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Comamonas testosteronan synthase, a bifunctional glycosyltransferase that produces a unique heparosan polysaccharide analog

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Glycosaminoglycans (GAGs) are linear hexosamine-containing polysaccharides. These polysaccharides are synthesized by some pathogenic bacteria to form an extracellular coating or capsule. This strategy forms the basis of molecular camouflage since vertebrates possess naturally occurring GAGs that are essential for life. A recent sequence database search identified a putative protein from the opportunistic pathogen *Comamonas testosteroni* that exhibits similarity with the *Pasteurella multocida* GAG synthase PmHS1, which is responsible for the synthesis of a heparosan polysaccharide capsule. Initial supportive evidence included glucuronic acid (GlcUA)-containing polysaccharides extracted from *C. testosteroni* KF-1. We describe here the cloning and analysis of a novel *Comamonas* GAG synthase, CtTS. The GAG produced by CtTS in vitro consists of the sugars D-GlcUA and *N*-acetyl-D-glucosamine, but is insensitive to digestion by GAG digesting enzymes, thus has distinct glycosidic linkages from vertebrate GAGs. The backbone structure of the polysaccharide product [-4-D-GlcUA-α1,4-D-GlcNAc-α1-]$_n$ was confirmed by nuclear magnetic resonance. Therefore, this novel GAG, testosteronan, consists of the same sugars as the biomedically relevant GAGs heparosan (*N*-acetylheparosan) and hyaluronan but may have distinct properties useful for future medical applications.

Keywords: Comamonas / glycosaminoglycan / glycosyltransferase / heparosan / polysaccharide

Introduction

Certain pathogenic microbes employ extracellular capsules of host-like glycans to evade host defenses and to increase virulence (DeAngelis 2002). Previous work in our laboratory and others has identified very distinct types of microbial glycosaminoglycan (GAG) synthases, the bifunctional enzymes that assemble GAG polysaccharides. These synthases include peripheral membrane-associated two domain enzymes such as the *Pasteurella multocida* GAG synthases PmHAS (hyaluronan, HA; DeAngelis et al. 1998), PmCS (chondroitin; DeAngelis and Padgett-McCue 2000), PmHS1 and PmHS2 (heparosan; DeAngelis and White 2002; DeAngelis and White 2004; Figure 1A) and KfoC (chondroitin) from *Escherichia coli* K4 (Ninomiya et al. 2002), as well as integral membrane proteins with unknown domain structures such as the *Streptococcus pyogenes* HA synthase (DeAngelis et al. 1993) and the *Chlorella* virus paramecium bursaria chlorella virus (PBCV)-1 HA synthase (DeAngelis et al. 1997). All the known GAG synthases employ uridine diphosphate (UDP)-sugar precursors to form the repeating disaccharide units (DeAngelis 2002). The *Streptococcus* HA synthase has some similarity with vertebrate HA synthases at the amino acid sequence level (Weigel and DeAngelis 2007), but the bacterial chondroitin and heparosan synthases (HSs) are quite different from their vertebrate counterparts (DeAngelis and Padgett-McCue 2000; DeAngelis and White 2002).

Recently, we reported the distinct catalytic phenotypes exhibited by the two *P. multocida* HSs (Sismey-Ragatz et al. 2007). As a part of our efforts to better understand the mechanism of these GAG synthases, including the structure/function relationship that manifests donor and acceptor specificity, a search of the NCBI sequence databases identified a potential bifunctional glycosyltransferase (GT) (ZP_03542636; 32% identity, Supplementary data, Figure S1) in the genome of the *Comamonas testosteroni* KF-1 isolate with a region of sequence similarity with the carbohydrate-active enzymes (CAZy) (http://www.cazy.org) GT45 family of GTs (Cantarel et al. 2009; Drummond et al. 2010). The CAZy GT45 family of proteins contains only eight members; as of January 2011, the CAZy GT database contained 92 families with ~65,000 GT modules. The bifunctional *P. multocida* HSs contain this relatively rare GT45 domain that in combination with a GT2 domain synthesizes the GAG heparosan that comprises the capsule of type D *P. multocida*.

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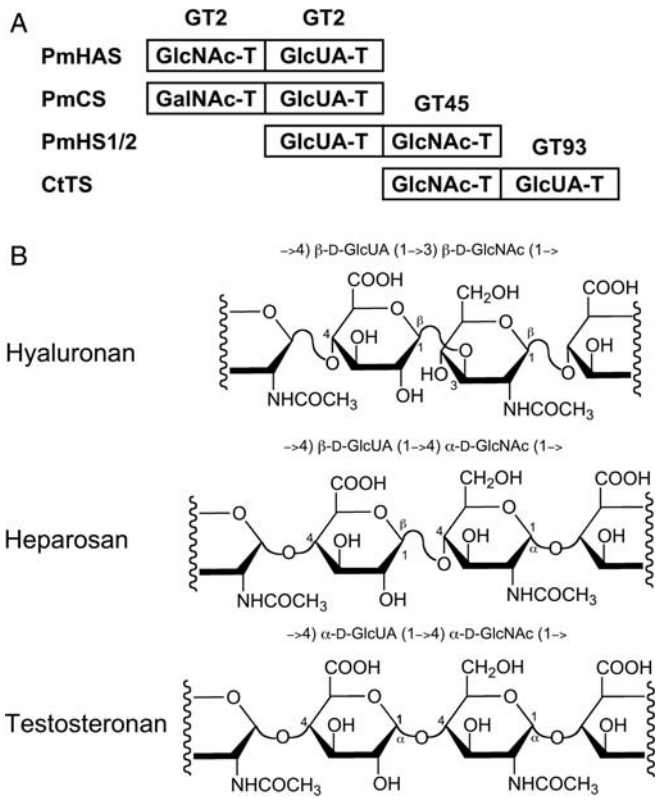

Fig. 1. Schematic alignment of the bifunctional *Pasteurella* GAG synthases with the *Comamonas* testosteronan synthase and their GAG products. (**A**) The PmHS GT45 domain is 32% identical to that of the *Comamonas* synthase (CtTS). There are only eight predicted members of the GT45 family. Bioinformatic analyses of the putative GlcUA transferase domain of the *Comamonas* synthase indicate that this is a new CAZy GT family member designated GT93 (Dr. Bernard Henrissat, personal communication). (**B**) The GAG repeat disaccharide structures of HA, heparosan and testosteronan are shown as Haworth structures; these GAGs all possess the same monosaccharide units, but in different glycosidic linkages (chondroitin has the same structure as HA, but with a GalNAc unit substituting for GlcNAc).

Another similar pair of proteins, the single-action GTs KfiA and KfiC of *E. coli* K5, together also make heparosan; the former is a GT45-containing enzyme. In the studied GT45 enzymes, the activity is a retaining GT. For PmHS1, PmHS2 and KfiA, an α1,4-linked D-GlcNAc is formed; thus, these catalysts have utility for generating a linkage found in heparosan, the precursor polysaccharide to heparan sulfate and heparin.

