound by reaction with other reagents in solution (Vinkenborg *et al.*, 2011; Tang *et al.*, 2012). Diagnosis of CPS structure with molecular beacons would be rapid, cheap, and easily performed by any healthcare worker.

Subsequent to diagnosis of the predominant CPSs, targeted therapeutic strategies can be implemented. One strategy for treatment of intestinal infections might involve ingestion of a probiotic strain that has been previously characterized to exclude or inhibit pathogens (Fig. 9a) (Lebeer *et al.*, 2010). The term "probiotics" describes microorganisms that confer health benefits to the host, particularly with respect to human health (Marco *et al.*, 2006). For instance, the probiotic strain *E. coli* Nissle 1917 has been shown to outcompete the encapsulated uropathogenic *E. coli* (UPEC) strain CFT073 in several growth conditions, including growth in urine (Hancock *et al.*, 2010), and it exhibits bactericidal activity against many other pathogenic microbes as well (Storm *et al.*, 2011). Prophylactic administration of Nissle 1917 in a porcine model was even shown to abolish secretory diarrhea upon challenge with an ETEC strain (Schroeder *et al.*, 2006). In addition to the ability of Nissle 1917 to outcompete pathogens, it has been shown in another experiment using a porcine model to persist in the gut for up to a month after inoculation (Barth *et al.*, 2009). Nissle 1917 is of particular interest in this review because of its non-immunogenic heparosan capsule, which might confer an advantage over other probiotics in outcompeting extraintestinal pathogens in, for example, the urinary tract. Furthermore, the ease of genetic manipulation of *E. coli* compared to other bacteria could make Nissle 1917 a model probiotic target for more complicated engineering strategies. The most common probiotics in use today are, however, members of the *Lactobacillus* and *Bifidobacterium* generas, which are utilized primarily in the GI tract. In addition, previous work has demonstrated the feasibility of engineering probiotic bacteria for vaccine delivery, as exemplified by a study in which nasal administration of *Lactococcus lactis*, recombinantly expressing pneumonococcal protective protein on its surface, induced protection against *Streptococcus pneumoniae* infection in young mice (Vintiñi *et al.*, 2010). A related but speculative therapeutic strategy would be engineering probiotic strains to detect pathogenic signals and secrete pathogen-specific CPS-depolymerizing enzymes, effectively presenting the surface of the pathogen to the host immune system in a strategy that might complement any other positive effects already conferred by the probiotic strain (Fig. 9c). This strategy would require engineering a sensor capable of controlling expression and secretion of a CPS-degrading enzyme. One candidate is known as a riboregulator, an RNA sequence capable of binding a ligand through an aptamer domain, which induces a conformational change sequestering an antisense domain and allowing expression of the cognate mRNA.

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Riboregulators have been used to control translation of proteins in response to various ligands as discussed elsewhere (Khalil & Collins 2010); thus, it is expected that incorporation of an RNA aptamer domain capable of binding a specific CPS could be incorporated into a riboregulator to control expression of a secretion-tagged, CPS-depolymerizing lyase or glycosidase. An alternative to engineering enzyme-secreting probiotic strains would be to simply purify recombinantly expressed CPS-cleaving enzymes from fermentations of genetically tractable, secretion-capable production strains and to administer the purified enzyme to the patient. In one study illustrating the potential of this strategy, intraperitoneal injection of an anti-K1 glycosidase (endosialidase E from a bacteriophage) to rat pups with previously induced blood-borne *E. coli* K1 infection significantly reduced mortality and also showed prophylactic efficacy (Mushtaq *et al.*, 2004). The glycosidase treatment was shown not reduce the viability of the pathogen, but rather to expose the bacteria to complement-mediated killing; bacteremia typically subsided within 24 h post injection and prevented death in most cases. Many studies have also demonstrated the promise of alginate lyase therapeutics for degrading *Pseudomonas aeruginosa* biofilms in the airways of cystic fibrosis patients, including a recent investigation of alginate lyase-PEG conjugates for degradation of *P. aeruginosa* biofilms on abiotic surfaces (Lamppa *et al.*, 2011).

Phage therapy is another approach that should be considered for its potential to combat pathogenic bacteria possessing non-immunogenic capsules (Fig. 9d). Interest in the use of bacteriophages, or viruses that infect bacteria, declined in the West after the advent of antibiotics, but the alarming trend of drug-resistant bacterial infections has provoked reconsideration throughout the last decade of phage therapy as a strategy to replace or potentiate antibiotic treatment. Despite the decreasing investigation of phage therapy in Europe and the Americas after the commercialization of antibiotics, much work devoted to this topic was continued in states of the former Soviet Union and Poland throughout the twentieth century, including the use of bacteriophage to treat human diseases (O'Flaherty *et al.*, 2009). An excellent review details the successful application of phage therapy for a wide range of infections and addresses the safety of phages from a medical standpoint (Sulakvelidze *et al.*, 2001). A practical example of phage treatment includes the U.S. Food and Drug Administration's approval in 2006 of the use of a phage cocktail to prevent adulteration of ready-to-eat meat products by *Listeria monocytogenes* (Shuren, 2006). Furthermore, promising recent work has demonstrated the ability to engineer bacteriophages like T7 to degrade infectious biofilms (Azeredo & Sutherland, 2008) by expressing CPS-specific (Scholl *et al.*, 2005) or EPS-specific (Lu & Collins, 2007) depolymerizing enzymes on their surface or tail spike, or by expressing these enzymes intracellularly during infection for subsequent release and targeting of neighboring bacterial cells after lysis. One strategy that phages have naturally evolved is the ability to “dig” for cell surface receptors that have been masked by cell surface biomolecules, such as CPS, by depolymerizing the coating. This is exemplified by K1 and K5 specific coliphages (Stummeyer *et al.*, 2004; Thompson *et al.*, 2010) that express evolved tail spike proteins capable of CPS-specific degradation. To date, various bacteriophages have been shown to possess CPS-degrading enzymes specific for heparosan (Thompson *et al.*, 2010), polysialic acid (Stummeyer *et al.*, 2004), and hyaluronan (Baker *et al.*, 2002), but no bacteriophages are known to possess chondroitin

