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Acceptor Specificity of the *Pasteurella* Hyaluronan and Chondroitin Synthases and Production of Chimeric Glycosaminoglycans*[S]

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The hyaluronan (HA) synthase, PmHAS, and the chondroitin synthase, PmCS, from the Gram-negative bacterium *Pasteurella multocida* polymerize the glycosaminoglycan (GAG) sugar chains HA or chondroitin, respectively. The recombinant *Escherichia coli*-derived enzymes were shown previously to elongate exogenously supplied oligosaccharides of their cognate GAG (*e.g.* HA elongated by PmHAS). Here we show that oligosaccharides and polysaccharides of certain noncognate GAGs (including sulfated and iduronic acid-containing forms) are elongated by PmHAS (*e.g.* chondroitin elongated by PmHAS) or PmCS. Various acceptors were tested in assays where the synthase extended the molecule with either a single monosaccharide or a long chain ($\sim 10^{2-4}$ sugars). Certain GAGs were very poor acceptors in comparison to the cognate molecules, but elongated products were detected nonetheless. Overall, these findings suggest that for the interaction between the acceptor and the enzyme (*a*) the orientation of the hydroxyl at the C-4 position of the hexosamine is not critical, (*b*) the conformation of C-5 of the hexuronic acid (glucuronic *versus* iduronic) is not crucial, and (*c*) additional negative sulfate groups are well tolerated in certain cases, such as on C-6 of the hexosamine, but others, including C-4 sulfates, were not or were poorly tolerated. *In vivo*, the bacterial enzymes only process unsulfated polymers; thus it is not expected that the PmCS and PmHAS catalysts would exhibit such relative relaxed sugar specificity by acting on a variety of animal-derived sulfated or epimerized GAGs. However, this feature allows the chemoenzymatic synthesis of a variety of chimeric GAG polymers, including mimics of proteoglycan complexes.

Glycosaminoglycans (GAGs),[2] polysaccharides containing a hexosamine as part of their repeat unit, serve essential biological functions in vertebrates, including adhesion, modulation of motility and proliferation, and coagulation (1–8). Certain pathogenic bacteria camouflage themselves with GAGs to increase virulence and enhance infection in their animal hosts. These capsular polysaccharides serve as molecular camouflage as well as a means to potentially hijack host functions (9). The backbones of the vertebrate GAGs, HA (-β1,4-GlcUA-β1,3-GlcNAc-), chondroitin sulfate (-β1,4-GlcUA-β1,3-GalNAc-), heparan sulfate (α1,4-GlcUA-β1,4-GlcNAc-), heparin (-α1,4-IdoUA-α 1,4-GlcNAc-), and keratan sulfate (-β1,3-Gal-β1,4-GlcNAc-), are sulfated except for HA. The bacterial GAG polymers are not known to be sulfated. The enzymes involved in the biosynthesis of all the GAG backbones, except for keratan, have been molecularly cloned.

Certain bacterial enzymes, including the Gram-negative *Pasteurella multocida* type A hyaluronan synthase, PmHAS, and the type F chondroitin synthase, PmCS, are particularly amenable to study because of their two active center architecture (10, 11), their ability to polymerize long chains *in vitro* (12), and their ability to elongate exogenously supplied acceptor oligosaccharides *in vitro* (13, 14). The *Escherichia coli* K4 chondroitin polymerase, KfoC (15), is ~60% identical to the PmHAS and PmCS; therefore, this system probably operates in a similar fashion. The streptococcal and animal HA synthase enzymes, however, do not readily utilize exogenously supplied acceptors and are more difficult to study (13). Heparosan synthases from *Pasteurella* type D, PmHS1 (16), and *Pasteurella* types A, D, and F, PmHS2 (17), promise to be interesting experimental models as well, but their overall primary structure differs substantially from PmHAS and PmCS.

The PmHAS and PmCS enzymes each possess independent hexosamine and glucuronic acid transfer sites as assessed by mutating various sequence motifs (10, 11). Kinetic studies suggest that there is a separate acceptor binding pocket for each of

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[2] The abbreviations used are: GAG, glycosaminoglycan; PmHAS, *P. multocida* hyaluronan synthase; HA, hyaluronan, hyaluronate, or hyaluronic acid; C, chondroitin; PmCS, *P. multocida* chondroitin synthase; HexNAc, *N*-acetylhexosamine; SEC, size exclusion chromatography; SAX-HPLC, strong anion exchange-high performance liquid chromatography; ESI-MS, electrospray ionization mass spectrometry; Me_2SO, dimethyl sulfoxide; Hep, heparin; MALLS, multiangle laser light scattering.

TABLE 1

Carbohydrate nomenclature, code and structure

Due to the presence of multiple sulfation isomers within any one commercial chondroitin sulfate preparation (*e.g.* CSA and CSB, etc.), the simple identification code designates the animal source of polymer (the source is underlined). The manufacturer assay values are presented for polysaccharides. The oligosaccharide structures were verified by mass spectrometry and/or NMR. R indicates functional group on hexosamine.

