

MINI REVIEW

Microbial glycosaminoglycan glycosyltransferases

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Glycosaminoglycans, a class of linear polysaccharides composed of repeating disaccharide units containing a hexosamine, are important carbohydrates found in many organisms. Vertebrates utilize glycosaminoglycans in structural, recognition, adhesion, and signaling roles. Certain pathogenic bacteria produce extracellular capsules composed of glycosaminoglycans or glycosaminoglycan-like polymers that enhance the microbes' ability to infect or to colonize the host. In the period from 1993 to 2001, bacterial enzymes were discovered that catalyze the polymerization of the repeating unit of hyaluronan, chondroitin, or N-acetyl-heparosan (unsulfated, unepimerized heparin). Depending on the specific carbohydrate and the microorganism, either a dual-action enzyme (synthase) that transfers two distinct monosaccharides or a pair of single-action transferases are utilized to synthesize the glycosaminoglycan polymer. Current views on the enzymology, structures, potential evolution, and the roles of the known glycosyltransferases from *Streptococcus*, *Pasteurella*, and *Escherichia* are discussed.

Key words: chondroitin/heparin/heparosan/hyaluronan or hyaluronic acid/polysaccharide

Bacterial capsules and glycosaminoglycans

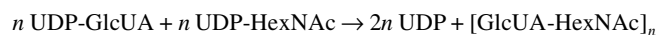
Pathogenic bacteria are notorious for their ability to surmount host defenses by producing a wide gamut of virulence factors that enhance microbial infectivity and/or persistence. One such factor is the capsule, an extracellular polymer coating surrounding the microbial cell (reviewed in Roberts, 1996). Pathogens that lose the ability to produce a capsule are often attenuated or avirulent. The majority of described capsules are composed of long anionic polysaccharide chains, but neutral polysaccharides as well as proteinaceous components are also observed in some cases.

Hundreds of structures have been reported from animal and plant pathogens, but of special interest are capsular polymers chemically identical or similar to host molecules. In particular, both vertebrates and certain microbes produce glycosaminoglycans (GAGs), linear polysaccharides composed of repeating disaccharide units containing a derivative of an amino sugar (either glucosamine or galactosamine).

Hyaluronan (HA), chondroitin, and heparan sulfate/heparin contain a uronic acid as the other component of the disaccharide repeat, and keratan contains a galactose (Table I). Vertebrates can contain all four types of GAGs, but the polysaccharide chain is often further modified after sugar polymerization. One or more modifications, including O-sulfation of certain hydroxyls, deacetylation and subsequent N-sulfation, or epimerization of glucuronic acid to iduronic acid, are found in most GAGs except HA. An amazing variety of distinct structures have been reported for chondroitin sulfate and heparan sulfate/heparin even within a single polymer chain (Esko and Lindahl, 2001). A few clever microbes also produce GAG chains, but sulfation or epimerization have not been yet described. The chondroitin and heparan sulfate/heparin chains in vertebrates are initially synthesized by elongation of a xylose-containing linkage tetrasaccharide attached to a variety of proteins. Keratan is either O-linked or N-linked to certain proteins depending on the particular molecule. HA and all of the known bacterial GAGs are not part of glycoproteins. This review focuses on the identity and the nature of the known microbial glycosyltransferases that produce GAG chains (Table II).

Basics of GAG glycosyltransferases

All of the known HA, chondroitin, and heparan sulfate/heparin glycosyltransferase enzymes that synthesize the alternating sugar repeat backbones in microbes and in vertebrates utilize uridine diphospho-(UDP)-sugar precursors and metal cofactors (e.g., magnesium and/or manganese ion) near neutral pH according to the overall reaction:



where HexNAc = GlcNAc or GalNAc. Depending on the specific GAG and the particular organism or tissue examined, the degree of polymerization, n , ranges from ~25 to ~10,000. The bacterial GAG glycosyltransferase polypeptides are associated with the cell membranes; this localization makes sense with respect to synthesis of polysaccharide molecules destined for the cell surface.