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lyases. However, bacterial enzymes have been successfully expressed by bacteriophage, so it could be valuable to explore bacteriophage surface or tail spike expression of chondroitinase to target and depolymerize the chondroitin capsules of certain pathogenic *P. multocida* and *E. coli* strains. Taken together, these studies not only highlight the potential to utilize highly specific phages as antibacterial agents against encapsulated bacteria, but they suggest that it should be possible to engineer bacteriophages to specifically target and lyse pathogenic microbes possessing non-immunogenic capsules.

Finally, small molecule therapeutics could be used to interfere with CPS biosynthesis, CPS translocation, or even CPS attachment to the outer leaflet of the outer membrane (Fig. 9b). In a recent publication, Seed *et al.* developed a high-throughput screening method to search large chemical libraries for capsule inhibitors (Goller & Seed, 2010). Using a CPS-dependent bacteriophage (that requires surface-exposed capsule for adsorption and subsequent ejection of phage nucleic acid into the host) as an indicator for presence of intact capsule, bacteria that had been incubated with library compounds were evaluated for capsule production. One compound, dubbed “C7”, inhibited capsule formation in UTI89, a UPEC K1 strain. In a fascinating turn of events, C7 also inhibited capsule formation in UPEC K5 strain DS17. The finding that assembly of two different CPS structures was inhibited by the same compound, coupled with data showing insignificant accumulation of intracellular or extracellular CPS, it was hypothesized that C7 inhibits an early stage of CPS biogenesis in all Group 2 capsular *E. coli.* This study exemplifies the approaches that could lead to incredibly valuable therapeutics against infectious pathogens. Very recently, another study demonstrated the use of glycomimetics, rationally designed to block export of an *E. coli* CPS by “plugging” Wza, a pore-like translocon protein in the Wzy-dependent transport pathway (Kong *et al.*, 2013). It is our hope that aforementioned strategies will contribute to a better understanding of capsule biosynthesis in a metabolic context and inspire discussion leading to CPS-targeting therapeutic and prophylactic solutions to a host of serious diseases caused by masquerading microbial pathogens.

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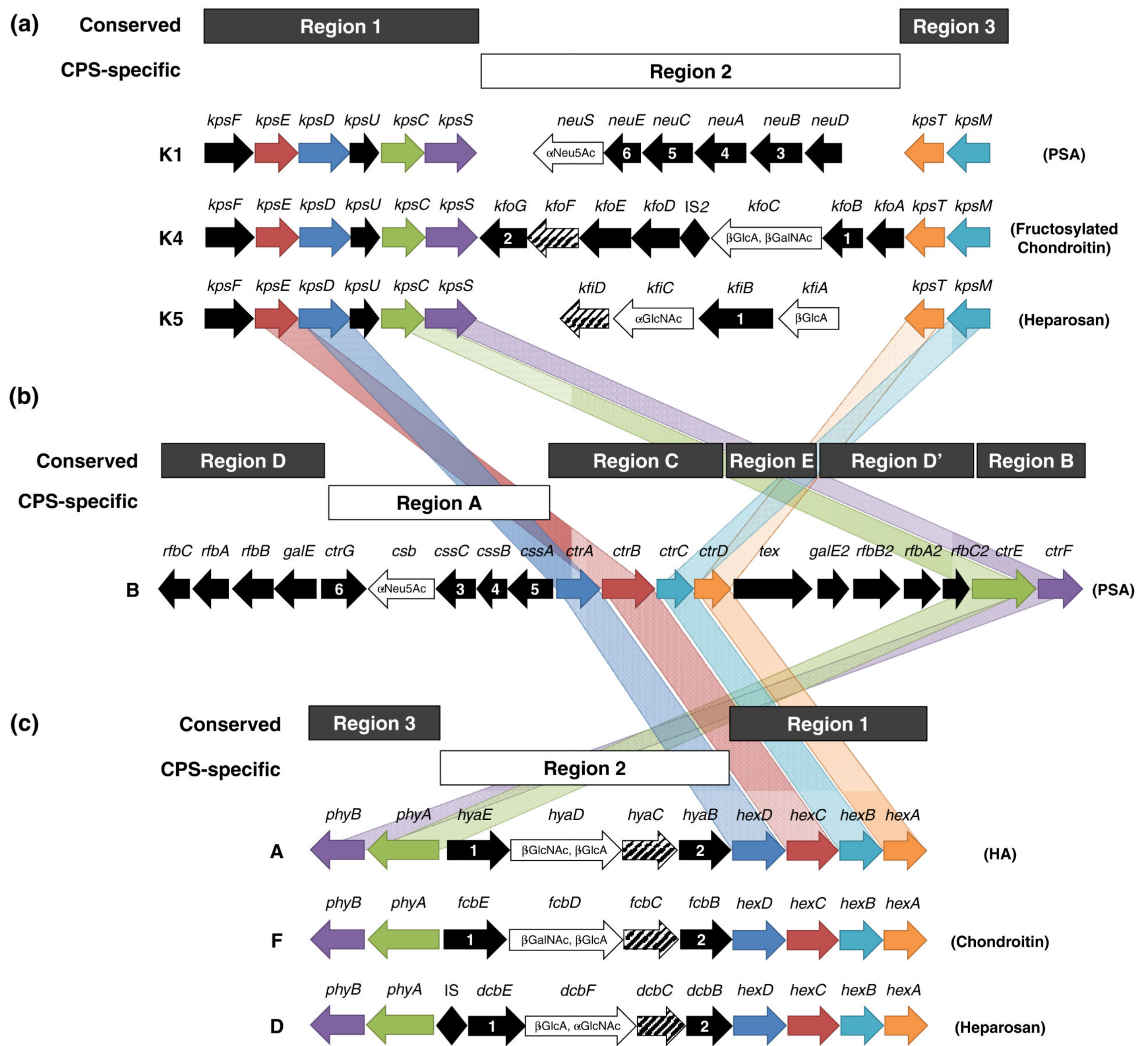