C. testosteroni is a Gram-negative aerobic bacteria that is found in diverse environments (Ma et al. 2009). Bacteria of the genus *Comamonas* are predominant in activated sewage sludge (Dias and Bhat 1964) and are defined by a poor ability to use carbohydrates; instead carbon is derived from molecules such as testosterone and other cyclic hydrocarbons (Linares et al. 2008; Horinouchi et al. 2010). *C. testosteroni* has recently been identified as an opportunistic human pathogen that has been found in various hospital infections including meningitis (Arda et al. 2003; Jin et al. 2008), bacteremia (Gul et al. 2007) and endophthalmitis (Reddy et al. 2009). The ability for *C. testosteroni* to survive and thrive in such diverse environments, as well as its potential use for cleaning up environmental contamination with xenobiotic compounds such as polychlorinated biphenyls and linear alkylbenzene sulfonate make it a particularly interesting organism (Schleheck et al. 2004, 2010). There is only one published study indicating the presence of a mucoid exopolysaccharide capsule of *C. testosteroni* A20 (Bossier and Verstraete 1996); however, there is no genomic information available for this strain and nature of the polysaccharide was not determined. We hypothesized that the ZP_03542636 gene might be responsible in part for forming a GAG-like polysaccharide in *C. testosteroni* KF-1.

In this work, we demonstrate that this *C. testosteroni* KF-1 gene product is a novel bifunctional GAG synthase (possessing an N-terminal GT45 domain and a new prototype GT family domain GT93 at the C-terminus) that we call "CtTS". The polysaccharide backbone produced by CtTS is a previously unidentified GAG, which we call "testosteronan", possessing the structure $[\text{-4-D-GlcUA-}\alpha\text{1,4-D-GlcNAc-}\alpha\text{1-}]_n$ (Figure 1B).

Results

Donor sugar specificity of CtTS

By sequence comparison with the *Pasteurella* HSs, CtTS is predicted to possess α-GlcNAc-transferase activity due to the presence of a GT45 family domain. However, both PmHAS and PmCS also exhibit high sequence identity, yet they transfer distinct monosaccharides from the donor molecules UDP-GlcNAc and UDP-GalNAc, respectively (Jing and DeAngelis 2000). In order to determine the preferred hexosamine sugar donor utilized for putative GAG-like heteropolysaccharide biosynthesis, we performed activity assays with clarified lysates from recombinant bacteria expressing CtTS using two different UDP-sugars simultaneously in polymerization reactions in vitro. Specifically, we employed radiolabeled UDP-[^{3}H]GlcUA (glucuronic acid) as a traceable precursor and either unlabeled UDP-GlcNAc, UDP-GalNAc or UDP-Glc as the second precursor. Significant activity was seen in the presence of UDP-GlcNAc, but not with UDP-GalNAc or UDP-Glc (Figure 2, top); without any UDP-hexosamine, the signal due to incorporation of [^{3}H] GlcUA was the same as vector-alone control lysates. This result was confirmed with the converse polymerization experiment which was performed with radiolabeled UDP-[^{3}H] GlcNAc and unlabeled UDP-GlcUA. To assess if this uronic acid was the preferred donor, we also performed radiochemical incorporation assays with UDP-[^{3}H]GlcNAc in the presence of either UDP-GlcUA, UDP-galacturonic acid, UDP-iduronic acid (Weïwer et al. 2008) or UDP-Glc as the second precursor; only the reaction mixtures containing UDP-GlcUA showed significant incorporation (Figure 2, bottom). Therefore, simultaneous incubation of lysates containing the recombinant *CtTS* gene product with both UDP-GlcUA and UDP-GlcNAc was required for the polymeric signal. In summary, we hypothesize that CtTS is a bifunctional enzyme capable of forming polysaccharide composed of GlcNAc and GlcUA in vitro. These are the same two sugars found in both HA and heparosan. By further

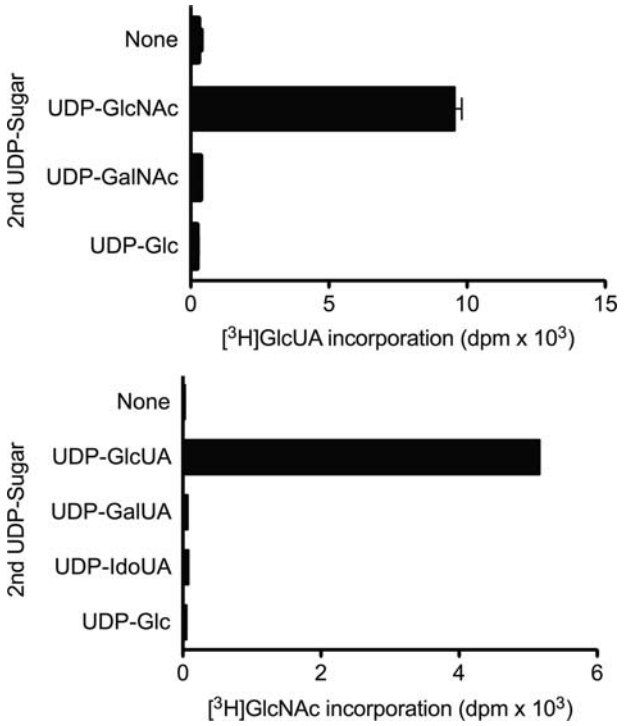

Fig. 2. Donor specificity of CtTS. Activity assays were performed using recombinant *Comamonas* testosteronan synthase with either radiolabeled UDP-[^{3}H]GlcUA (top panel) or UDP-[^{3}H]GlcNAc (bottom panel) and the indicated unlabeled UDP-sugar or no precursor (None). Vector only lysates in the presence of UDP-[^{3}H]GlcUA or UDP-[^{3}H]GlcNAc gave values of ~200 dpm. These activity assays reveal a preference for UDP-GlcNAc as the hexosamine sugar, and UDP-GlcUA as the uronic acid sugar, the identical precursors employed by both heparosan and HA synthases.

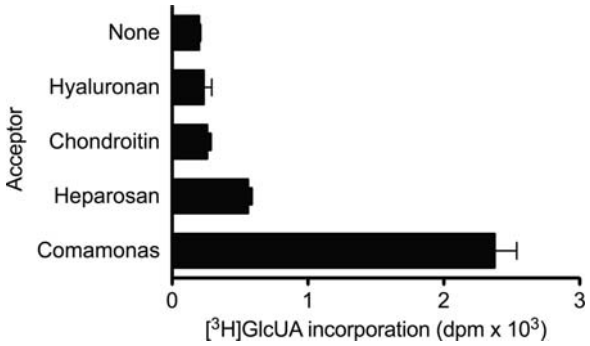

Fig. 3. Acceptor specificity of CtTS. Extension of exogenous polysaccharide by recombinant *Comamonas* testosteronan synthase was measured. Activity assays were performed with radiolabeled UDP-sugars in the presence of HA, unsulfated chondroitin, heparosan or sonicated *Comamonas* polysaccharide. This result was reproduced with HA and heparosan tetrasaccharide acceptors; only the heparosan tetrasaccharide gave a substantial signal. The *Comamonas* synthase shows a preference for both heparosan and the *Comamonas* polysaccharide as acceptor molecules.

studying acceptor specificity and using GAG-degrading enzymes that are specific for these GAGs, we set forth to characterize the nature of the polysaccharide formed by CtTS in vitro.