Various names for the GAG glycosyltransferases have been used in the literature over the last four decades. The dual-action enzymes required for the production of the HA chain have been called synthases (or, in early reports, synthetases). The enzymes that elongate the repeating chondroitin or the repeating heparan sulfate/heparin backbone have been called various names, including co-polymerases, co-transferases, polymerases, synthases, or the individual component activities

Table I. Structures of the vertebrate and microbial GAG repeating backbones

Polymer	Disaccharide repeat	Postpolymerization modifications	
		Vertebrates	Bacteria
Hyaluronan	β 3GlcNAc β 4GlcUA	none	none
Chondroitin	β 3GalNAc β 4GlcUA	O-sulfated, epimerized	none or fructose(β 1,3)GlcUA
Heparan sulfate/ heparin	α 4GlcNAc β 4GlcUA	O-, N-sulfated, epimerized	none
Keratan	β 4GlcNAc β 3Gal	O-sulfated	not reported

Table II. Microbes, diseases, GAGs, and glycosyltransferases

Bacteria (disease) ^a	Hyaluronan	Chondroitin	Heparosan	Enzyme [size ^b]/GenBank
<i>Streptococcus</i>				
Group A (pharyngitis, scarlet fever, necrotizing fasciitis, impetigo, and more)	X			spHAS [419]/L20853
Group C (mastitis*, strangles*)	X			seHAS [418]/AF023876
<i>Escherichia coli</i>				
K4 (diarrhea)		X ^c		not reported
K5 (urinary tract and extraintestinal infections, sepsis)			X	Kfi A [238] + Kfi C [520] complex/X77617
<i>Pasteurella multocida</i>				
Type A (fowl cholera*, shipping fever*, sepsis from animal bites)	X			pmHAS [972]/AF036004
Type D (atrophic rhinitis*)			X	pmHS [617]/AF425591
Type F (fowl cholera*)		X		pmCS [965]/AF195517

^aAnimal diseases denoted with a asterisk (remainder are human diseases).

^bNumber of amino acid residues in the deduced open reading frame.

^cFructosylated polymer.

were directly termed (e.g., glucuronic acid [GlcUA]-transferase, GlcNAc-transferase, or GalNAc-transferase).

Hyaluronan synthases of *Streptococcus* and *Pasteurella*

The HA extracellular capsules of Gram-positive Group A *Streptococcus* (Kendall *et al.*, 1937) and Gram-negative Type A *Pasteurella multocida* (Carter and Annau, 1953) were shown to be identical to HA of vertebrates. As the vertebrate HA synthases (HASs) were (and remain) relatively difficult to study biochemically, more initial progress was made on the “simpler,” higher specific activity membrane preparations of streptococcal enzymes (Stoolmiller and Dorfman, 1969; Sugahara *et al.*, 1979).

Transposon insertional mutagenesis was utilized to tag and to identify the genes for the microbial HASs of both Group A *Streptococcus* (*S. pyogenes* spHAS or HasA; DeAngelis *et al.*, 1993; Dougherty and van de Rijn, 1994) and *P. multocida* Type A (pmHAS; DeAngelis *et al.*, 1998). The parental wild-type strains are so heavily encapsulated that the colonies on agar media appeared large and wet by visual inspection, but acapsular mutants form smaller, dry colonies. The transposon-disrupted DNA was used to generate probes for obtaining intact synthase genes from genomic DNA libraries. Degenerate

polymerase chain reaction (PCR)-based on the Group A *Streptococcus* HAS sequence was used to obtain the homologous enzyme sequence from a Group C organism (*S. equisimilis* seHAS; Kumari and Weigel, 1997). In all the known cases (including the vertebrate and viral enzymes; reviewed in Weigel *et al.*, 1997; DeAngelis, 1999a), the HA polysaccharide is polymerized by a single polypeptide, the HAS.

The microbial HASs contain two distinct glycosyltransferase activities as demonstrated by expression in foreign hosts (e.g., *Escherichia coli*) and various biochemical analyses (DeAngelis *et al.*, 1993, 1998; DeAngelis and Weigel, 1994; Kumari and Weigel, 1997). Recombinant preparations of the microbial HASs rapidly form HA chains with elongation rates of ~10–150 sugars/second *in vitro*. The sugar transfer specificity of the enzymes is exquisite, and only the authentic HA sugars, GlcUA and GlcNAc, are incorporated into polymer. The enzymes do not utilize significantly the C4 epimer precursors, UDP-galacturonic acid or UDP-GalNAc, or the unsubstituted analog, UDP-glucose.