Fig. 1.

Schematic cross-sectional representation of layers constituting the bacterial cell wall of a typical Gram-negative bacterium. The thick external CPS layer conceals the bacterium to prevent desiccation, bacteriophage infection, complement-mediated killing, and opsonophagocytosis. The black and white inset (top left) shows a quick-freeze, deep-etch scanning electron micrograph of the Gram-negative organism *Bacteroides thetaiotaomicron* (Martens *et al.*, 2009); this SEM image was originally published in The Journal of Biological Chemistry. Martens EC, Roth R, Heuser JE & Gordon JI. Cover image. *J Biol Chem.* 2009; **284**(27):cover. © the American Society for Biochemistry and Molecular Biology.

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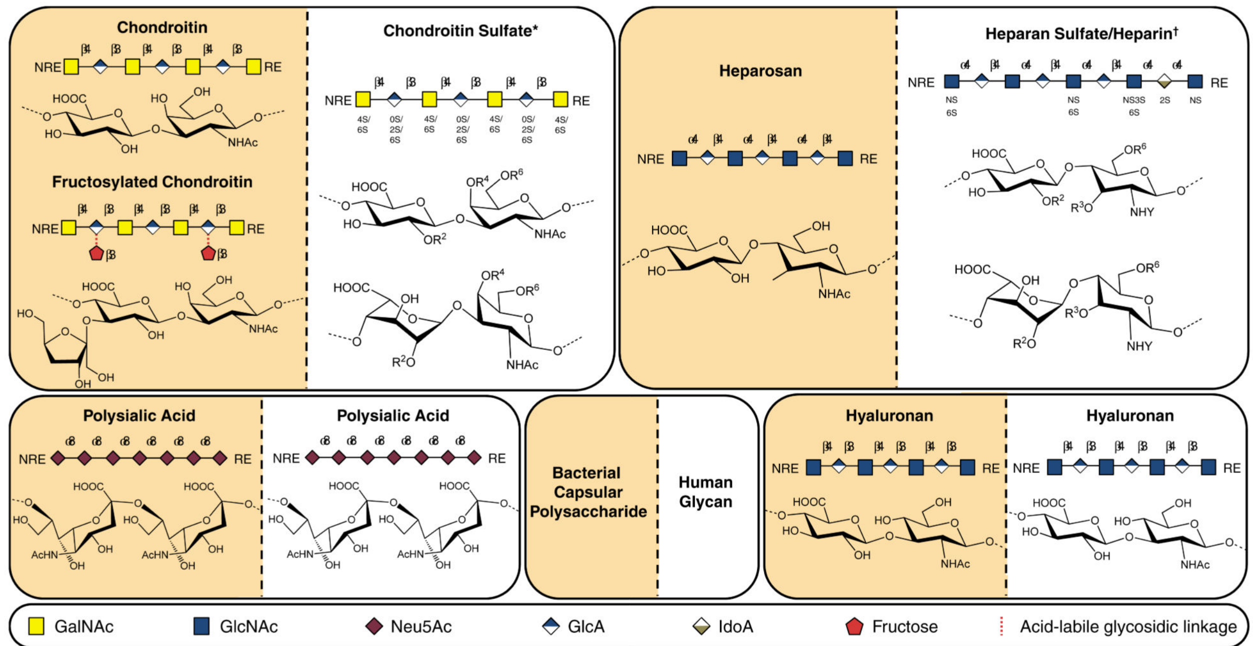

Fig. 2.