Acceptor specificity of CtTS

To identify the acceptor preference of CtTS, we performed radiolabeled sugar polymerization assays in the presence of various GAG acceptors. In many, but not all, GAG synthases, exogenous cognate polysaccharide will increase the signal in sugar incorporation assays by bypassing the slower de novo initiation phase (DeAngelis 1999; Jing and DeAngelis 2004); the elongation phase is much more rapid thus higher activity is observed. Typically, non-cognate GAG polymers with different glycosidic linkage patterns are very poor or non-functional acceptors for the known GAG synthases in vitro. HA, unsulfated chondroitin and heparosan as well as polysaccharide extract from *C. testosteroni* were tested. HA and heparosan tetrasaccharides were also tested for their abilities to act as acceptors. CtTS showed a clear preference for the *Comamonas* polysaccharide, but was also able to use heparosan (Figure 3). To confirm that CtTS is able to directly extend a heparosan acceptor so as to rule out an artifactual stabilizing or conformational effect in the radioassays, we also tested an ^{125}I-labeled tetrasaccharide heparosan acceptor in vitro in the presence of only UDP-GlcNAc for single sugar addition, or UDP-GlcNAc and UDP-GlcUA for polymerization. The *C. testosteroni* synthase was able to extend ^{125}I-labeled heparosan tetrasaccharide (Supplementary data, Figure S2).

In summary, lysates containing the recombinant *CtTS* gene product are capable of utilizing heparosan and *Comamonas* polysaccharide as acceptors, but not HA or chondroitin. The failure to extend HA, which consists of the same sugars as heparosan (which is extended), indicates that the glycosidic linkages are not compatible with a structure required for polysaccharide extension. This could be due to either lack of acceptor binding or binding in a mode that does not orient the acceptor in an appropriate configuration with respect to the donor sugar for extension. The chondroitin acceptor contains a GalNAc sugar in place of GlcNAc and may also be unable to bind, or binds in a way that it cannot be extended. The finding that heparosan is extended by CtTS in vitro, but not as well as the *Comamonas* extract, suggests that the two polysaccharides share some structural features, but are not identical.

Metal dependence of CtTS

Many GTs require a divalent cation (e.g. Mg^{2+}, Mn^{2+}, Ca^{2+} etc.) in order to coordinate the UDP-sugar donor molecule for nucleophilic attack by the acceptor molecule. The *Streptococcus* HA synthase prefers Mg^{2+}, whereas the *Pasteurella* HA and HSs prefer Mn^{2+} (DeAngelis 1996). The difference in metal preference in vitro may be an indication of differences in coordination geometry at the active site structures and/or in the reaction mechanisms.

Using radiolabeled sugar incorporation assays performed in the presence of one or both of these divalent cations, we observed that lysates containing the recombinant *CtTS* gene product prefer Mg^{2+}. The presence of 5 mM Mn^{2+} supported GT activity, but activity achieved in the presence of 5 mM

Mg^{2+} was roughly five times as for Mn^{2+} (Supplementary data, Figure S3). Metal is required as the chelator ethylenediaminetetraacetic acid (EDTA) eradicated the polymerization signal. Control assays in which lysates were pretreated with 2 mM EDTA then excess (7 mM) Mg^{2+} or Mn^{2+} were added back confirm that a divalent cation is required for CtTS activity.

Analysis of C. testosteroni *polysaccharide*

The native target polysaccharide yield from 1 L culture of *C. testosteroni* in chemically defined medium (CDM) after 24 h was ~2 mg. Using complete acid hydrolysis of the purified polysaccharide extract into monosaccharides, followed by anion exchange chromatography, we observed GlcUA and GlcNAc (data not shown); the presence of GlcUA was consistent with the presence of a UDP-glucose 6-dehydrogenase gene adjacent to the *CtTS* gene in the *C. testosteroni* KF-1 genome. Gel analysis of the cetylpyridinium chloride (CPC) precipitated the native polysaccharide and the synthetic polysaccharide made with recombinant CtTS in vitro revealed products with apparent molecular weights of ~60 kDa based on the HA standards.

The preference for heparosan acceptor over HA and chondroitin in the radiochemical incorporation assays (Figure 3) as well as the sequence similarity with PmHS1 and PmHS2 initially suggested that CtTS was akin to HS. However, both the native polysaccharide and the synthetic polysaccharide were insensitive to degradation by heparin lyase III or ovine testicular hyaluronidase (Figure 4) as well as *Streptomyces hyalurolyticus* HA lyase or *Proteus vulgaris* chondroitinase ABC. The synthetic polysaccharide has no possibility of any potential post-polymerization modifications made by *Comamonas* in vivo that could block the action of the GAG digesting enzymes. Because of this finding, we believe that while CtTS is able to utilize heparosan as an acceptor molecule due to a hypothetical shared structural feature, this GAG is not the native substrate.

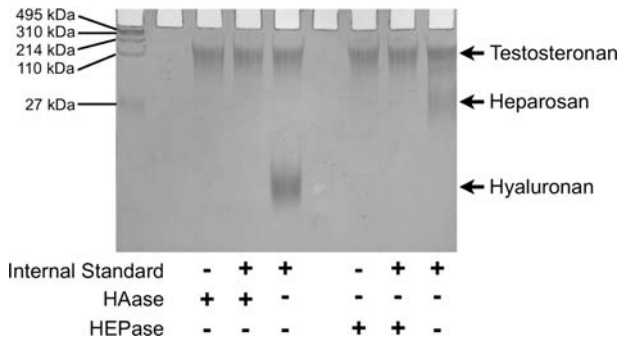

Fig. 4. GAG-degrading enzyme challenge of synthetic *Comamonas* polysaccharide. In vitro synthesized polysaccharide was subjected to heparin lyase III from *Pedobacter heparinus* (HEPase) or ovine testicular hyaluronidase (HAase) digestion; as a control, in parallel reactions sensitive polysaccharides (30 kDa heparosan or 10 kDa HA, respectively) were co-incubated in the appropriate reaction as internal standards. Reactions were subsequently run on 6% polyacrylamide gel and stained with Alcian Blue dye. The *Comamonas* polysaccharide is insensitive to digestion by both enzymes; similar results were observed for the native polysaccharide.

Due to our inability to digest the *C. testosteroni* polysaccharide with known GAG-degrading enzymes, partial acid hydrolysis was employed to produce oligosaccharides that would be suitable for further analysis. Thin-layer chromatography (TLC) was used to optimize the conditions (not shown) and confirmed the presence of sugar oligomers after hydrolysis. MALDI-ToF (matrix-assisted laser desorption/ionization-time of flight) mass spectrometry analysis yielded a ladder pattern of mass peaks that is indicative of a backbone with a repeating disaccharide pattern (i.e. 1:1 ratio of *N*-acetyl-hexosamine to uronic acid; Figure 5). Indeed, the fragment mass values were virtually identical to those seen with both heparosan and HA. The in vitro synthesized polymer mass spectral data were virtually identical to the native polysaccharide; however, it should be noted that such acidic conditions would also potentially remove labile modifications of the backbone. Under our conditions, many *N*-acetyl groups are removed (as in heparosan or HA). Therefore, in theory, the native polysaccharide could contain derivatives of the more well-known monosaccharide units, but such putative modifications may escape detection after hydrolysis.