The streptococcal enzymes and the *Pasteurella* enzyme produce the same polymer product from identical precursors, but these synthases possess quite distinct sequences and enzymological characteristics. The streptococcal HASs are integral membrane proteins with several transmembrane or membrane-associated regions (DeAngelis *et al.*, 1993;

Heldermon *et al.*, 2001). Vertebrate HASs have similar sequence motifs and predicted structure to the streptococcal enzymes (reviewed in Weigel *et al.*, 1997). On the other hand, the *Pasteurella* enzyme appears to contain a carboxyl-terminal region that allows docking with a membrane-bound partner because deletion of the region results in the expression of a functional soluble, cytoplasmic form of the enzyme (Jing and DeAngelis, 2000). As discussed later, recombinant pmHAS can elongate exogenously supplied HA-oligosaccharide acceptors, but the streptococcal and vertebrate enzymes have not been shown to perform similar reactions (Stoolmiller and Dorfman, 1969; DeAngelis, 1999b). In summary, two classes of HAS enzyme have been discovered thus far; Class I includes the streptococcal, vertebrate, and viral HASs, and the only Class II member is the enzyme from *Pasteurella* (DeAngelis, 1999a).

Chondroitin synthases of *Pasteurella* and *Escherichia*

The chondroitin chain is chemically identical to HA except that GalNAc is substituted for GlcNAc. Certain distinct isolates of *Pasteurella multocida*, now called Type F, were speculated to produce a chondroitin-like polymer based on the sensitivity of the bacterial capsule to chondroitin ABC lyase (Rimler, 1994). The capsular polysaccharide contains GalNAc and a uronic acid (DeAngelis and Padgett-McCue, 2000) and is unsulfated chondroitin as assessed by structural analyses (DeAngelis and Linhardt, unpublished data). Experiments utilizing pmHAS DNA probes and PCR primers indicated that a novel homologous synthase existed. An open reading frame, called pmCS, with ~90% identity at the gene and protein level to pmHAS was shown to have chondroitin synthase activity *in vitro* (DeAngelis and Padgett-McCue, 2000). Recombinant pmCS polymerizes long chains (~1000 sugars) composed of GalNAc and GlcUA that are sensitive to chondroitin ABC lyase but not HA lyase. The pmCS enzyme, like pmHAS, is a selective glycosyltransferase; only the authentic precursors, UDP-GalNAc and UDP-GlcUA, serve as donors *in vitro*. The recently discovered vertebrate chondroitin synthase is not very similar at the DNA or protein sequence level to pmCS (Kitagawa *et al.*, 2001).

Gram-negative *E. coli* K4 produces a capsule that is composed of a chondroitin backbone with a β -linked fructose attached to the C3 position of the GlcUA residue (Rodriguez *et al.*, 1988). The capsular locus from *E. coli* K4 was cloned by Drake *et al.* (1990), but the sequence has not been deposited in GenBank and the identity of the glycosyltransferase(s) involved in chondroitin biosynthesis has not been reported. Biochemical analysis of glycosyltransferase activity *in vitro* has demonstrated the addition of single sugars to acceptor molecules (i.e., a single GalNAc added to a nonreducing terminal GlcUA residue of a chondroitin-derived oligosaccharide acceptor), but long polymers were not formed (Lidholt and Fjelstad, 1997). It appears that the fructose branch is added to the K4 polymer chain after the GAG repeat is formed because defructosylated K4 oligosaccharide but *not* the intact native K4 oligosaccharide served as an acceptor. A puzzling question concerns the fructose branch as it makes the chondroitin polymer more antigenic; at this time, an *E. coli* capsular type without the fructose moiety has not been reported.

Heparosan glycosyltransferases of *Escherichia* and heparosan synthase of *Pasteurella*

Heparan sulfate/heparin and related polymers contain alternating α - and β -glycosidic linkages and thus are quite distinct from the entirely β -linked HA and chondroitin polymers. The UDP-sugar precursors are α -linked, therefore, heparin biosynthesis exhibits two types of reaction pathway: a retaining mechanism to produce the α -linkage and an inverting mechanism that results in a β -glycosidic linkage. A logical prediction is that two distinct polypeptides or a fusion of two distinct types of catalytic domain into a single polypeptide would be required to produce the heparin chain; both cases are in fact observed in the bacterial world as exemplified by heparosan biosynthesis.