Glycan-centric schematic of typical Gram-negative cell wall components. Membrane proteins and other cell-wall constituents are neglected for simplicity. (a) Cell wall cross-section. (b) Lipopolysaccharide. (c) Capsular polysaccharide. Abbreviations are as follows: Lyso-PG = lyso-phosphatidylglycerol, GlcNAC = *N*-acetylglucosamine, MurNAc = *N*-acetylmuramic acid, GalNAc = *N*-acetylgalactosamine, KDO = 3-deoxy-D-mannooctulosonic acid, PPEtn = Pyrophosphoethanolamine, GlcA = glucuronic acid.

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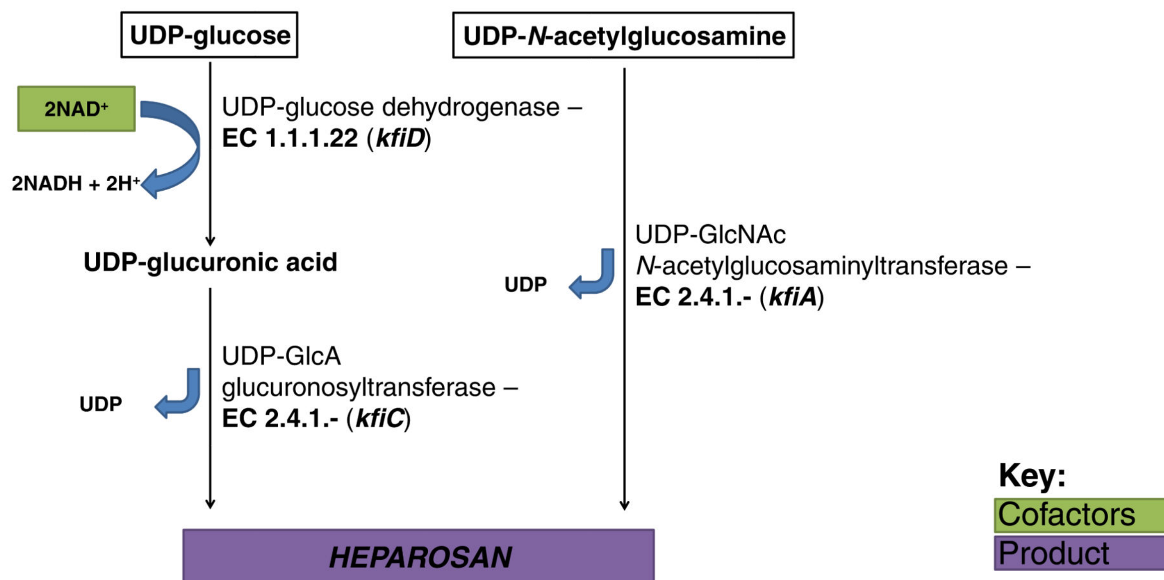

Fig. 3.

Symbolic representations and chemical structures of glycans described in this review. Non-immunogenic bacterial CPSs and structurally related animal glycans exhibited side-by-side to demonstrate similarity between backbones. In the case of chondroitin sulfate (CS) and heparan sulfate/heparin, bacterial CPS structures are identical to precursors of the mature human glycans depicted here. Of note, a related GAG known as dermatan sulfate also shares the unsulfated chondroitin backbone as a biosynthetic precursor, but, unlike CS, some glucuronic acid residues in the chain are epimerized to iduronic acid. CS type B possesses iduronic acid residues, so it is sometimes classified as dermatan sulfate. Conversely, HA and PSA structures are identical in microbial CPS and mature human GAGs. $^{*}R^{2,4,6}$ = H or SO_3^{-}; $^{\dagger}R^{2,3,6}$ = H or SO_3^{-}, Y = SO_3^{-} or Ac (Ac = $COCH_3$). Detailed disaccharide structures have been reported elsewhere (Sugahara & Mikami, 2007; Chang *et al.*, 2012b).