These mass spectral data, in combination with the in vitro synthesis of this GAG and the inability to digest both the in vitro and native polysaccharide with various known GAGases led us to conclude that this polysaccharide backbone was in fact a new GAG with distinct glycosidic linkages from those of both HA and heparosan. In keeping with the tradition of naming GAGs based on their initial origin, we have called this new polysaccharide backbone "testosteronan".

Nuclear magnetic resonance determination of a novel GAG

One-dimensional ^{1}H and ^{13}C spectroscopy were initially used to evaluate the structure of both the native and the synthetic polysaccharides. These polysaccharides had similar but not identical spectral properties with 14 carbon signals, consistent

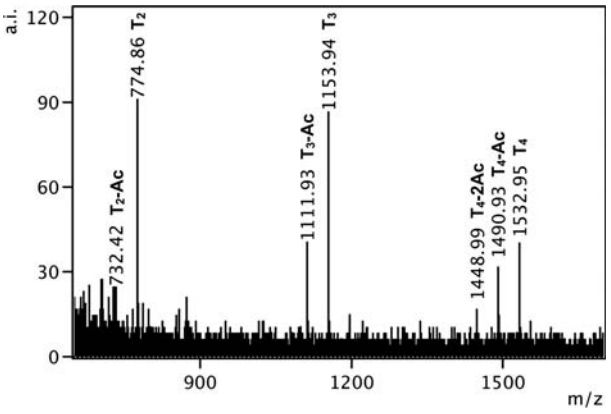

Fig. 5. MALDI-TOF mass spectra of acid hydrolyzed native *Comamonas* polysaccharide. Purified native *Comamonas* polysaccharide was subjected to partial acid hydrolysis by treatment with 1 M HCl at 95°C for 15 min. The resulting ladder pattern of mass peaks is virtually identical to that seen when acid hydrolysis is performed with either HA or heparosan or synthetic polysaccharide (not shown). Note the presence of deacetylation peaks (−42 Da) which is also diagnostic of these GAGs. T = [GlcUA-GlcNAc]; Ac = acetyl group; a.i. = arbitrary intensity.

with a repeating disaccharide unit of GlcUA and GlcNAc. The anomeric signals of each type of monosaccharide residues were assigned based on their characteristic downfield positions (Supplementary data, Figure S4). Next, two-dimensional COSY (COrrelation SpectroscopY; ^{1}H–^{1}H) and HMQC (Heteronuclear Multiple-Quantum Coherence experiment; ^{13}C–^{1}H) (Supplementary data, Figures S5, S6, S10 and S11) experiments were used to assign all signals to each proton and carbon within the two polysaccharides (Table I). Two-dimensional ge-HMQC-TOCSY (TOtal Correlation SpectroscopY; Figure 6) confirmed all of these assignments. Figure 6 shows the HMQC (green) spectrum of native *Comamonas* polysaccharide overlaid onto ge-HMQC-TOCSY (red) spectrum. Two-dimensional TOCSY (^{1}H–^{1}H) (Supplementary data, Figures S7 and S12) was used to obtain all possible correlations within each spin system. Finally, two-dimensional NOESY (Nuclear Overhauser Effect SpectroscopY; Figures 7 and 8) was used to assign nuclear overhauser effect (NOE) signals between the anomeric protons and H-4 protons of the adjacent saccharide residue across the glycosidic linkage to provide linkage positions.

Based on these spectral data, the structure of the native polysaccharide backbone could be definitively assigned as [-4-D-GlcUA-α1,4-D-GlcNAc-α1-]$_n$ but the proton at the 3-position of the GlcNAc residue was shifted downfield by 1.354 ppm, compared with the synthetic polysaccharide to 5.222 ppm, suggesting it carried a deshielding modification. One hypothesis is that during biosynthesis of testosteronan, the polysaccharide is polymerized by the CtTS synthase and then during a post-polymerization step or reaction, the C3 hydroxyl is modified. An alternative model is that a novel hexosamine unit is used by this organism in vivo but that the enzyme tolerates GlcNAc as observed in our in vitro tests. To date, we have been unable to determine the nature of the modification that causes this unusual shift in the C3 atom position. We have ruled out the possibility of sulfation [by X-ray photoelectron spectroscopy (XPS)] and phosphorylation [by ^{31}P NMR (nuclear magnetic resonance) and XPS]. We analyzed the sample at pH 9, but the unusual NMR signal remained thus ruling out the possibility of a potential base-labile *O*-acetyl unit or an inter-residue lactone.

Table I. Chemical shift values (pD 7.0, 25°C) and assignments of ^{1}H and ^{13}C NMR shifts for *C. testosteroni* polysaccharides

Residue/position	Native polysaccharide		Synthetic polysaccharide	
	^{13}C	^{1}H	^{13}C	^{1}H
GlcUA 1	99.994	5.210	99.685	5.356
GlcUA 2	71.306	3.465	71.995	3.508
GlcUA 3	73.280	3.805	73.850	3.819
GlcUA 4	76.166	3.646	75.262	3.646
GlcUA 5	71.723	4.025	73.130	4.020
GlcNAc 1	97.409	5.301	96.722	5.349
GlcNAc 2	51.957	4.109	53.470	3.861
GlcNAc 3	73.432	5.222	71.275	3.868
GlcNAc 4	73.869	4.008	76.536	3.625
GlcNAc 5	68.398	4.001	70.472	3.771
GlcNAc 6a,b	61.114	3.770	60.136	3.745
GlcNAc (CH_3)	20.040	2.023	21.931	1.983

The synthetic polysaccharide was produced in vitro with pure precursors, UDP-GlcUA and UDP-GlcNAc. The ^{1}H and ^{13}C signals of the GlcUA residue in synthetic polysaccharide resonated at nearly identical chemical shift values as those observed for the native polysaccharides. The chemical shift values for the ^{1}H and ^{13}C signals for the GlcNAc residue were different for the two polysaccharides, particularly those at the 3-position. Moreover, overlap (even at 800 MHz field strength) between the proton at the 4-positions of both the GlcNAc and GlcUA residues in the synthetic polysaccharide initially made it impossible to definitively assign linkage positions. When the probe temperature was elevated up to 338 K, the GlcUA and GlcNAc anomeric signals could be partially separated (Supplementary data, Figure S8) but their linkage positions could still not be assigned by NOESY due to peak broadening. This problem was overcome by reducing the pD (the pH equivalent in deuterated water) of the synthetic polysaccharide sample from 6.9 to 3.6 (Supplementary data, Figure S9) allowing its structure to be definitely assigned as [-4-D-GlcUA-α1,4-D-GlcNAc-α1-]$_n$.

Discussion

GAGs are hydrophilic polysaccharides that can play signaling, structural and protective roles in the human body. These properties make GAGs desirable molecules for therapeutics and tissue engineering. In the current work, we have identified a bifunctional GAG synthase, CtTS, which is responsible, in part, for forming an extracellular polysaccharide in the bacteria *C. testosteroni* KF-1. The GAG backbone formed by the action of CtTS, testosteronan, consists of the same chemical composition as both heparosan and HA, but has distinct glycosidic linkages. Heparosan is [-4-D-GlcUA-β1,4-D-GlcNAc-α1-]$_n$ while HA is [-4-D-GlcUA-β1,3-D-GlcNAc-β1-]$_n$. Our NMR studies have been able to confirm that this new GAG backbone has the structure [-4-D-GlcUA-α1,4-D-GlcNAc-α1-]$_n$. Therefore, the testosteronan backbone has identically configured GlcNAc units as heparosan; this observation probably explains why heparosan served as an acceptor for CtTS in vitro, but not as efficiently as the native polymer found in *Comamonas* extracts.