Code	Oligosaccharide (source)	Structure
HA_4	Hyaluronan tetrasaccharide (*S. zooepidemicus*)	$(\beta1,3\text{-GlcNAc-}\beta1,4\text{-GlcUA})_2$
CS_{bt3}	Chondroitin 4-sulfate trisaccharide (bovine trachea)	$(GalNAc\text{-}4SO_4)\text{-}\beta1,4\text{-GlcUA-}\beta1,3\text{-}(GalNAc\text{-}4SO_4)$
CS_{s3}	Chondroitin 6-sulfate trisaccharide (shark cartilage)	$(GalNAc\text{-}6SO_4)\text{-}\beta1,4\text{-GlcUA-}\beta1,3\text{-}(GalNAc\text{-}6SO_4)$
CS_{s5}	Chondroitin 6-sulfate pentasaccharide (shark cartilage)	$(C_{s3})\text{-}\beta1,4\text{-GlcUA-}\beta1,3\text{-}(GalNAc\text{-}6SO_4)$
CS_{pII3}	C-5-epimerized chondroitin 4-sulfate trisaccharide (porcine intestinal mucosa, Prep II; Celsus)	$(GalNAc\text{-}4SO_4)\text{-}\beta1,4\text{-IdoUA-}\alpha1,3\text{-}(GalNAc\text{-}4SO_4)$
CS_{pII5}	C-5-epimerized chondroitin 4-sulfate pentasaccharide (porcine intestinal mucosa, Prep II; Celsus)	$(CS_{pII3})\text{-}\beta1,4\text{-IdoUA-}\alpha1,3\text{-}(GalNAc\text{-}4SO_4)$
dCS_{pII3}	Desulfated C-5-epimerized chondroitin sulfate trisaccharide (porcine intestinal mucosa, Prep II; Celsus)	$GalNAc\text{-}\beta1,4\text{-IdoUA-}\alpha1,3\text{-GalNAc}$
Hep_5	Synthetic heparin pentasaccharide (Arixtra®)	(GlcNR′-6OR)-α1,4-(GlcUA)-β1,4-(GlcNR′-3OR-6OR)-α1,4-(IdoUA-2OR)-α1,4-(GlcNR′-6OR)) methyl glycoside, where R, R′ = SO_3
H_{e5}	Heparosan pentasaccharide (*E. coli* K5)	$GlcNAc\text{-}(\alpha1,4\text{-GlcUA-}\beta1,4\text{-GlcNAc})_2$
Code	**Polysaccharide (source)**	**Structure**
HA	Hyaluronic acid (*S. zooepidemicus*)	$(\beta1,3\text{-GlcNAc-}\beta1,4\text{-GlcUA})_{\sim200}$
C	Unsulfated chondroitin (*Pasteurella* type F)	$(\beta1,3\text{-GalNAc-}\beta1,4\text{-GlcUA})_{\sim40}$
CS_{bt}	Chondroitin sulfate (bovine trachea)	$(\beta1,3\text{-}(GalNAc\text{-}4SO_4$ or $\text{-}6SO_4)\text{-}\beta1,4\text{-GlcUA})_{\sim50}$, where 70% CSA = 4-$SO_4$, 30% CSC = 6-$SO_4$
CS_{pI}	C-5-epimerized chondroitin sulfate (porcine intestinal mucosa, Prep I; Sigma)	$(\beta1,3\text{-}(GalNAc\text{-}4SO_4)\text{-}\alpha1,4\text{-IdoUA})_{\sim50}$, where 90% CSA = 4-$SO_4$; ? % epimerized
CS_{pII}	C-5-epimerized chondroitin 4-sulfate (porcine intestinal mucosa, Prep II; Celsus)	$(\beta1,3\text{-}(GalNAc\text{-}4SO_4)\text{-}\alpha1,4\text{-IdoUA})_{\sim60}$, ~95% epimerized
dCS_{pII}	Desulfated C-5-epimerized chondroitin sulfate (porcine intestinal mucosa, Prep II; Celsus)	$(\beta1,3\text{-GalNAc-}\alpha1,4\text{-IdoUA})_{\sim25}$, ~95% epimerized
CS_s	Chondroitin sulfate (shark cartilage)	$(\beta1,3\text{-}(GalNAc\text{-}4SO_4$ or $\text{-}6SO_4)\text{-}\beta1,4\text{-GlcUA})_{\sim130}$, where 90% CSC = 6-$SO_4$, 10% CSA = 4-$SO_4$
CS_{sf}	Chondroitin sulfate (shark fin)	$((\beta1,3\text{-GalNAc-}6SO_4)\text{-}(\beta1,4\text{-GlcUA/IdoUA-}2SO_4))_{\sim100}$[a]
CS_q	Chondroitin sulfate (squid cartilage)	$((\beta1,3\text{-GalNAc-4,6-di}SO_4)\text{-}\beta1,4\text{-GlcUA/IdoUA})_{\sim350}$[a]
Hep	Heparin (porcine intestinal mucosa)	$(\alpha(\beta)1,4\text{-GlcNAc-}\alpha1,4\text{-IdoUA/GlcUA})_{\sim45}$[a,b] with various *O*- and *N*-sulfation patterns
H	Heparosan (*P. multocida* type D)	$(\beta1,4\text{-GlcNAc-}\alpha1,4\text{-GlcUA})_{\sim500}$

[a] The major disaccharide unit of the polysaccharide. Percentages for purity and/or contaminants were not assessed.
[b] Molecular weight was based on an estimated typical literature value of ~18 kDa.

these glycosyltransferase activities that apparently interacts with three or four saccharide units of the nascent HA chain (18). The sugar nucleotide specificity of native sequence enzymes has been evaluated with naturally occurring UDP-sugar donors; only the authentic monosaccharide molecules are transferred efficiently by the native enzymes (*e.g.* C-4 epimers will not substitute). All known *Pasteurella* enzymes add single sugars in a repetitive stepwise fashion to the non-reducing terminus of their cognate acceptor oligosaccharides (13, 17, 19). Here we report that PmHAS and PmCS will elongate a range of acceptor molecules in addition to their cognate sugars; this finding sheds light on the nature of the synthase active sites as well as significantly expanding the potential repertoire of oligosaccharide and polysaccharide targets that may now be synthesized.

EXPERIMENTAL PROCEDURES

Natural GAGs—All GAG polysaccharides, except for HA, are heterogeneous in nature with respect to composition and sulfation pattern. It is currently impossible to isolate GAG polysaccharides from animal sources in one pure isomeric form. Therefore, throughout the text all polysaccharides are defined with respect to their source and are given a simple nomenclature code (Table 1).