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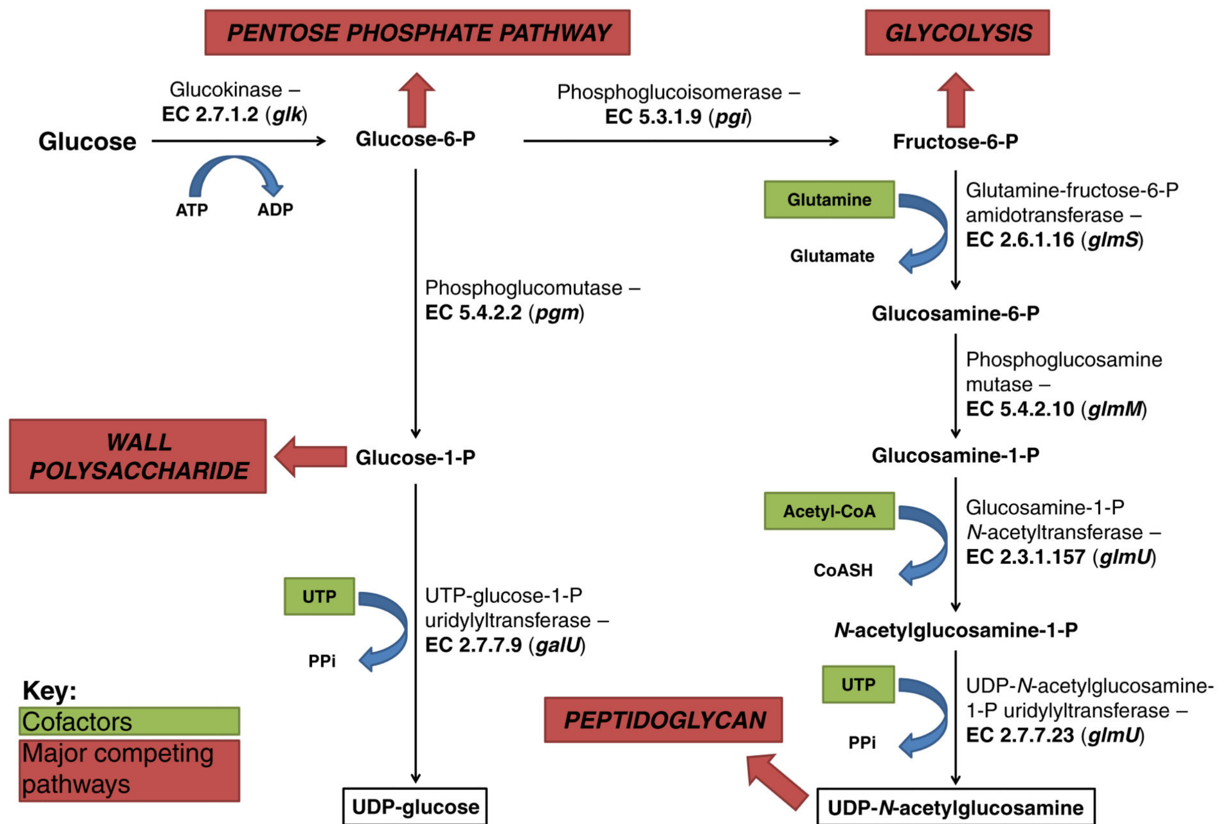

Fig. 4.

CPS gene loci in Gram-negative bacteria expressing ABC-transporter dependent CPS assembly pathways. (a) Gene loci encoding enzymes and transport proteins required for assembly of Group 2 *E. coli* K-antigens K1 (polysialic acid), K4 (chondroitin), and K5 (heparosan). Genes encoded by Regions 1 and 3 are well-conserved within Group 2 *E. coli* strains and encode enzymes required for CPS translocation across the cell wall, while Region 2 encodes CPS-specific glycosyltransferases and other biosynthetic enzymes. (b) Gene locus encoding proteins required for assembly of *N. meningitidis* serogroup B CPS. Region A encodes CPS-specific biosynthetic enzymes and varies between serogroups, while Regions B-E are highly conserved in all *N. meningitidis* serogroups. Regions B and C encode CPS translocation proteins homologous to genes in Regions 1 and 3 of Group 2 *E. coli* (homologous genes connected with gray bands), Regions D and D′ encode LPS assembly genes, and Region E has no known function. (c) Gene loci encoding *P. multocida* type A, D, and F CPS biosynthetic and transport proteins. Region 1 and 3 encode translocation genes whose functions are relatively well conserved in *P. multocida*, and Region 2 encodes CPS biosynthetic enzymes unique to the serotype specified. Homologous inter-species transport genes are color-coded and connected by bands of matching colors. Genes encoding glycosyltransferases are illustrated as white arrows with black outline and glycosyltransferase activity denoted within (bifunctional glycosyltransferases are labeled as found in nature, with N-terminal domain displayed at 5′ end of gene and C-terminal domain displayed at 3′ end of gene). UDP-glucose dehydrogenase is frequently encoded in CPS-specific biosynthetic clusters and is indicated here with diagonal lines. Other genes with

known and putative homologs are designated with matching numbers. Note: genes and operons are not drawn to scale.

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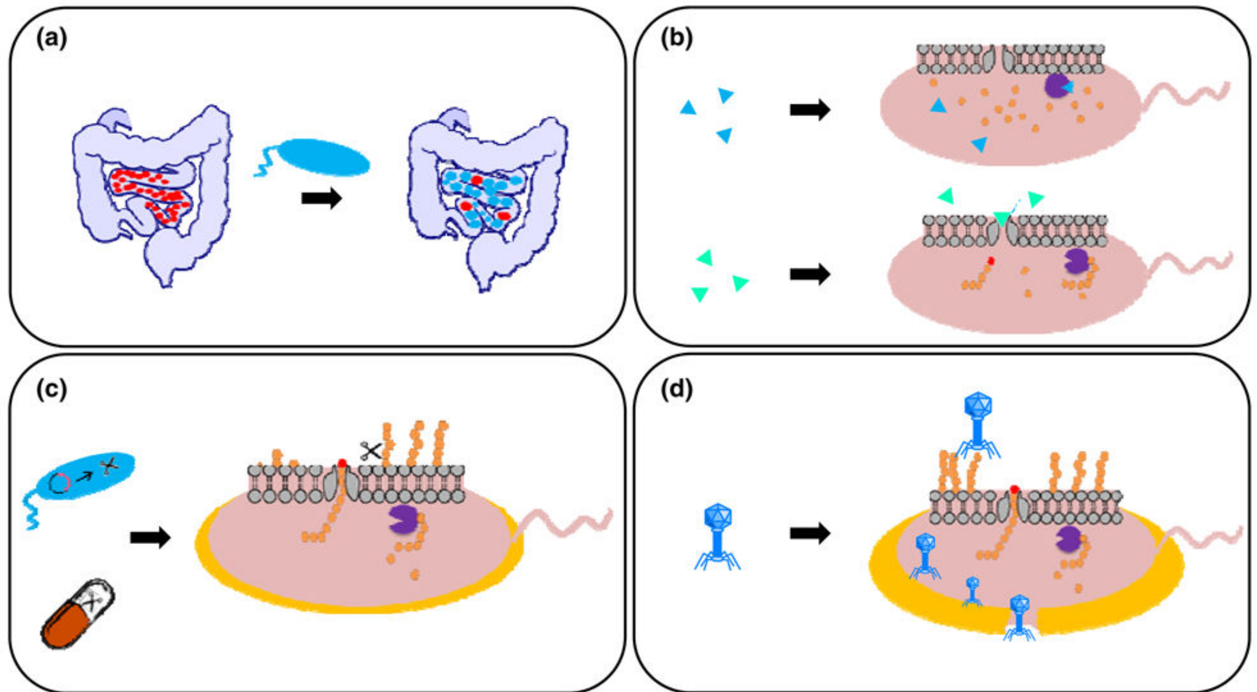