Some bacterial pathogens utilize polysaccharide capsules with molecular structures similar or identical to their host organism to avoid host defenses, such as antibodies, phagocytes or complement. Although the differences in the testosteronan structure may appear to be detrimental for the *Comamonas* microbe's strategy of molecular mimicry, some capsular glycans are not identical to host GAGs, but are still employed as virulence factors. Specific examples include: (i) heparosan of *P. multocida* type D and *E. coli* K5, which is employed without sulfation or epimerization, and (ii) a fructosylated version of chondroitin is used by *E. coli* K4 (DeAngelis 2002).

The glycosidic linkages of testosteronan are responsible for the polysaccharide's insensitivity to digestion by all GAG-degrading enzymes tested thus far, but it should be susceptible to vertebrate lysosomal exoglycosidases. This property of testosteronan may prove useful for generating longer-lasting polysaccharides or biomaterials provided the

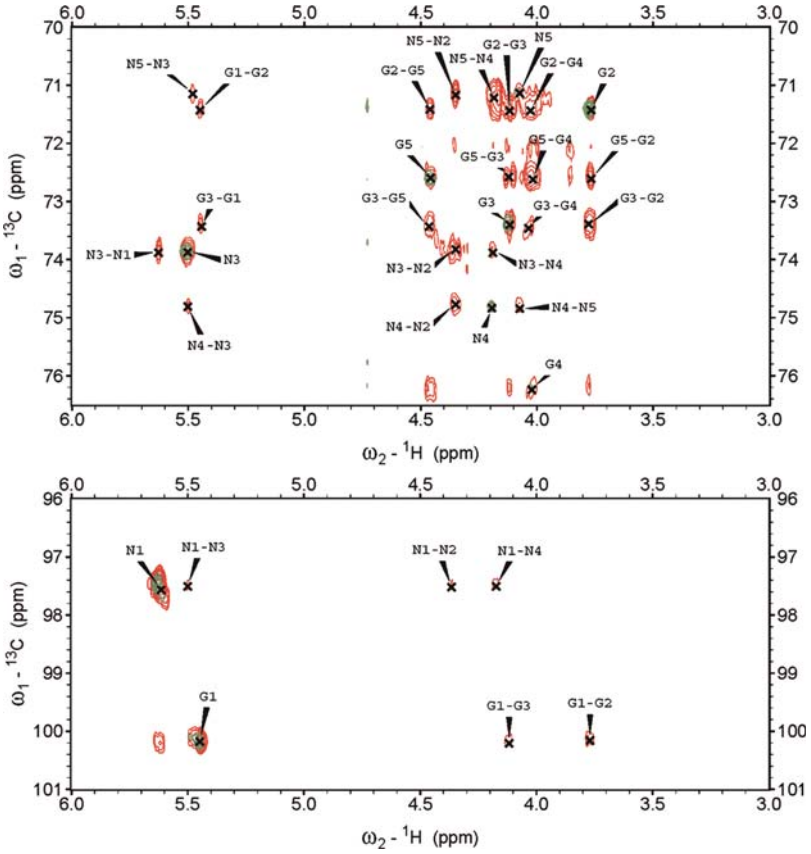

Fig. 6. Strip plots from two-dimensional ge-HMQC-TOCSY spectrum of the native *Comamonas* polysaccharide at pD 6.9; T 328 K. HMQC spectrum (green in online version) was overlaid onto HMQC-TOCSY spectrum (red in online version). The spectrum was recorded at 600 MHz on a Bruker Avance II spectrometer with probe temperature of 328 K. Mixing time 50 ms; delay time 1 s; acquisition time 0.232 s.

molecule does not promote an immunological response in the human body. Additionally, it is intriguing to consider the potential for this molecule to possess anticoagulant or anti-proliferative activity after chemical or enzymatic sulfation (Kuberan et al. 2003; Chen et al. 2005; Liu R et al. 2010). This is due to the similar structure to that of heparosan, which is the precursor molecule to heparin, the highly sulfated, epimerized form of the same molecule. The only difference between heparosan and testosteronan is the change from β- to α-linkage configuration between the GlcUA and the GlcNAc residues. Therefore, any binding protein or factor that relies on this structure (or the conformation it assumes) may not interact with the testosteronan backbone as well as with that of heparosan, but conversely, other proteins that do not rely on this structure may be minimally affected. If such a scenario exists, then the basis for more selective therapeutics exists. Future studies will assess this and other possible applications for testosteronan.

CtTS serves as the source of a new GT domain (GT93) for protein engineering, as well as expanding the pool of characterized GT45-containing enzymes. By mining genomes for related or similar sequences to CtTS and other GAG synthases, we may both elucidate GT mechanisms as well as generate new catalytic tools capable of producing novel polysaccharides.

Materials and methods

Materials

Wild-type *C. testosteroni* KF-1 (DSM# 14576) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The strain was grown in CDMs which is free of other polysaccharides and animal extracts (van De Rijn and Kessler 1980; JRH Biosciences, Lenexa, KS). All reagents were from Sigma (St Louis, MO) unless otherwise noted. Oligonucleotide primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA).

Isolation and analysis of capsular polysaccharide

Wild-type *C. testosteroni* KF-1 was grown in CDM for 24 h at 30°C with shaking at 250 rpm. Cells were then removed from the culture by centrifugation. Spent media was treated with 0.1 μg/μL DNase/RNase for 2 h at room temperature to degrade nucleic acids. The anionic polysaccharide fraction was precipitated by addition of 1% CPC for 1 h at room temperature. The pellet was collected by centrifugation (2000 × *g*, 60 min), washed with water and resuspended in 1 M NaCl. The solution was clarified by centrifugation and the supernatant was ethanol precipitated (70% v/v final). The ethanol precipitation/1 M NaCl dissolution procedure was repeated twice and then the pellet was washed with 70% ethanol.