A variety of GAGs were obtained from Sigma, including *Streptococcus zooepidemicus* hyaluronan (HA), bovine trachea chondroitin sulfate (CS_{bt}) (sold as "chondroitin sulfate A"; mainly composed of chondroitin 4-sulfate), C-5 epimerized porcine intestinal mucosa chondroitin sulfate (CS_{pI}) (also referred to as dermatan sulfate and sold as "chondroitin sulfate B"), shark cartilage chondroitin sulfate (CS_s) (sold as "chondroitin sulfate C"; mainly composed of chondroitin 6-sulfate), and porcine intestinal heparin (Hep). Shark fin chondroitin sulfate (CS_{sf}) (sold as "chondroitin sulfate D") and squid cartilage chondroitin sulfate (CS_q) (sold as "chondroitin sulfate E") were obtained from Associates of Cape Cod/Seikagaku America (Falmouth, MA). In addition, a highly C-5 epimerized (>95% 4S-GalNAc-IdoUA repeats) chondroitin sulfate (CS_{pII}; a high purity dermatan sulfate from porcine intestinal mucosa similar to CS_{pI} above, but better characterized) and heparan sulfate (porcine intestinal mucosa) were obtained from Celsus Laboratories (Cincinnati, OH). Unsulfated chondroitin polysaccharide (C) and unsulfated heparosan polysaccharide was prepared from cultures of either type F or type D *P. multocida*, respectively (20). The degree of polymerization of the HA and the unsulfated chondroitin was reduced to ~80 and ~20 kDa, respectively, by autohydrolysis (121 °C, 20 p.s.i., 20 min) to be more comparable with the smaller molecular weight chondroitin sulfates (~15–40 kDa) and heparin (~17–19 kDa). In addition, 80-kDa monodisperse HA was prepared by synchronized stoichiometrically controlled chemoenzymatic reactions (12).

To avoid the heterogeneity problem intrinsic to natural GAG polysaccharides, a series of defined oligosaccharides of known

TABLE 2

Oligosaccharide derivative preparation strategy

The various polysaccharides were cleaved by GAG digestive enzymes (HAase, testicular hyaluronidase; ABCase, chondroitin ABC lyase; HepIIIase, heparin lyase III) and/or desulfated by solvolysis. Each oligosaccharide was purified by gel filtration and/or anion exchange chromatography (chrom).

HA	CS_{bt}	CS_{pII}		CS_s	Heparosan
↓ HAase ↓ chrom	↓ ABCase ↓ Hg^{2+} ↓ chrom	↓ H^+/MeOH ↓ NaOH	↓ ABCase ↓ Hg^{2+} ↓ chrom	↓ ABCase ↓ Hg^{2+} ↓ chrom	↓ HepIIIase ↓ Hg^{2+} ↓ chrom
HA_4	CS_{bt3}	dCS_{pII}	CS_{pII3} CS_{pII5} ↓ H^+/MeOH ↓ NaOH dCS_{pII3}	CS_{s3} CS_{s5}	H_{e5}

TABLE 3

Electrospray ionization mass spectral data for chondroitin sulfate derived oligosaccharides

	CS_{bt3}, CS_{s3}, CS_{pII3}	CS_{s5}, CS_{pII5}	dCS_{pII3}
Parent ion[a]	$[M - 3Na + H]^{2-} = 379.1$ (calculated, 379.1)	$[M - 2Na]^{2-} = 641.5$ (calculated, 641.5)	$[M - Na]^{-} = 599.1$ (calculated, 599.2)
Molecular ion	$[M - Na]^{-} = 803.0$ (calculated, 803.1)	$[M - Na]^{-} = 1306.0$ (calculated, 1306.1)	$[M - Na]^{-} = 599.1$ (calculated, 599.2)
Mass	826.1	1329.1	622.2

[a] Highest abundance ion is shown.

structure and length (Table 1) was prepared as described below and outlined in Table 2.

Hyaluronic Acid Tetrasaccharide (HA_4)—The tetrasaccharide HA_4 was generated by exhaustive degradation of streptococcal HA polymer with ovine testicular hyaluronidase (type V; Sigma) and purified by extensive chloroform extraction, ultrafiltration, and SEC on Bio-Gel P2 resin (Bio-Rad) (14).

Chondroitin 4-Sulfate Trisaccharide (CS_{bt3})—Chondroitin 4-sulfate from bovine trachea, CS_{bt} (1.0 g), was treated with chondroitin ABC lyase (EC 4.2.2.4, *Proteus vulgaris*; Sigma; 1 unit) in 50 mM Tris-HCl/sodium acetate buffer at 37 °C to obtain a mixture of unsaturated oligosaccharides. The resulting oligosaccharide products were fractionated on a Bio-Gel P6 (Bio-Rad) column eluted with 100 mM sodium chloride. Sized oligosaccharides were then desalted by SEC on a Bio-Gel P2 column. Charge separation of the tetrasaccharide fraction was carried out by semi-preparative strong anion exchange-high performance liquid chromatography (SAX-HPLC) (21). The major peaks were pooled, lyophilized, and desalted on a Bio-Gel P2 column. The unsaturated chondroitin sulfate tetrasaccharide containing two 4-*O*-sulfonated GlcNAc residues (1 mg) was dissolved in distilled water (1 mg/ml). To remove the nonreducing terminal unsaturated GlcUA residue, equal volumes of the oligosaccharide solution and mercuric acetate reagent (35 mM, adjusted to pH 5 using acetic acid in distilled water) were added and stirred for 15 min at room temperature. The reaction mixture was then passed through a pre-washed Dowex 50W-X8 H^+ column, neutralized with saturated sodium bicarbonate solution, and then applied to a Bio-Gel P2 column to obtain the pure trisaccharide CS_{b3}. The structure of this trisaccharide was confirmed by ESI-MS analysis (Table 3) (21).

Chondroitin 6-Sulfate Trisaccharide (CS_{s3}) and Pentasaccharide (CS_{s5})—Chondroitin 6-sulfate from shark cartilage, CS_s (50 mg), was treated with chondroitin ABC lyase (0.05 units) as above. The resulting oligosaccharide products were fractionated on a Bio-Gel P6 column, and each oligosaccharide was desalted on a Bio-Gel P2 column. The unsaturated chondroitin tetrasaccharide (1 mg) and hexasaccharide (1 mg) were treated with mercuric acetate reagent as described earlier to obtain the trisaccharide CS_{s3} and pentasaccharide CS_{s5}. The structures of these products were determined by ESI-MS analysis (Table 3).