E. coli K5 produces a capsule composed of an unsulfated, unepimerized N-acetyl-heparosan (heparosan or desulfoheparin) (Vann *et al.*, 1981). The *E. coli* K5 capsular locus contains open reading frames *KfiA-D* (also called the *Kfa* locus in some reports; Petit *et al.*, 1995). Biochemical analyses of the glycosyltransferase activities in membrane preparations or in lysates from both the native K5 and recombinant bacteria have been reported (Finke *et al.*, 1991; Griffiths *et al.*, 1998). However, it was difficult to ascertain that two distinct enzymes were actually required for the synthesis of the repeating GAG chain in part due to the lack of continued polymerization by recombinant enzymes *in vitro*; only the addition of single sugars to oligosaccharide acceptors was observed. At first, KfiC was stated to be a dual-action glycosyltransferase responsible for the alternating addition of both GlcUA and GlcNAc to the heparosan chain (Griffiths *et al.*, 1998). This report also concluded that the enzyme's GlcUA-transferase activity was inactivated by the removal of a segment of the carboxyl terminus, but the GlcNAc-transferase activity remained intact. However, a later report by the same group reported that another protein, KfiA, encoded by the same operon was actually the α -GlcNAc-transferase required for heparosan polymerization (Hodson *et al.*, 2000). Therefore, at least these two enzymes, KfiA and KfiC, work in concert to form the disaccharide repeat. Another deduced protein in the operon, KfiB, was suggested to stabilize the enzymatic complex during elongation *in vivo* but not participate directly in catalysis.

The Type D *P. multocida* capsular polysaccharide is also N-acetylheparosan as measured by compositional and structural analyses (DeAngelis and Linhardt, unpublished data). In this microbe, however, the polymer is synthesized by a dual-action glycosyltransferase, the heparosan synthase or pmHS (DeAngelis and White, unpublished data). The recombinant *E. coli*-derived pmHS enzyme polymerizes both GlcNAc and GlcUA *in vitro*, but the biochemical details are still emerging.

One region of the pmHS protein is similar to *E. coli* K5 KfiA, and another region of pmHS is similar to KfiC, suggesting that a two-domain structure exists in the *Pasteurella* enzyme. The sequence of pmHS, however, is very different from other *Pasteurella* GAG synthases, pmHAS and pmCS. The overall organization of the capsule loci of Type A, D, and F *P. multocida*, on the other hand, are quite similar based on recent sequence comparisons (Townsend *et al.*, 2001). Most notably, highly homologous UDP-glucose dehydrogenase genes (92–98% identical) follow the synthase genes in all three capsular types. The exostosin proteins, EXT1 and 2, the vertebrate enzymes responsible for biosynthesis of the

heparan sulfate/heparin backbone, are not similar to the bacterial heparosan glycosyltransferases at the sequence level (reviewed in Duncan *et al.*, 2001).

Molecular directionality and mode of polysaccharide polymerization

The sugars of a linear polymer chain can be added to either the nonreducing or the reducing terminus. Definitive proof of nonreducing end elongation has been shown for pmHAS (DeAngelis, 1999b), pmCS (DeAngelis and Padgett-McCue, 2000), the *E. coli* K4 activity (Lidholt and Fjelstad, 1997), and KfiA and KfiC (Hodson *et al.*, 2000). A series of experiments based on adding a single monosaccharide from a UDP-sugar to a variety of oligosaccharide acceptor molecules with known termini were crucial for deciphering the molecular directionality question. The native (Stoolmiller and Dorfman, 1969) and the recombinant (DeAngelis, 1999b) streptococcal enzyme, spHAS, however, does not elongate any of the tested exogenous oligosaccharide acceptors. The streptococcal enzyme was shown by indirect means (e.g., selective labeling and degradation) to add to the nonreducing terminus (Stoolmiller and Dorfman, 1969), but more recent efforts on the vertebrate HA synthases (which has a similar overall predicted structure) have suggested that reducing end elongation occurs (reviewed in DeAngelis, 1999a). Further analysis will be required to resolve the question of molecular directionality in these cases.

With respect to polymerization mode, if a pair of glycosyltransferases were utilized to synthesize a GAG polymer, then each enzyme would be expected to add single sugars in a concerted fashion to produce the disaccharide repeat structure. However, in the case of a dual-action synthase, other polymerization modes are also possible. It has been theorized that certain polysaccharide polymerizing systems composed of an enzyme on the cell surface (including microbial enzymes that make HA or cellulose) would be required to synthesize the polymer in disaccharide units due to topological or energetic constraints (Saxena *et al.*, 1995). The newly formed disaccharide unit would then translocate out of the catalytic site and the process is repeated to form longer chains. However, no direct experimental evidence has been reported to support the simultaneous sugar addition hypothesis. Experiments with *Pasteurella* pmHAS, however, demonstrated that single sugars are added to the growing chain sequentially; the intrinsic fidelity of each transfer step ensures the production of the GAG repeat structure (DeAngelis, 1999b). The homologous chondroitin synthase, pmCS, behaves in a similar fashion. Experiments that define the mode of polymerization of streptococcal HASs have not been reported, but the single addition of a radiolabeled monosaccharide to the nascent HA chain of native streptococcal HAS preparations was observed (Stoolmiller and Dorfman, 1969).