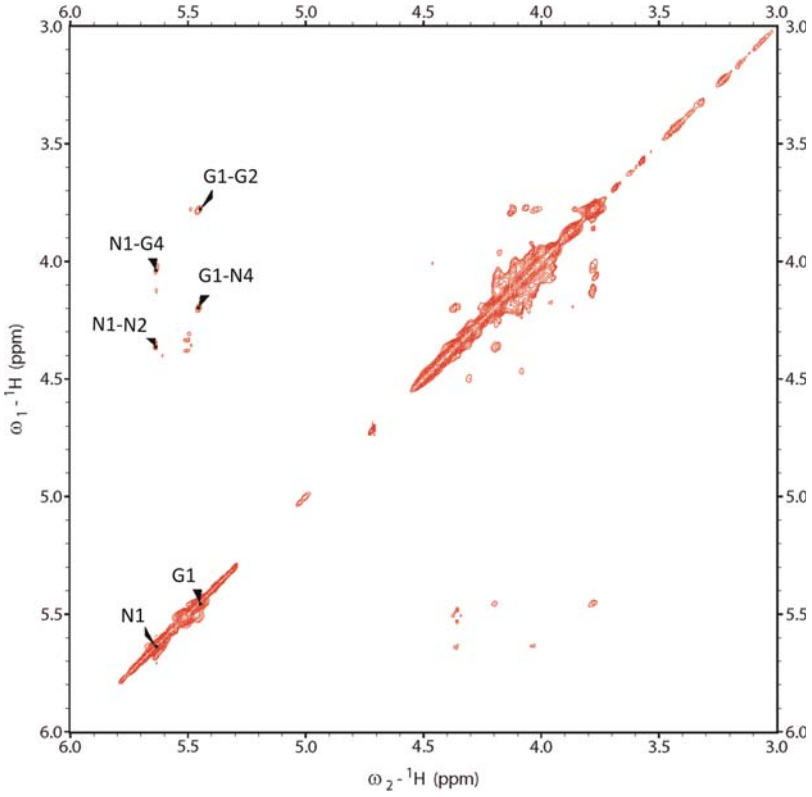

Fig. 7. ^{1}H–^{1}H NOESY spectrum of the native *Comamonas* polysaccharide at pD 6.9; T 328 K. The GlcUA residues are labeled G and the GlcNAc residues are labeled N. The spectrum was recorded at 800 MHz on a Bruker Avance II spectrometer with probe temperature of 328 K. Mixing time 400 ms; delay time 1.5 s; acquisition time 0.852 s.

Finally, the pellet was resuspended in water, treated with 0.1 µg/µL DNase/RNase for 1 h (20 mM Tris, pH 7.2, 1 mM $MgCl_2$) and extracted with $CHCl_3$ to remove proteins from the sample. This polysaccharide extract was further fractionated by anion exchange chromatography (HiTrap Q HP 1 mL column, GE LifeSciences, Uppsala, Sweden) using a 0.05–2 M ammonium formate gradient (1 mL/min for 93 min). The resulting fractions were analyzed by polyacrylamide gel electrophoresis [PAGE; 1× tris-borate-EDTA (TBE), 6% acrylamide] with staining by Alcian Blue. Fractions containing the target polysaccharide (~0.6–0.75 M ammonium formate) were pooled and lyophilized three times from water to remove the volatile ammonium formate. The polysaccharide pool was then treated with proteinase K (1 µg/µL enzyme, 50 mM NaOAc, pH 7.4, overnight at 30°C) to destroy any contaminating proteins. In certain preparations, as noted, other HA-like polysaccharides were digested with ovine testicular hyaluronidase prior to proteinase K digestion. Digest reactions were $CHCl_3$ extracted to remove enzymes and exchanged via ultrafiltration into water (Microcon 30 kDa, 3× with 500 µL rinses). The retentate containing polysaccharide was purified on a PD-10 column (GE LifeSciences) to remove remaining low-molecular-weight contaminants. Briefly, the column was equilibrated, loaded and eluted as per manufacturer's instructions using 0.2 M ammonium formate buffer. The void volume fractions containing polysaccharide were pooled and lyophilized 3×. Uronic acid content was measured by the carbazole assay (Bitter and Muir 1962) with GlcUA standard. Resulting polysaccharide was used for NMR and monosaccharide analysis.

For monosaccharide analysis, polysaccharides were subjected to acid hydrolysis; 30–100 µg of polysaccharide was incubated in 300 µL of 2-*N*-trifluoroacetic acid (TFA) for 6 h at 100°C. Finally, reactions were cooled to room temperature and dried in a rotary evaporator. Samples were dissolved in 50 µL of water and 20 µL was subjected to anion exchange chromatography with pulsed amperometric detection on a Dionex DX600 instrument as described previously (DeAngelis et al. 2002; Dionex, Inc., Sunnyvale, CA).

Cloning and expression of C. testosteroni *synthase*

Genomic DNA was extracted from the wild-type *C. testosteroni* microbe grown in CDMs employing the UltraClean Microbial DNA Sample Kit (MO BIO, Carlsbad, CA) and used as a template for polymerase chain reaction (PCR) using primers designed to amplify the predicted 1923 bp coding sequence (sense: ATGAGCGGCATGTTTAAGGTT GCCAATG; antisense: TCATTTCACCATCATCTTTTTAA TTCTGAG). The resulting PCR product was cloned into the pTrcHis-TOPO vector (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and transformed into *E. coli* TOP-10F' cells with selection on LB/ampicillin plates at 30°C. The plasmids of transformants were screened by

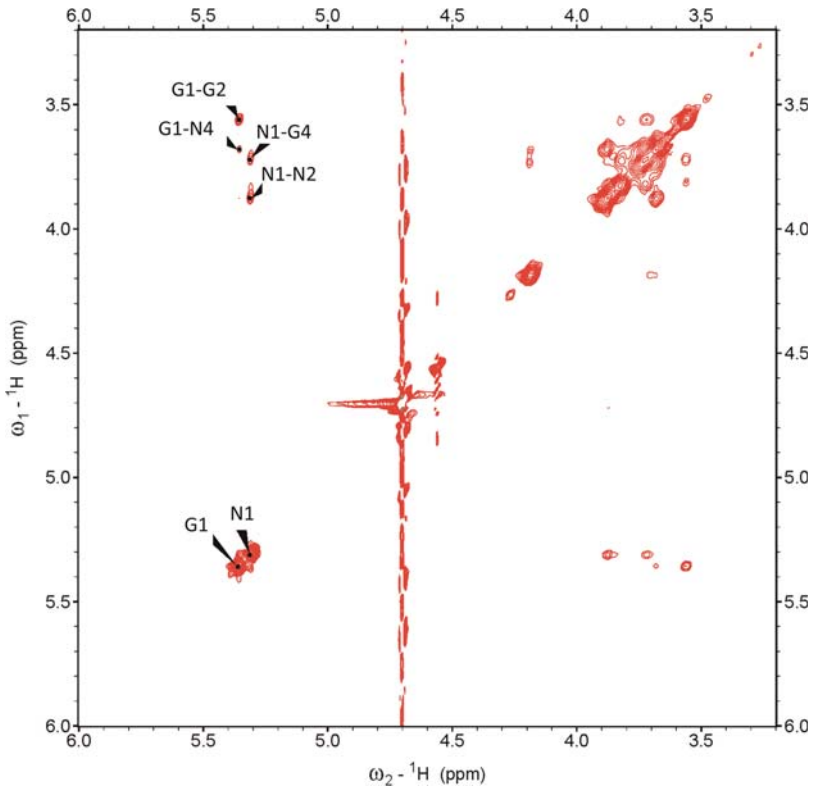

Fig. 8. ^{1}H–^{1}H NOESY spectrum of the synthetic *Comamonas* polysaccharide at pD 3.6; T 328 K. The GlcUA residues are labeled G and the GlcNAc residues are labeled N. The spectrum was recorded at 600 MHz on a Bruker Avance II spectrometer with probe temperature of 328 K. Mixing time 400 ms; delay time 1.5 s; acquisition time 0.852 s.

restriction digest and DNA plasmids positive for the correctly oriented insert were confirmed by sequencing both strands (Oklahoma Medical Research Foundation sequencing facility). Plasmid was then transformed into phage lysin-expressing freeze/thaw lysis *E. coli* XJa cells according to the manufacturer's instructions (Zymo Research, Orange, CA). For protein production, cultures of *E. coli* XJa in Superior Broth (AthenaES, Baltimore, MD) with ampicillin (50 μg/mL), carbenicillin (50 μg/mL) and L-arabinose (3.25 mM final; to induce the lysin enzyme) were grown at 30°C. Expression of target protein was induced by addition of isopropyl β-D-1-thiogalactopyranoside (0.2 mM final) at OD_{600} 0.35. At 1 h post-induction, growth was supplemented with fructose (12.8 mM final) and growth proceeded for ∼16 h before cells were harvested by centrifugation (3000 × *g*, 30 min at 4°C). The cell pellet was resuspended in 50 mM Tris, pH 7.2, with protease inhibitors pepstatin, benzamidine, *N*-[*N*-(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucyl]-agmatine, leupeptin on ice and subjected to two freeze/thaw cycles to allow the phage lysin to degrade the cell walls. The lysates were then clarified by centrifugation (20,000 × *g*, 30 min at 4°C). Protein content was measured by the Bradford assay with a BSA standard (Thermo Fisher Scientific, Waltham, MA).