Fig. 5.

Central biosynthetic pathway for CPS precursor production in *E. coli.* This metabolic model is representative of early CPS biosynthesis for many bacteria, including those of interest in this review.

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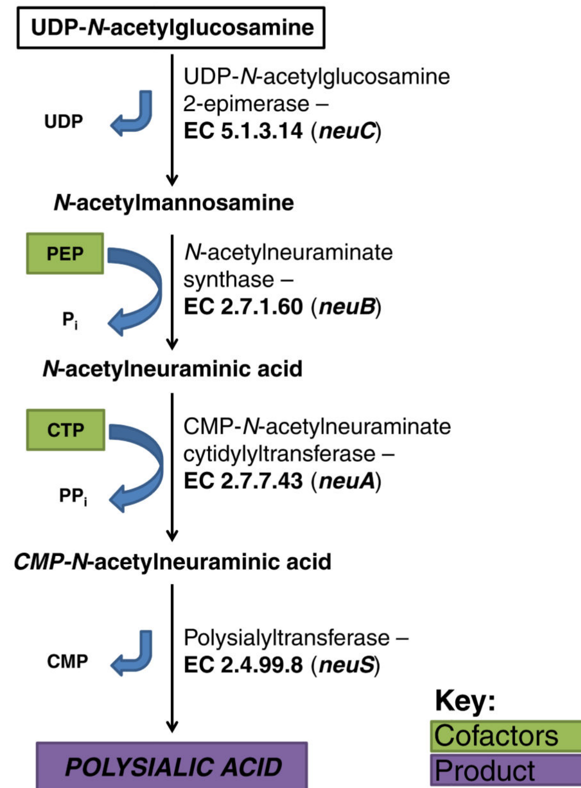

Fig. 6.

Biosynthetic pathway for PSA production in *E. coli* K1.

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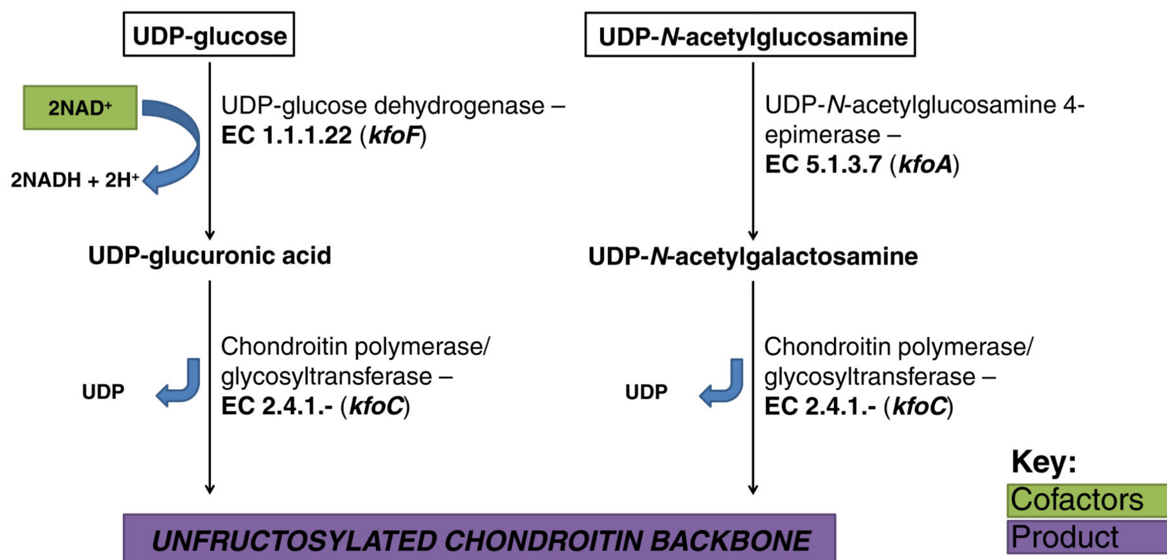

Fig. 7.

Biosynthetic pathway for production of K4 CPS, chondroitin, in *E. coli* K4.