C-5-epimerized Chondroitin 4-Sulfate Trisaccharide and Pentasaccharide (CS_{pII3} and CS_{pII5})—C-5-epimerized chondroitin sulfate from porcine intestinal mucosa, CS_{pII} (Celsus; 10 g), was treated with chondroitin ABC lyase (20 units) as above. The resulting oligosaccharide products were fractionated on a Bio-Gel P6 column, and each oligosaccharide was desalted by SEC on a Bio-Gel P2 column. Charge separation of sized oligosaccharide fractions was carried out by SAX-HPLC. The major peaks were pooled, lyophilized, and desalted on a Bio-Gel P2 column. The unsaturated C-5-epimerized chondroitin sulfate tetrasaccharide (6 mg) and hexasaccharide (2 mg) were treated with mercuric acetate reagent to obtain the pure C-5-epimerized chondroitin sulfate trisaccharide, CS_{pII3}, and pentasaccharide, CS_{pII5}. The structures of these oligosaccharides were confirmed by ^1H NMR and ESI-MS analysis (Table 3) (21).

Desulfated C-5-epimerized Chondroitin Sulfate (dCS_{pII}) Polysaccharide—The sodium salt of C-5-epimerized chondroitin sulfate (high purity dermatan sulfate from porcine intestinal mucosa; Celsus), CS_{pII} (250 mg), was treated with 250 ml of acidic methanol (1.25 ml of acetyl chloride in 250 ml of methanol) for 3 days at room temperature. The recovered solid product (methyl ester of desulfated epimerized chondroitin sulfate) was treated with 10 ml of 0.1 M sodium hydroxide for 24 h at room temperature to obtain free carboxylate, *O*-desulfated epimerized chondroitin sulfate dCS_{pII} (22). This structure was confirmed by ^1H NMR spectroscopy (23).

Desulfated C-5-epimerized Chondroitin Sulfate Trisaccharide (dCS_{pII3})—The dCS_{pII} polymer (100 mg) was treated with chondroitin ABC lyase (1 unit) as described earlier. The resulting oligosaccharide products were fractionated on a Bio-Gel P6 column, and each oligosaccharide was desalted on a Bio-Gel P2 column. Charge separation of sized oligosaccharide fractions was carried out by SAX-HPLC. The major peaks were pooled,

lyophilized, and desalted on a Bio-Gel P2 column. C-5-epimerized chondroitin tetrasaccharide without sulfate groups at GlcNAc residues (1 mg) was treated with mercuric acetate reagent to obtain the trisaccharide. The structure of this trisaccharide was confirmed by ESI-MS analysis (Table 3).

Synthetic Heparin Pentasaccharide (Hep_5)—The synthetic analog of the anti-thrombin III binding pentasaccharide, fondaparinux sodium (Arixtra®), was obtained from the pharmacy. The contents of 10 syringes (25 mg in 5 ml of isotonic saline total) were collected and dialyzed (1000-Da cutoff) against water for 2 days at 4 °C and lyophilized to obtain the pentasaccharide Hep_5 in quantitative yield.

Heparosan Pentasaccharide (H_{e5})—Heparosan polysaccharide was isolated from *E. coli* K5 and purified according to the published procedure (24). Using heparin lyase III from *Flavobacterium heparinum*, a controlled partial depolymerization was performed. The oligosaccharide mixture was size-fractionated using a Bio-Gel P6 column. Each fraction was concentrated and desalted on a Bio-Gel P2 SEC column. The unsaturated hexasaccharide was treated with mercuric acetate reagent to obtain heparosan pentasaccharide H_{e5}. Analysis by ESI-MS confirmed the structure of this product (data not shown).

Sugar Quantitation—All carbohydrates were assayed by carbazole method (25) for uronic acid using GlcUA as a standard.

Synthase Purification—The recombinant *E. coli* cells expressing the truncated, soluble dual-action catalysts PmHAS-(1–703) or PmCS-(46–695) were extracted with the permeabilization agent octylthioglucoside (1% w/v) in 1 M ethylene glycol, 50 mM Tris, pH 7.2, containing protease inhibitors (14). The extract was clarified by centrifugation and applied to a pseudoaffinity column (Tosoh Toyopearl AF-Red-650) equilibrated in the same buffer except 50 mM Hepes was substituted for Tris. The protein eluted with a NaCl gradient (0–1.5 M NaCl for 120 min), and the enzyme peak was determined by Coomassie Blue staining of SDS-polyacrylamide gels. The pooled enzyme (~95% pure PmHAS or PmCS) was concentrated with an ultrafiltration device (Amicon Ultra 15 ml, 50-kDa cutoff). The protein was quantitated by the Bradford assay with a bovine serum albumin standard (Pierce).

Synthase Assays and Acceptor Utilization Tests—PmHAS or PmCS-catalyzed polymerization was measured with radiolabeled sugar incorporation assays. UDP-GlcUA and UDP-HexNAc polymerization was recorded by monitoring incorporation of UDP-[^{3}H]GlcUA as denoted in Equation 1,

$$\text{acceptor} + n\ \text{UDP-[}^3\text{H]GlcUA} + n\ \text{UDP-HexNAc} \rightarrow 2n\ \text{UDP} + (\text{[}^3\text{H]GlcUA-HexNAc})_n - \text{acceptor} \quad \text{(Eq. 1)}$$

Typically, 25-μl reactions containing ~1 μg (~13 pmol) of enzyme, PmHAS-(1–703) or PmCS-(46–695), and ~0.01 nmol to 3.75 nmol of a given acceptor were employed. A "no acceptor" control was used for all analyses; this background value corresponding to *de novo* polymer initiation was subtracted from the value obtained from the assay with acceptor. Assays with an HA polymer acceptor were employed in every data set to allow for normalization. Polymerization reactions contained 50 mM Tris, pH 7.2, 1 M ethylene glycol, 2 mM $MnCl_2$, 0.05 mM UDP-[^{3}H]GlcUA (1 μCi; PerkinElmer Life Sciences), and 0.2 mM UDP-HexNAc (UDP-GlcNAc for PmHAS *or* UDP-GalNAc for PmCS) and were incubated at 30 °C for either 3 min (polysaccharide acceptors) or 30 min (oligosaccharide acceptors). Reactions were stopped by placing on ice and adding SDS (2% final w/v). Descending paper chromatography was used to separate the unincorporated radiolabel from the elongated acceptors (10). The assays were performed in duplicate and were linear with respect to enzyme concentration and time; less than 5% UDP-sugar substrate was consumed.