GT activity assays

Radiolabeled sugar incorporation assays (25 μL, 30 min at 22°C) were performed using clarified lysates (60 μg total protein) in the presence of 0.05 mM UDP-GlcUA or UDP-GlcNAc as carrier with UDP-[^{3}H]GlcUA or UDP-[^{3}H]GlcNAc (0.1 μCi/assay; NEN Perkin Elmer, Waltham, MA) with various unlabeled UDP-sugars (1 mM) or no second sugar as a negative control. The reaction buffer was 50 mM Tris, pH 7.2, 5 mM $MgCl_2$; these conditions were obtained after limited optimization trials. Certain reactions used acceptors (1 μg) to bypass de novo initiation including HA, heparosan or *C. testosteroni* polysaccharide (sonicated 10 min on ice to increase the number of termini).

Reactions were incubated at the indicated temperatures for the times noted, and then stopped with 2% final sodium dodecyl sulfate and separated by descending paper chromatography (65:35 ethanol/1 M ammonium acetate buffer, Whatman 3MM paper). This method enables the separation of GAG polysaccharides with greater than ∼14 sugar units, which remain at the origin, from smaller oligomers and unincorporated nucleotide sugars that migrate down the strip. The origin of the strip was cut out and subjected to liquid scintillation counting (Biosafe, RPI Corp., Mt Prospect, IL).

Production of synthetic polysaccharide

To produce larger amounts of the polysaccharide synthesized by the recombinant enzyme in vitro, 200 μL reaction mixtures were employed containing 10 mM each of unlabeled UDP-GlcNAc and UDP-GlcUA, 5 mM $MgCl_2$ and 50 mM Tris, pH 7.2, with 40 ng/μL heparosan tetrasaccharide as

acceptor at 30°C overnight using clarified lysates (3 mg/mL total protein). Synthetic polysaccharide was purified using the same methods as the native polysaccharide extract following (and including) the final $CHCl_3$ extraction, ultrafiltration and PD-10 column; the yield was 0.6 mg.

Digestion of native and synthetic polysaccharide with GAG-degrading enzymes

Purified synthetic or native polysaccharide was treated with heparin lyase III from *Pedobacter heparinus* (previously *Flavobacterium heparinum*; kindly supplied by Jian Liu, University of North Carolina; 0.2 mg/mL, 50 mM Tris, pH 7.2, at 30°C) or ovine testicular hyaluronidase (0.4 mg/mL, 30 mM ammonium acetate, pH 5.5, at 30°C) to help characterize the polysaccharide being produced by CtTS. Polysaccharides were analyzed post-treatment with either 6% PAGE (1× TBE) stained with Alcian Blue (Min and Cowman 1986) or 2% agarose gels [1× tris-acetate-EDTA (TAE)] with Stains-all detection (Lee and Cowman 1994). Nearly monodisperse HA standards (Hyalose, LLC, Oklahoma City, OK) were used to estimate size (Jing and DeAngelis 2004). As a positive control for complete and specific digestion, we employed internal standards of authentic HA or heparosan as appropriate; the use of GAGs with molecular weights differing from the *C. testosteroni* polysaccharides allowed analysis of the test and standard samples in the same reaction and gel lane.

Acid hydrolysis of Ct native polysaccharide extract

Purified native *Comamonas* polysaccharide was partially fragmented with 1 M HCl at 95°C for 15 min. The resulting hydrolyzed oligosaccharides were then analyzed by either TLC (silica plates with *n*-butanol, acetic acid, H_2O, 2:1:1 and staining by naphthoresorcinol) or MALDI-ToF mass spectrometry. MALDI-ToF mass spectrometry was performed in a reflector negative mode using an Ultraflex II instrument (Bruker Daltonics, Billerica, MA), with the matrix 6-aza-2-thiothymine at a concentration of 5 mg/mL in 50% acetonitrile, 0.1% TFA. HA oligosaccharides were employed as mass calibrants. Spectra were analyzed using mMass 3 (Strohalm et al. 2010).

NMR studies

The native *Comamonas* and synthetic polysaccharides were analyzed by one-dimensional ^{1}H and two-dimensional COSY, HMQC, TOCSY, NOESY and ge-HMQC-TOCSY experiments to elucidate their structure. All NMR experiments were acquired on Bruker Avance II Ultrashield 600 MHz (14.1 T) and 800 MHz (18.8 T) NMR instruments equipped with an ultrasensitive HCN cryoprobe with a *z*-axis gradient. The spectra were mostly acquired at a probe temperature of 298 and 328 K. Polysaccharides (~0.5–1.5 mg) were dissolved in 0.4 mL of 99.996% deuterium oxide (^2H_2O; Sigma) and freeze-dried to remove exchangeable protons. The residual water peak served as a reference (HOD, 4.76 ppm); typical silane standards were not employed due to water insolubility or lack of volatility that would interfere with subsequent analyses. The chemical shift of the water peak yields reliable chemical shift values for polysaccharides (Liu Z et al. 2010). For one-dimensional ^{1}H-NMR spectra, sweep width of 20.5 ppm and acquisition time of 2.65 s were employed. For the ^{1}H–^{1}H COSY, ^{1}H–^{1}H TOCSY and NOESY spectra, 512 experiments resulting in 4096 data points for a spectral width of 10 ppm were measured. Proton-detected HMQC experiments used 10 and 78 ppm spectral widths in the ^{1}H dimension and ^{13}C dimension, respectively. A mixing time of 400 ms with 1.5 s relaxation delay and a mixing time of 50 ms with 1 s relaxation delay were used in NOESY and ge-HMQC-TOCSY experiments, respectively. The two-dimensional NMR data sets were processed by Topspin version 2.1.4 and cross-peak assignments were carried out using an NMR assignment software Sparky (Goddard and Kneller 2001).

XPS studies

XPS measurements (1 mg sample/test) were carried out in a PHI 5400 instrument (Physical Electronics, Chanhassen, MN) with a 200 W AI Kalpha mono probe beam. The spectrometer was configured to operate at high resolution with pass energy of 117.40 eV.

Supplementary data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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Conflict of interest statement

None declared.