Domain structure of synthases

The pmHAS enzyme has been shown to contain two independent glycosyltransferase sites by biochemical analysis of various mutants (Jing and DeAngelis, 2000). The GlcNAc-transferase or the GlcUA-transferase activities of the *Pasteurella* enzyme can be assayed separately *in vitro* by supplying the appropriate

acceptor oligosaccharide and only one of the UDP-sugar precursors. Two tandemly repeated sequence elements are present in pmHAS (Figure 1). Each element of pmHAS contains a set of two short sequence motifs: a DGS followed by a DXD (X = S or C) about 45 residues downstream. The DXD motif is particularly widespread in other known glycosyltransferases.

Based on crystallographic structural evidence from another putative glycosyltransferase, SpsA of *Bacillus subtilis* (Charnock and Davies, 1999), the DGS motif may interact with the uracil ring of the UDP-sugar. The DXD motif is probably involved in the coordinating the metal ion bound to the UDP-sugar and interacting with the ribose based on the structures of SpsA and GlcAT-I, the mammalian GlcUA-transferase responsible in part for creation of the linkage tetrasaccharide connecting GAGs to the protein core (Pedersen *et al.*, 2000). The DXD motif may be important for binding the sugar nucleotide directly and/or assisting the leaving group departure after sugar transfer (Charnock and Davies, 1999). Experiments utilizing photoactivatable nucleotide analogs to assess the affinity of an enzyme mutated at the DXD motif for UDP-sugar suggest different roles are possible. For example, the large clostridial cytotoxin glycosyltransferase apparently uses the DXD to bind precursor (Busch *et al.*, 1998), but this is not the case for the GM2 ganglioside synthase (Li *et al.*, 2001).

Mutation of the aspartate residue in any one DGS or DXD motif of pmHAS converts the dual-action synthase into a single-action glycosyltransferase (Jing and DeAngelis, 2000 and unpublished data). The GlcNAc-transferase and the GlcUA-transferase activities are relatively independent based on kinetic comparisons of the various mutants to the wild-type pmHAS enzyme. In the case of the *E. coli* K5 KfiA and KfiC glycosyltransferases that co-polymerize heparosan, mutation of the DXD motif of either enzyme results in substantial loss of activity (Griffiths *et al.*, 1998; Hodson *et al.*, 2000). The specific

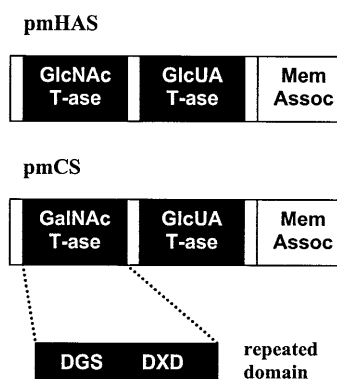


Fig. 1. Schematic diagram of *Pasteurella* HA and chondroitin synthases. The hyaluronan (pmHAS) and the chondroitin synthases (pmCS) contain two distinct, relatively independent transferase sites (T-ase; black boxes) that elongate the GAG chain by repeated step-wise addition of single sugars. Each site contains a pair of short sequence motifs (DGS and DXD; X = C in the hexosamine T-ase or X = S in the GlcUA T-ase) with essential aspartate residues. The carboxyl-terminus of the synthase is required to associate with the membrane fraction (Mem Assoc), but not essential for catalytic activity *in vitro*. The catalytic domain structure of the streptococcal HA synthases is not yet known.

catalytic roles of the aspartate residues for the GAG-synthesizing enzymes have not yet been determined.

The *Pasteurella* chondroitin synthase, pmCS, contains separate GalNAc-transferase (a slightly mutated version of the GlcNAc-site of pmHAS) and GlcUA-transferase sites (Figure 1). The *Pasteurella* heparosan synthase, pmHS, also appears to contain at least two domains (DeAngelis and White, unpublished data). These regions, however, resemble the *E. coli* KfiA and C components and are not very similar to the sequence elements of the pmHAS and pmCS enzymes. The site of nascent GAG chain binding and/or polymer growth is not yet known for any *Pasteurella* synthase, but it should be proximal or accessible to the two UDP-sugar binding sites.

Detailed information on the active sites of the other bacterial GAG glycosyltransferase enzymes is not available. Mutational analysis of the streptococcal HAS has not been particularly fruitful as a result of the inability to perform the half-assay of sugar transfer. A combination of biochemical, enzymological, topological, and sequence analyses suggest that the catalytic residues reside in a central cytoplasmic domain (Heldermon *et al.*, 2001), but further work to localize the active site is required.