Single sugar additions were also monitored by reverse phase HPLC ESI-MS (26). Briefly, similar reaction conditions were utilized as described above, except only single unlabeled UDP-sugar (2–6 mM final) was employed. The enzyme adds a monosaccharide unit onto the nonreducing end of various acceptors as shown in Equation 2 or 3.

$$\text{acceptor} + \text{UDP-GlcUA} \rightarrow \text{UDP} + (\text{GlcUA} - \text{acceptor}) \quad \text{(Eq. 2)}$$

$$\text{acceptor} + \text{UDP-HexNAc} \rightarrow \text{UDP} + (\text{HexNAc} - \text{acceptor}) \quad \text{(Eq. 3)}$$

Chimeric GAG Synthesis—The purified enzymes PmHAS-(1–703) or PmCS-(46–695) (~1 μg) were used to add unlabeled HA or chondroitin chains, respectively, to various GAG polysaccharide acceptors (~8–225 μg). Reactions (25 μl) contained the same reaction buffer as above except that 2–4 mM UDP-GlcUA and 2–4 mM UDP-HexNAc (UDP-GlcNAc for PmHAS *or* UDP-GalNAc for PmCS) were utilized at 30 °C for varying times (see Equation 1, but without the ^{3}H label).

Size Analysis of Polysaccharides—Polymers were analyzed using 1–1.2% 1× TAE-agarose gels (30 V, 5 h, Stains-All detection) (27). The specific *Streptomyces* hyaluronate lyase from Sigma was employed to destroy and thus identify authentic HA chains. Defined HA molecular weight standards were from (Hyalose L.L.C., Oklahoma City, OK) (12). Kilobase DNA standards were from Stratagene (La Jolla, CA).

Analytical high performance SEC was performed with PLaquagel-OH 60, -OH 50, -OH 40 columns in tandem (15 μm, 7.5 × 300 mm, Polymer Laboratories Amherst, MA) eluted with 50 mM $NaPO_4$, 150 mM NaCl, pH 7, at 0.4–0.5 ml/min. Multiangle laser light scattering (MALLS) detection was performed to quantify absolute molecular weights (12).

RESULTS AND DISCUSSION

The finding that recombinant PmHAS, and later PmCS, was able to elongate certain small oligosaccharides, including HA_4 and chondroitin sulfate hexasaccharides, respectively (13, 19), led to a broader investigation of the potential range of acceptor usage (Tables 4 and 5). In contrast, the other distinct bacterial HA synthase enzymes from group A and C *Streptococcus* cannot be tested at this time because of their inability to elongate exogenously added acceptors.

E. coli KfoC, chondroitin polymerase, was shown previously to elongate certain polysaccharides and oligosaccharides, but the polymers were not rigorously defined with respect to sulfation isomers (15). Also, the desulfated chondroitin preparations may have suffered oxidative degradation that hindered enzy-

TABLE 4

PmHAS and PmCS polysaccharide acceptor specificity

Polymerization assays with each acceptor (at least three experiments in duplicate) were performed. The value of no acceptor control (~300–600 dpm) was subtracted from each point. The averaged values for PmHAS or PmCS are presented. The ratio (nanomoles of test acceptor/nmol of HA standard) was employed to normalize to the HA signal (30,000 dpm for 0.02 nmol of HA), which was set to 100%. For all results, * = $p < 0.05$ and ** = $p < 0.01$ in a Student's one-tailed *t* test for comparison of a given test acceptor to the no acceptor control assay. ND indicates not determined. Both enzymes may utilize either HA or chondroitin polymers, but heparin and heparosan are very poor acceptors or slightly inhibitory.

Polysaccharide	Relative PmHAS activity normalized to HA	Relative PmCS activity normalized to HA
	%	%
HA	100**	100**
C	54**	40**
CS_{bt}	12**	4.1**
CS_{pI}	0.4**	0.2**
CS_{pII}	0.5**	0.3**
dCS_{pII}	2.4**	0.7**
CS_s	10**	5.0**
CS_{sf}	2.4**	1.4**
CS_q	5.1**	0.9**
Hep	0.03*	−0.1*
H	0.1**	ND

TABLE 5

PmHAS and PmCS oligosaccharide acceptor specificity

Polymerization assays with each acceptor (at least three experiments in duplicate, except for H_{e5}) were performed. The value of no acceptor control (~300–600 dpm) was subtracted from each point. The averaged values for PmHAS or PmCS are presented. The ratio (nanomoles of test acceptor/nmol of HA_4) was employed to normalize to the HA_4 signal (62,000 dpm for 0.15 nmol of HA_4), which was set to 100%. For all results, * = $p < 0.05$ and ** = $p < 0.01$ in a Student's one-tailed *t* test for comparison of a given test acceptor to the no acceptor control assay. ND indicates not determined. Both enzymes efficiently utilize the 6-sulfated chondroitin oligosaccharide but not the 4-sulfated isomer. Again, the heparin and heparosan oligosaccharide are very poor acceptors or slightly inhibitory.

Oligosaccharide	Relative PmHAS activity normalized to HA_4	Relative PmCS activity normalized to HA_4
	%	%
HA_4	100**	100**
CS_{bt3}	3.6**	5.0*
CS_{s3}	80*	190*
CS_{s5}	61*	140**
CS_{pII3}	0.3*	3.9*
CS_{pII5}	0.4*	4.1*
Hep_5	−0.1**	ND
H_{e5}	1.6	ND

matic utilization (15). Our data show that both HA and unsulfated chondroitin polysaccharides are very good acceptors for HA or chondroitin polymerization by PmHAS or PmCS, respectively (Table 4). The difference between the HA and the chondroitin backbone is the identity of the hexosamine residue (Fig. 1). HA contains GlcNAc while chondroitin contains the C-4 epimer, GalNAc. For both PmHAS and PmCS, it appears that the acceptor site or nascent chain retention site of the enzyme probably does not recognize the C-4 hydroxyl group by a direct bonding interaction or does not form a close association with this region of the pyranose ring. As mentioned earlier, KfoC was reported to elongate chondroitin worse than chondroitin sulfate, but as those authors admitted, the solvolytic desulfation reaction may have damaged the polymer. On the other hand, the *Pasteurella* chondroitin tested here is an unsulfated polymer thus eliminating any issues of potential damage because of side reactions during solvolysis. Both PmHAS and PmCS proteins also utilize the small HA tetrasaccharide, HA_4, with high efficiency (Table 5).