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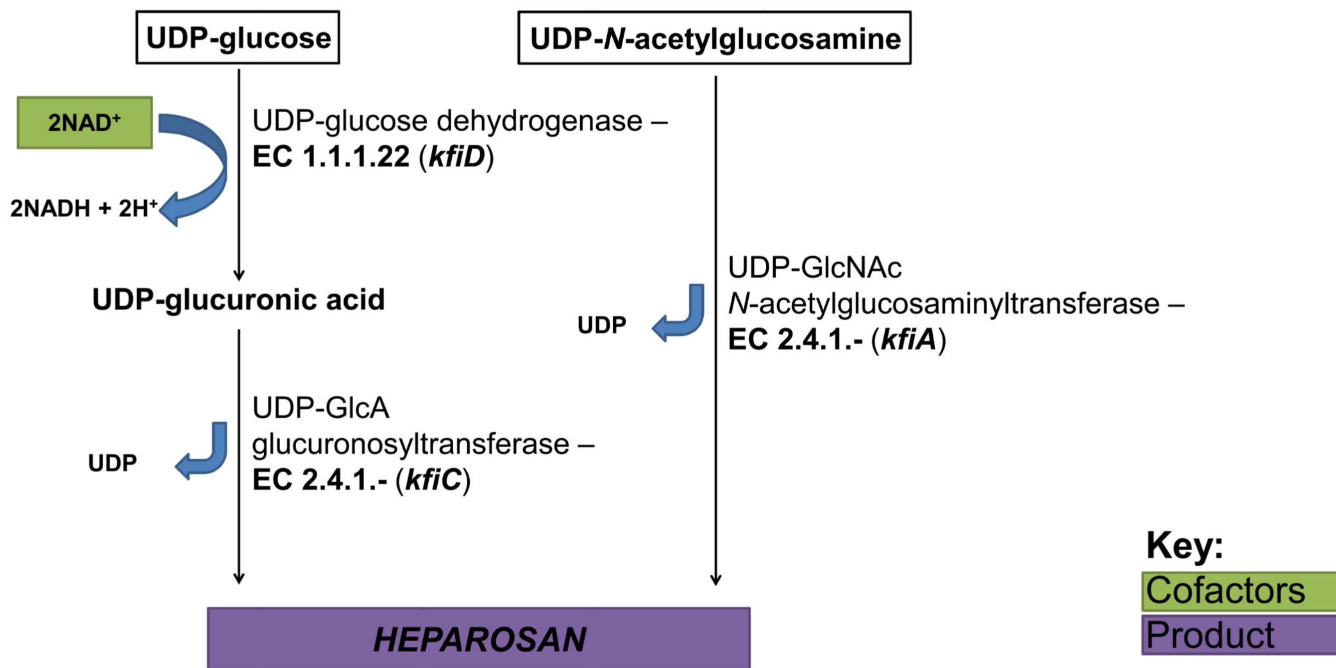

Fig. 8.

Biosynthetic pathway for production of K5 CPS, heparosan, in *E. coli* K5.

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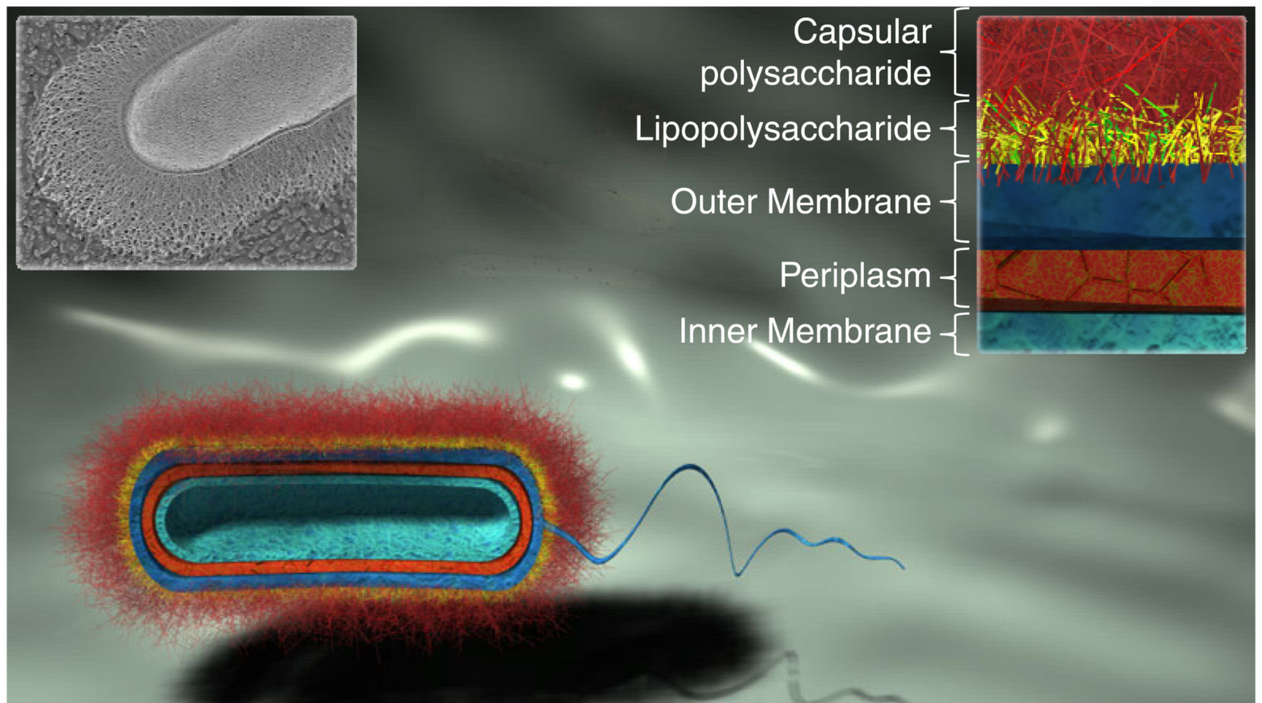