Evolution of dual-action synthases

One model of the genesis of the *Pasteurella* GAG synthases involves some combination of events including gene duplication of an ancestral single-action glycosyltransferase and mutation of the resulting pair of enzymes to form different sugar specificities. With respect to the historical evolutionary path, it has not been determined whether a gene fusion event or a gene scission event forged the dual-action synthases and the two-enzyme systems. Another model for the creation of synthases is driven by gene flux between similar or disparate microbial species. In certain cases, a pair of genes encoding compatible single-action transferases may have ended up on the same chromosome and teamed up to form heteropolysaccharides. It would not be surprising if examples of both models occurred in the past. The efficiency and convenience of combining the two required enzyme activities into a single polypeptide seems clear, but as a counterexample the *E. coli* KfiA and KfiC proteins remain separate entities.

The highly homologous pmHAS and pmCS enzymes probably diverged after a few key mutations in the hexosamine-transferase domain, but it is not yet possible to decipher if the HA synthase or the chondroitin synthase was the ancestral enzyme. The pmHAS and pmHS enzymes both perform the task of polymerizing the identical monosaccharides, as HA and heparosan only differ with respect to their linkages. The creation of different anomeric linkages probably requires very distinct active sites due to the disparity between a retaining and an inverting sugar transfer mechanism. The pmHS protein may be the result of the fusion of two ancestral single-action enzymes that did not resemble pmHAS or pmCS.

The evolution of HASs in *Streptococcus*, vertebrates, and an algal virus is quite a perplexing problem (Weigel *et al.*, 1997; DeAngelis, 1999a). Two obvious alternative explanations for highly similar HA synthases occurring in disparate organisms are (1) horizontal gene transmission between kingdoms or (2) functional convergent evolution. The streptococcal HAS genes are more similar to the capsule genes of certain *Streptococcus*

pneumoniae isolates (especially Type 3, which makes a GlcUA-glucose capsule) than to the vertebrate HASs; therefore, the Group A and C *Streptococcus* probably did not acquire directly a vertebrate HAS gene. The engineering of biosynthetic enzymes for the production of a GAG capsule was probably worth retooling an existing glycosyltransferase; the obvious case is existence of the very distinct Class I streptococcal HA synthases and the Class II pmHAS.

Potential role of glycosyltransferases in transport during capsular biosynthesis

Glycosyltransferases catalyze the formation of the repeating GAG backbone, but in certain cases, these same polypeptides may also play roles in transporting the polymer across the cell membrane. The Gram-positive Group A and C *Streptococcus* possess only one lipid membrane and the capsule operon encodes the synthase and two enzymes for UDP-GlcUA production, UDP-glucose dehydrogenase and UPP-glucose pyrophosphorylase (~4 kb of DNA; Crater and van de Rijn, 1995). Topological analyses of a series of streptococcal spHAS fusion proteins containing reporter enzymes indicate that this synthase spans the bilayer at least four times and is intimately associated with the membrane (Heldermon *et al.*, 2001) (Figure 2). From biochemical and biophysical analyses, it appears that a complex composed of a monomer of the spHAS or seHAS polypeptide and ~16 lipid molecules catalyzes the transfer of both UDP-sugars to the nascent HA chain (Tlapak-Simmons *et al.*, 1998). It was speculated that spHAS or seHAS, small integral membrane polypeptides, would require the assistance of the lipids to facilitate transport of the growing HA polymer