The conundrum of an impure bulk GAG polymer population

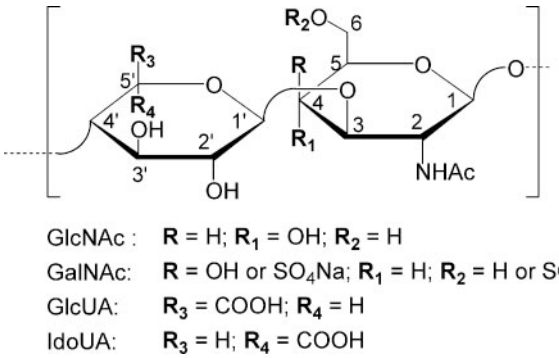

FIGURE 1. **Structures of GAG repeats.** The various HA or chondroitin-based acceptors contain repeats delineated in the Haworth representation. The *R* groups are the positions that were probed for their role in function with the *Pasteurella* synthases in this study.

found in natural extracts was addressed here by employing defined (*i.e.* HPLC-purified, mass spectrometry, and NMR-validated) oligosaccharides with known sulfation patterns. The nature of the sulfation pattern seems to be an important characteristic on the extent of utilization of the modified acceptor by both PmCS and PmHAS. For example, the 6-sulfated chondroitin trisaccharide (CS_{s3}) and chondroitin pentasaccharide (CS_{s5}) served as relatively good acceptors for PmHAS. In contrast, the 4-sulfated chondroitin trisaccharide (CS_{bt3}) preparation was a poor acceptor (Table 5). In fact, the signals in the radiolabeled incorporation assay of CS_{bt3} appear to be due entirely to a trace amount of pentasaccharide with mixed 4- and 6-sulfates contaminating the preparation based on ESI-MS of the PmHAS-extended mixture. The starting CS_{bt3} trisaccharide peak (calculated 760.12 Da; observed 759.1) was not extended into the predicted tetrasaccharide product (calculated 936.15 Da); a major fraction of the pentasaccharide, however, was extended into a hexasaccharide possessing the appropriate mass.

The bulk 4-sulfated chondroitin polysaccharide (CS_{bt}) did serve as an acceptor for PmHAS and PmCS, but this polymer is not composed entirely of the 4-sulfated isomer; these preparations are actually a 70:30 mixture of GalNAc-4/6SO_4 isoforms (Table 1). The 6-sulfated sequences may be responsible for the signal observed. In summary, as long as the nonreducing terminal GalNAc residue either possesses a 6-sulfate group or lacks a sulfate group, both *Pasteurella* enzymes should elongate the polymer quite readily. These findings suggest that a bulky negative group at the C-4 position of the hexosamine is causing an unfavorable interaction (steric and/or charge repulsion) with the synthase polypeptide because the HA and unsulfated chondroitin are good acceptors. The 6-sulfate trisaccharide is better tolerated suggesting that the C-6 hydroxyl of the HexNAc (Fig. 1) is neither in close proximity nor in a bonding interaction with the synthase.

Two other sulfated chondroitin polysaccharides containing GalNAc-6SO_4, CS_{sf} and CS_q, show some activity as acceptors (Table 4), but both CS_{sf} and CS_q also have an additional sulfate group; CS_{sf} contains GlcUA-2SO_4, whereas CS_q contains GalNAc-6/4-disulfate. These polymers serve as modest acceptors (2.4 and 5.1%, respectively, for PmHAS) but are not as good as CS_{bt} (12%) and CS_s (10%). Again, the relative activity of the various natural chondroitin preparations to HA may be affected by the fact that these polymers are not 100% pure and can contain additional minor sequences at the nonreducing termini.

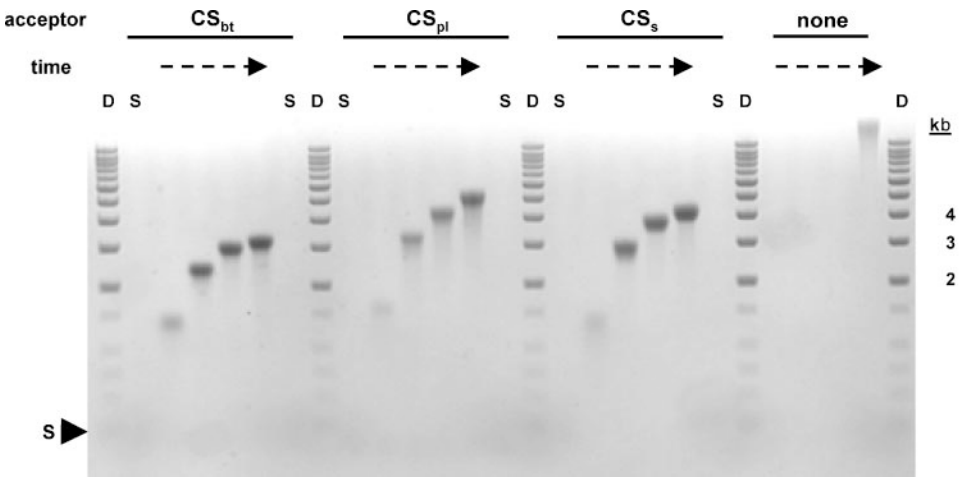

FIGURE 2. **PmHAS-catalyzed addition of HA chains onto various sulfated chondroitin acceptors.** Reactions with various acceptors (CS_{bt}, chondroitin sulfate, bovine trachea; CS_{pI}, C-5-epimerized chondroitin sulfate, porcine intestinal mucosa, preparation I; CS_s, chondroitin sulfate, shark cartilage) were incubated for various times (2, 4, and 6 h or overnight) and separated on a 1% agarose gel with Stains-All detection. Additional aliquots of each UDP-sugar (2 mM final) were added at the 2- and 4-h time points. The chondroitin sulfate starting materials (*S*) for each reaction were run on both sides of the 2-h and overnight time points (migration distance indicated with an *arrowhead*). A no acceptor control denoting *de novo* initiation of HA is shown as well; only a small amount of high molecular weight HA polysaccharide forms after extended incubation times without acceptor present. *D*, kb DNA ladder, 12, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1.5, 1, 0.75, 0.5, 0.25 kb from *top* to *bottom*.