Fig. 9.

Strategies to combat capsule-bearing pathogens. Probiotic or engineered strains are designated in blue, and pathogenic strains are colored red with or without an orange capsule. (a) Ingestion of wild-type probiotic or engineered strains that outcompete pathogenic bacteria. (b) Small molecule inhibitors of CPS biosynthetic enzymes (upper) or CPS translocation proteins (lower). Triangles represent small-molecule inhibitors; purple circular ("Pac-Man") symbol represents polysaccharide glycosyltransferase. (c) Treatment with probiotic bacteria engineered to secrete lyases or glycosidases (drawn as scissors) with activity against pathogen's CPS (upper) or treatment with purified enzyme (lower). (d) Treatment with natural or engineered bacteriophage to lyse bacteria bearing specific CPS type.

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Table 1

Pathogenic bacteria possessing non-immunogenic CPSs that are identical to human and animal glycans

CPS	GA G	Organism*	Serotype/Capsule Type	Disease(s) (Organism)	Reference(s)
Polysialic Acid	No	*Escherichia coli*	K1	Meningitis, Urinary Tract Infection, diarrhea, septicemia (human)	(Silver *et al.*, 1988)
Polysialic Acid	No	*Neisseria meningitidis*	B	Meningitis (human)	(Finne *et al.*, 1983)
Polysialic Acid	No	*Moraxella nonliquefaciens*		Endopthlamitis, sepsis, meningitis, endocarditis (human)	(Bøvre *et al.*, 1983; Devi *et al.*, 1991; Rafiq *et al.*, 2011)
Polysialic Acid	No	*Mannheimia* (formerly *Pasteurella*) *haemolytica*	A2	Bovine respiratory disease (bovine)	(Adlam *et al.*, 1987; Rice *et al.*, 2007)
Chondroitin†	Yes	*Escherichia coli*	K4	Urinary Tract Infection, diarrhea (human); diarrhea (bovine)	(Rodriguez *et al.*, 1988; Orskov *et al.*, 1985; Moxley & Francis, 1986)
Chondroitin	Yes	*Pasteurella multocida*	type F	Fowl cholera (avian)	(Rimler & Rhoades, 1987)
Chondroitin	Yes	*Avibacterium paragallinarum*	genotype I	Coryza (avian)	(Wu *et al.*, 2010; Zhao *et al.*, 2010)
Heparosan	Yes	*Escherichia coli*	K5	Urinary Tract Infection (human)	(Minshew *et al.*, 1978; Zingler *et al.*, 1990)
Heparosan	Yes	*Pasteurella multocida*	type D	Pneumonia (porcine)	(Ewers *et al.*, 2006)
Heparosan	Yes	*Avibacterium paragallinarum*	genotype II	Coryza (avian)	(Wu *et al.*, 2010)
Hyaluronan	Yes	*Streptococcus pyogenes*		Scarlet fever, pharyngitis (human)	(Wessels *et al.*, 1991; Ralph & Carapetis 2013)
Hyaluronan	Yes	*Streptococcus equi* subsp. *zooepidemicus*		Septicemia, meningitis, endocarditis and arthritis (bovine, porcine, ovine, and canine)	(Wibawan *et al.*, 1999; Wei *et al.*, 2012)
Hyaluronan	Yes	*Streptococcus dysgalactiae subsp. equisimilis*		Streptococcal Toxic Shock Syndrome (human)	(Calvinho *et al.*, 1998; Hashikawa *et al.*, 2004)
Hyaluronan	Yes	*Streptococcus uberis*		Mastitis (bovine)	(Almeida & Oliver, 1993; Almeida *et al.*, 2013)
Hyaluronan	Yes	*Streptococcus equi* subsp. *equi*		Upper respiratory tract infection (equine)	(Anzai *et al.*, 1999)
Hyaluronan	Yes	*Pasteurella multocida*	type A	Respiratory disease (bovine, feline)	(Borrathybay *et al.*, 2003b; Ewers *et al.*, 2006)
Hyaluronan	Yes	*Avibacterium paragallinarum*		Coryza (avian)	(Sawata & Kume, 1983; Byarugaba *et al.*, 2007)

*In recent years, there has been much confusion regarding delineation of *Streptococcus* species and subspecies due to imprecise, muddled, and archaic classification systems (Jensen & Kilian, 2012). Similar scenarios occurred with *Avibacterium* and *Mannheimia*. Organism names are provided as originally reported unless a clear indication of misclassification was detected.

†Fructosylated