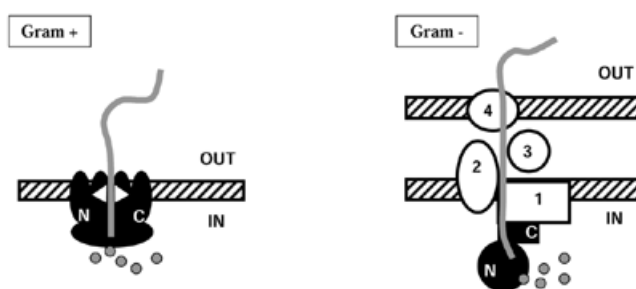


Fig. 2. Schematic models of GAG biosynthesis in Gram-positive and Gram-negative bacteria. Monosaccharides from the UDP-sugar precursors (gray spheres) inside the cell are polymerized by the GAG glycosyltransferases into long polymer chains (gray line). The polymers are transported out of the cell to form the extracellular capsule. The Gram-positive bacteria (left) possess integral membrane synthases (e.g., spHAS, seHAS, black; lipid bilayer, hatched) that may both catalyze sugar addition to the growing chain as well as transport the hydrophilic polymer across the membrane. Lipid molecules (white triangles) may facilitate pore formation. The approximate locations of the amino- (N) and the carboxyl- (C) termini of the synthase are depicted in white text. The Gram-negative bacteria (right) possess a more complicated polysaccharide transport apparatus (numbered white items; adapted from Whitfield and Roberts, 1999) composed of many proteins to translocate the capsular polymer (1, KpsC,M,S,T; 2, KpsE; 3, KpsD; 4, porin). The enzymes that catalyze the sugar addition are either a dual-action synthase (e.g., pmHAS or pmCS, black in this example) or a pair of single-action glycosyltransferases (e.g., KfiA and KfiC) that probably dock with the transport apparatus during polymer synthesis.

chain across the hydrophobic core of the bilayer by creating a protein/lipid pore.

On the other hand, the Gram-negative bacteria capable of GAG biosynthesis, *E. coli* and *P. multocida*, possess two lipid membranes, and their capsule loci encode many transport-associated proteins in addition to the glycosyltransferases and the UDP-GlcUA forming enzymes (~10–18 kb; Roberts, 1996; Townsend *et al.*, 2001). Although many details are not well understood, in the best-studied model, the *E. coli* Group II capsular system, it appears that transport of the nascent polymer chain requires an apparatus composed of at least seven distinct polypeptide species (Whitfield and Roberts, 1999; Silver *et al.*, 2001). Briefly, a complex containing KpsC,M,S,T assembles on the inner membrane and interacts with the KfiA,B,C catalytic complex. KpsM and T form the ATP-binding cassette (ABC) transporter. A periplasmic protein, KpsD, and a dimer of another inner membrane protein, KpsE, help transport the polymer across the periplasmic space (Arrecubieta *et al.*, 2001). A porin complex in the outer membrane is recruited to transport the growing polysaccharide chain out of the cell. Certain *Kps* mutants polymerize the capsular polysaccharide chain, but possess faulty translocation resulting in polymer accumulation in the cytoplasm or periplasm. *P. multocida* is also thought to have a Group II-like transport system based on the sequence similarities and gene arrangement of its putative transport proteins to the *E. coli* proteins.

In the case of pmHAS and pmCS, the carboxyl-terminal tail is likely to contain a docking segment that interacts with the transport mechanism (Jing and DeAngelis, 2000) (Figure 2). However, the region(s) of *E. coli* K5 enzymes responsible for docking to the transport apparatus is not known, and there is no obviously similar sequence to the carboxyl-terminus of the *Pasteurella* enzymes. Polymer transport across membranes is a difficult phenomenon to study and definitely requires more research.

Contributions of a GAG capsule to pathogenesis: molecular mimicry or molecular hitchhiking?

Some of the earliest recognized roles for capsules in infection are avoiding phagocytosis and resisting the action of complement. Other potential functions for the capsule include enhancing adhesion to host tissues, avoiding desiccation, modulating host physiology, or protecting the microbe from noxious environments. Various *Streptococcus* (Wessels *et al.*, 1994), *Pasteurella* (Chung *et al.*, 2001) and *Escherichia* (Svanborg-Eden *et al.*, 1987) mutants lacking the GAG capsule are less virulent or less infectious than the encapsulated wild-type strain in bioassays. The particular model strains in the studies above possess relatively large capsules and probably rely heavily on this virulence factor; the microbes succumb to host defenses if capsular biosynthesis is disrupted. In most cases, resistance to phagocytosis and/or complement-mediated killing is reduced in these acapsular mutants because capsules are slippery ultrastructures that physically shield the microbial surface from attack. It has been noted, however, that certain isolates are virulent but produce only small or virtually undetectable amounts of capsule. If there were multiple virulence factors in a single bacterium, it is likely that the loss of the

capsule will not be totally incapacitating. The differences in virulence among isolates probably stem from the great diversity of the organisms after eons of mutational drift and gene swapping in the microbial world; taxonomic grouping of bacteria into a species does not always mean that the genotypes and phenotypes are identical.