Chondroitin sulfate B polysaccharides (CS_{pI} and CS_{pII}) contain IdoUA, the C-5 epimer of GlcUA (Fig. 1), and contain GalNAc-4SO_4 residues; these polymers possess very low activity (0.4 and 0.5%; see Table 4). Both polymers also contain a low percentage (4%) of an additional sulfate, IdoUA-2SO_4, which may contribute to their reduced activity as acceptors (Table 4). To further assess the roles of IdoUA and 4-SO_4, pure chondroitin sulfate B oligosaccharides with specified modification patterns were analyzed as acceptors. CS_{pII3} (C-5-epimerized chondroitin 4-sulfate trisaccharide) and CS_{pII5} (C-5-epimerized chondroitin 4-sulfate pentasaccharide) oligosaccharides verified the results seen with CS_{pI} and CS_{pII} (Table 5); these oligosaccharides are poor acceptors.

Desulfated C-5-epimerized chondroitin sulfate B (dCS_{pII}) was also assayed to determine whether the IdoUA or sulfate groups (Fig. 1) were the problematic structures for the synthases. The desulfated C-5-epimerized polysaccharide, dCS_{pII}, showed 2.4% activity relative to HA, higher than that of the 4-sulfated epimerized chondroitin polymers (CS_{pI} and CS_{pII}, 0.4 and 0.5%, respectively; see Table 4) but lower than that of the unsulfated C polymer (54%). We hypothesized that PmHAS and PmCS may be able to tolerate IdoUA to some degree due to the increase in signal in dCS_{pII} (after de-sulfation) when compared with CS_{pI} and CS_{pII}. There is a chance, however, that IdoUA was not well tolerated. The observed signal may have actually been because of the nonreducing terminus of some chains in the polysaccharide population containing an accessible GlcUA, the native uronic acid for the synthase, instead of IdoUA (*i.e.* CS_{pII}, the original material that was de-sulfated to make dSC_{pII}, contains ~95% IdoUA, and thus there is an ~5% chance of the GlcUA residing at the nonreducing terminus of the polymers). To further address this issue, a defined IdoUA-containing trisaccharide, dCS_{pII3}, was subjected to a single sugar addition reaction (Eq. 2) with PmHAS. Reverse phase HPLC-ESI-MS analysis verified that the trisaccharide (calculated 600.2 Da; observed 599.2 Da) was converted to the expected tetrasaccharide (calculated 776.23 Da; observed 775.1 Da). Therefore, IdoUA is tolerated by PmHAS. An exact comparison of the GlcUA *versus* IdoUA utilization rates will need to await the preparation of larger amounts of defined dCS_{pII3} oligosaccharide. The difference between the GlcUA and the IdoUA epimers is the conformation of the sugar ring. Even though both monosaccharide units have the carboxylate in the equatorial position, two different chair forms ($^4C_1$ *versus* $^1C_4$ for GlcUA or IdoUA, respectively) are present and changes the linkage from β to α upon epimerization.

Two antithrombotics, the synthetic heparin pentasaccharide, Arixtra (Hep_5), and bulk heparin polysaccharide (Hep), show very low (0.03%, Hep) relative acceptor activity to HA or were inhibitory (−0.1%, Hep_5) with PmHAS (Tables 4 and 5). The unsulfated heparosan oligosaccharide (1.6%, H_{e5}) and polysaccharide (0.1%, H) tested show slightly more activity than the sulfated heparin oligosaccharide and polysaccharide, respectively. It may be that the repeated GlcUA groups among all acidic GAGs can partially compensate for the improper sugar backbone linkages, albeit poorly. However, too many additional negative groups (*i.e.* sulfate groups) may also be detrimental.

Overall, PmCS exhibited similar acceptor specificity relative to PmHAS (Tables 4 and 5) except that the various chondroitin oligosaccharides were better acceptors for the chondroitin synthase, perhaps reflecting its optimization for chondroitin biosynthesis. A few minor potential differences among the various acceptors were noted, but at this time limiting amounts of sugar reagents precluded further ranking.

Perhaps it is unexpected that the PmHAS and PmCS enzymes would elongate noncognate or sulfated GAGs, but it is not illogical. Some glycosyltransferases have been reported to be promiscuous with respect to their substrate recognition. Also, in living bacteria, the synthase enzymes in the cell interior are not expected to be confronted with noncognate acceptor molecules; thus these catalysts do not need to be selective. On the other hand, the synthase donor sites (especially the UDP-hexosamine pocket) are exposed to structurally similar UDP-sugars, and thus more stringent selectivity for the authentic substrate is expected and is observed.

A potential biotechnological utility of the relaxed acceptor usage (*i.e.* the ability of these particular synthases/polymerases to accept unnatural substrates) is the creation of GAG molecules with two or more types of sugar repeating units. A variety of chimeric polymers consisting of two distinct GAG polysaccharides fused together were produced (Figs. 2 and 3). The synthase-catalyzed reactions were rapid (complete within 2–6 h). The length of the added HA or chondroitin chain may be con-