It has been hypothesized that producing a GAG capsule was a strategy to avoid the formation of protective antibodies that normally foil microbial attack (Quinn and Sing, 1957). The GAGs produced by *Streptococcus*, *Escherichia*, and *Pasteurella* are similar or identical to vertebrate polymers, thus anticapsule immunoglobulins are not generated to combat the microbes. Other bacteria with non-GAG capsules usually infect the host once (but never again) due to humoral and cellular responses. The anticapsule immunoglobulins serve as anchors or handles to grasp and to destroy the microbes. Unfortunately, some pathogenic species, such as *S. pneumoniae* or *E. coli*, have more than 80 discrete capsular types. The host is bombarded with a parade of different antigens over a lifetime before a “complete” repertoire of protective antibodies is induced, unless one is immunized with a polyvalent vaccine composed of a mixture of various capsular polymers (Lee, 1987).

Evidence for GAG-mediated microbial adhesion phenomena is emerging. Mimicry may be a great benefit to the microbe, but it is also probable that capsular GAGs allow the pathogen to tap into the intercellular adhesive system of the host body. The numerous and omnipresent GAG-binding host proteins may serve to anchor the pathogen to the host during infection. The HA-encapsulated *Streptococci* appear to utilize this strategy based on decreased persistence or colonization of acapsular mutants (Husmann *et al.*, 1997). Adhesion of *Pasteurella* to various phagocytic cell types has been shown; it was speculated that microbes utilized a surface HA-receptor for invasion (Pruimboom *et al.*, 1999). Encapsulated *E. coli* K5 colonize the intestine better than acapsular mutants but the mechanism is not known (Herias *et al.*, 1997).

It is interesting to speculate that GAG-coated microbes may also induce errant signaling or modified physiology that favors infection. Many scenarios are possible because GAGs are important in many processes in the healthy body, and clever microbes will certainly take any possible advantage. For example, benefits for the bacteria may include the weakening of host defenses through alterations in (1) cytokine production, (2) neutrophil recruitment, (3) hemostasis and coagulation, or (4) surface sequestering of growth factors. In the case of chondroitin or heparosan polymers, one might expect that the lack of postpolymerization modifications of the microbial GAGs would reduce the carbohydrate's effect on certain proteins that require sulfation and/or iduronic acid to interact with high affinity. However, the sheer abundance of a lower-affinity GAG polymer on the bacterial surface may counteract this deficiency to some degree.

In addition to the complicated host/pathogen interactions described, other benefits derived from the physical properties of GAG capsular polymers are possible. Acapsular Group A *Streptococcus* mutants do not grow well in laboratory media due to the accumulation of toxic levels of hydrogen peroxide, an unavoidable metabolite from respiration that cannot be decomposed by this microbe's innate pathways (Cleary and Larkin, 1979). In this case, the HA capsule was thought to act as

a shield to protect the bacteria from oxygen, thus circumventing peroxide buildup.

Future research aims and utility of bacterial GAGs

The GAG glycosyltransferases are revealing secrets on the nature of polysaccharide biosynthesis. As model systems, the GAG polymer repeating disaccharide backbone is ideal to study in comparison to homopolysaccharides that cannot be easily stopped midstream during chain elongation. GAG enzymes can be examined in a step-wise fashion by controlling UDP-sugar availability. No complicated sugar block pattern or branching is present. It is now obvious from studies of bacterial glycosyltransferases that there is more than one way to polymerize a GAG chain. Major future hurdles are to obtain the three-dimensional structures and elucidate the detailed reaction mechanisms for these fascinating GAG glycosyltransferases.

With respect to applied medical sciences, understanding microbial capsule biosynthesis may also assist the uncloning of bacterial camouflage without disrupting host GAG synthesis. Host defenses can more readily destroy a pathogen lacking a capsule. This antimicrobial approach may be quite valuable as more bacterial strains become resistant to the existing arsenal of conventional antibiotics.

GAG-producing bacteria or recombinant derivatives should serve as nonanimal sources of important medicinal products in the future. Group C *Streptococcus* is a commercial source of HA polysaccharide for surgical, ophthalmic, and viscoelastic applications. Heparin is an effective anticoagulant, and dermatan sulfate (epimerized chondroitin sulfate) shows promise as well. These GAGs are currently harvested from animal tissues, but in the future, the bacterial polymers should be able to substitute in many treatments after appropriate processing now that all the modifying enzymes have been cloned (Esko and Lindahl, 2001).

As more bacteria are characterized and their genomes are sequenced, more GAG synthases and glycosyltransferases will undoubtedly be identified to entice our imaginations and, with some tinkering, to fill our molecular toolboxes.

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Abbreviations

GAG, glycosaminoglycan; GlcUA, glucuronic acid; HA, hyaluronan or hyaluronic acid; HAS, hyaluronan synthase; PCR, polymerase chain reaction; UDP, uridine diphospho.

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