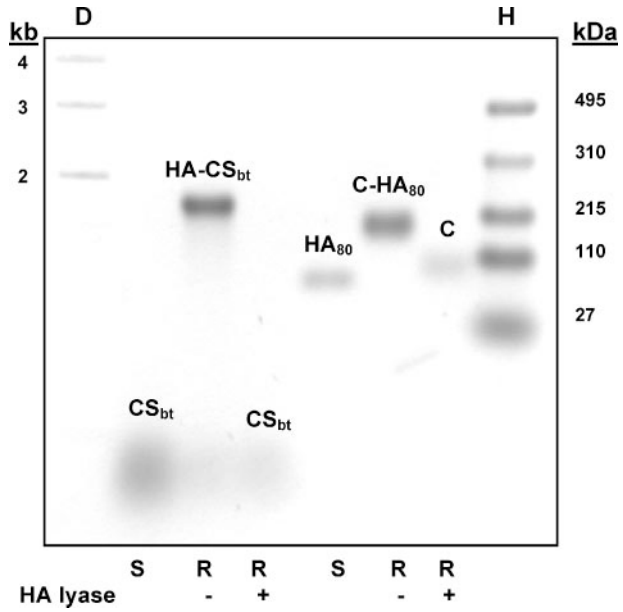

FIGURE 3. **Analysis of HA-CS$_{bt}$ and C-HA$_{80}$ chimeric GAGs.** In the first example, CS$_{bt}$ (chondroitin sulfate, bovine trachea) was extended with HA by PmHAS to produce the HA-CS$_{bt}$ chimeric product (~430 kDa by SEC-MALLS; the chondroitin sulfate component makes the chimeric product run faster on agarose gels than HA alone). HA-CS$_{bt}$ was then treated with the specific *Streptomyces* HA lyase (will not degrade chondroitin or chondroitin sulfate) to remove the HA chain, recreating the original CS$_{bt}$. In the complementary fashion, monodisperse HA (HA$_{80}$, 80-kDa HA polymer) was extended with unsulfated chondroitin to form the C-HA$_{80}$ chimeric product (~280 kDa by SEC-MALLS). The C-HA$_{80}$ molecule was then treated with HA lyase leaving the ~100-kDa (or ~500 saccharide units) chondroitin (*C*) extension remaining. *S.* starting material; *R.* reaction mixture; *D*, 4-, 3-, and 2-kb DNA standards; *HA*, Hyalose Select-HA Lo Ladder.

trolled by altering the stoichiometry of UDP-sugar to acceptor (12). For example, chondroitin sulfate was extended by PmHAS with HA chains ranging from ~400 kDa (~2,000 saccharides; Fig. 3) to ~1,600 kDa (~8,000 saccharides) depending on the donor/GAG acceptor ratio. In addition, the chimeric polymers appear to be relatively monodisperse populations (for example, in Fig. 3, polydispersity Mw/Mn values: HA-CS$_{bt}$, 1.006 ± 0.02; C-HA, 1.003 ± 0.02; for reference, "1" is the ideal polymer preparation).

We verified that HA was not contaminating the chondroitin sulfate preparations by pre-treating with HA lyase; this enzyme produces HA oligosaccharides with defective unsaturated nonreducing ends that cannot be extended by *Pasteurella* synthases (data not shown). In addition, the Stains-All detection method for agarose gels can distinguish chondroitin sulfate from HA. The sulfated polymers yield a purple or yellow color (depending on sulfation level), whereas the HA is blue; the chimeric molecules have a purple color readily distinguishable from the HA alone (supplemental data, Fig. S1). Furthermore, the chimeric nature of the new GAGs is demonstrated by treatment with specific HA lyase (Fig. 3). For example, an HA chain was extended with a chondroitin chain via the action of PmCS. The chimeric molecule was then treated with HA lyase, digesting the HA chain, leaving the chondroitin chain component intact.

We did note that the entire bulk chondroitin sulfate polymer starting material was not incorporated into chimeric molecules even after repeated addition of synthase and UDP-sugars; this observation suggests that certain nonreducing termini (especially the 4-sulfated chondroitin units) cannot be efficiently extended by native sequence PmHAS or PmCS enzymes. At the start of a reaction utilizing a poorly functioning noncognate acceptor, the *Pasteurella* synthase slowly extends the polymer, but once the catalyst adds on a short cognate sugar extension to the nonreducing terminus, the nascent molecule is transformed rapidly into an excellent acceptor. At longer times (*e.g.* approximately >2 h), the differences because of the initial lag period are obscured, and thus the differences among the various chondroitin sulfate preparations are less apparent.

The only other reported methods for chemoenzymatically constructing chimeric GAGs involved the use of testicular hyaluronidase to (*a*) transglycosylate GAG chains (28, 29) or (*b*) couple oxazoline monomers (30). Testicular hyaluronidase catalyzes the cleavage of HA, chondroitin, or chondroitin sulfate using a water hydroxyl with an optimum at pH 5. However, the hyaluronidase can also use a polymer hydroxyl group thus adding saccharide units instead of cleaving in a reaction called transglycosylation with an optimum at pH 7. Unfortunately, the transglycosylation method is difficult to control and has low yields; a mixture of products (*i.e.* a series of structurally similar chimeric GAGs with ~6–22 saccharide units) result, and the degrading enzyme will actually cleave the products. In the latter elegant method, oxazoline sugar analogs (*e.g.* employing mixtures of HA, chondroitin, or chondroitin sulfate disaccharides, etc.) mimicking the transition state are coupled by the hyaluronidase. However, these analogs are unstable in water resulting in a potential overabundance of "dead-end" acceptors (the oxazoline analog degrades to an ordinary disaccharide) compared with the activated donor that drives down the size distribution of the final products (~10–20 kDa or ~50–100 sugars reported). In addition, the product degradation by hyaluronidase problem mentioned above in method *a* also exists. Also, the exact placement of desired sugars within a given target structure (especially for small chains) is difficult or impossible for the oxazoline process. On the other hand, the HA and chondroitin synthases and their mutants may be utilized in a stepwise fashion to make defined GAG oligosaccharides (14).

Proteoglycan-like molecules with two distinct high molecular weight GAG components, but missing the protein bridge (*e.g.* core and link proteins), can be assembled by our method. These molecules may potentially serve the field of tissue engineering as scaffolds to assemble various cell types into tissues or organs. The lack of any polypeptide in these artificial chimeric molecules removes the issues of protease susceptibility and concerns of both immunogenicity and allergenicity (*e.g.* when an animal-derived product is used in humans). Furthermore, the threat of adventitious agents (*e.g.* prions, virus) from animal sources may be diminished or eliminated.

On a smaller scale, novel oligosaccharides with HA-like and/or chondroitin-like structures may also be constructed using the *Pasteurella* synthases.[3] We are exploring if these molecules bind with different selectivity or affinity to hyaladherins, HA-binding proteins, or receptors. In addition to mapping out the oligosaccharide binding requirements of hyaladherins, we

[3] B. S. Tracy, A. E. Sismey, and P. L. DeAngelis, unpublished observations.

may be able to generate compounds that will selectively inhibit the binding of HA to one particular hyaladherin species without perturbing other species. Such sugar molecules may have future utility as selective therapeutics with minimal side effects for diseases such as cancer, autoimmune disease, inflammation, and infection.

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