Abbreviations

CAZy, Carbohydrate-Active enZymes; CDM, chemically defined medium; COSY, COrrelation SpectroscopY; CPC, cetylpyridinium chloride; EDTA, ethylenediaminetetraacetic acid; GAG, glycosaminoglycan; ge, gradient-enhanced; GlcNAc, *N*-acetyl-glucosamine; GlcUA, glucuronic acid; HA, hyaluronan; HAS, hyaluronan synthase; HMQC, Heteronuclear Multiple-Quantum Coherence experiment; HOD, chemical structure; HS, heparosan synthase; KF, strain

name; MALDI-ToF, matrix-assisted laser desorption/ionization-time of flight; NMR, nuclear magnetic resonance; NOE, nuclear overhauser effect; NOESY, Nuclear Overhauser Effect SpectroscopY; PAGE, polyacrylamide gel electrophoresis; PBCV, paramecium bursaria chlorella virus; PCR, polymerase chain reaction; TAE, tris-acetate-EDTA; TBE, tris-borate-EDTA; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; TOCSY, TOtal Correlation SpectroscopY; UDP, uridine diphosphate; XPS, X-ray photoelectron spectroscopy.

References

Arda B, Aydemir S, Yamazhan T, Hassan A, Tünger A, Serter D. 2003. *Comamonas testosteroni* meningitis in a patient with recurrent cholesteatoma. *APMIS*. 111:474–476.

Bitter T, Muir HM. 1962. A modified uronic acid carbazole reaction. *Anal Biochem*. 4:330–334.

Bossier P, Verstraete W. 1996. *Comamonas testosteroni* colony phenotype influences exopolysaccharide production and coaggregation with yeast cells. *Appl Environ Microbiol*. 62:2687–2691.

Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V, Henrissat B. 2009. The Carbohydrate-Active EnZymes database (CAZy): An expert resource for glycogenomics. *Nucleic Acids Res*. 37:D233–238.

Chen J, Avci FY, Muñoz EM, McDowell LM, Chen M, Pedersen LC, Zhang L, Linhardt RJ, Liu J. 2005. Enzymatic redesigning of biologically active heparan sulfate. *J Biol Chem*. 280:42817–42825.

DeAngelis PL. 1996. Enzymological characterization of the *Pasteurella multocida* hyaluronic acid synthase. *Biochemistry*. 35:9768–9771.

DeAngelis PL. 1999. Hyaluronan synthases: Fascinating glycosyltransferases from vertebrates, bacterial pathogens, and algal viruses. *Cell Mol Life Sci*. 56:670–682.

DeAngelis PL. 2002. Microbial glycosaminoglycan glycosyltransferases. *Glycobiology*. 12:9R–16R.

DeAngelis PL, Gunay NS, Toida T, Mao W-J, Linhardt RJ. 2002. Identification of the capsular polysaccharides of Type D and F *Pasteurella multocida* as unmodified heparin and chondroitin, respectively. *Carbohydr Res*. 337:1547–1552.

DeAngelis PL, Jing W, Drake RR, Achyuthan AM. 1998. Identification and molecular cloning of a unique hyaluronan synthase from *Pasteurella multocida*. *J Biol Chem*. 273:8454–8458.

DeAngelis PL, Jing W, Graves MV, Burbank DE, Van Etten JL. 1997. Hyaluronan synthase of chlorella virus PBCV-1. *Science*. 278:1800–1803.

DeAngelis PL, Padgett-McCue AJ. 2000. Identification and molecular cloning of a chondroitin synthase from *Pasteurella multocida* type F. *J Biol Chem*. 275:24124–24129.

DeAngelis PL, Papaconstantinou J, Weigel PH. 1993. Molecular cloning, identification, and sequence of the hyaluronan synthase gene from group A *Streptococcus pyogenes*. *J Biol Chem*. 268:19181–19184.

DeAngelis PL, White CL. 2002. Identification and molecular cloning of a heparosan synthase from *Pasteurella multocida* type D. *J Biol Chem*. 277:7209–7213.

DeAngelis PL, White CL. 2004. Identification of a distinct, cryptic heparosan synthase from *Pasteurella multocida* types A, D, and F. *J Bacteriol*. 186:8529–8532.

Dias FF, Bhat JV. 1964. Microbial ecology of activated sludge. I. Dominant bacteria. *Appl Microbiol*. 12:412–417.

Drummond A, Ashton B, Buxton S, Cheung M, Cooper A, Heled J, Kearse M, Moir R, Stones-Havas S, Sturrock S, et al. 2010. *Geneious 4.7.6*. Auckland, New Zealand. http://www.geneious.com.

Goddard TD, Kneller DG. 2001. *SPARKY*, 3rd ed. San Francisco (CA): University of California.

Gul M, Ciragil P, Bulbuloglu E, Aral M, Alkis S, Ezberci F. 2007. *Comamonas testosteroni* bacteremia in a patient with perforated acute appendicitis. Short communication. *Acta Microbiol Immunol Hung*. 54:317–321.

Horinouchi M, Kurita T, Hayashi T, Kudo T. 2010. Steroid degradation genes in *Comamonas testosteroni* TA441: Isolation of genes encoding a Δ4 (5)-isomerase and 3α- and 3β-dehydrogenases and evidence for a 100 kb steroid degradation gene hot spot. *J Steroid Biochem Mol Biol*. 122:253–263.

Jin L, Perper JA, Cina SJ. 2008. *Comamonas testosteroni* meningitis in a homeless man. *J Forensic Sci*. 53:1198–1199.

Jing W, DeAngelis PL. 2000. Dissection of the two transferase activities of the *Pasteurella multocida* hyaluronan synthase: Two active sites exist in one polypeptide. *Glycobiology*. 10:883–889.

Jing W, DeAngelis PL. 2004. Synchronized chemoenzymatic synthesis of monodisperse hyaluronan polymers. *J Biol Chem*. 279:42345–42349.

Jing W, Haller FM, Almond A, DeAngelis PL. 2006. Defined megadalton hyaluronan polymer standards. *Anal Biochem*. 355:183–188.

Kuberan B, Beeler DL, Lawrence R, Lech M, Rosenberg RD. 2003. Rapid two-step synthesis of mitrin from heparosan: A replacement for heparin. *J Am Chem Soc*. 125:12424–12425.

Lee HG, Cowman MK. 1994. An agarose gel electrophoretic method for analysis of hyaluronan molecular weight distribution. *Anal Biochem*. 219:278–287.

Linares M, Pruneda-Paz JL, Reyna L, Genti-Raimondi S. 2008. Regulation of testosterone degradation in *Comamonas testosteroni*. *J Steroid Biochem Mol Biol*. 112:145–150.

Liu Z, Masuko S, Solakyildirim K, Pu D, Linhardt RJ, Zhang F. 2010. Glycosaminoglycans of the porcine central nervous system. *Biochemistry*. 49:9839–9847.

Liu R, Xu Y, Chen M, Weïwer M, Zhou X, Bridges AS, DeAngelis PL, Zhang Q, Linhardt RJ, Liu J. 2010. Chemoenzymatic design of heparan sulfate oligosaccharides. *J Biol Chem*. 285:34240–34249.

Ma Y-F, Zhang Y, Zhang J-Y, Chen D-W, Zhu Y, Zheng H, Wang S-Y, Jiang C-Y, Zhao G-P, Liu S-J. 2009. The complete genome of *Comamonas testosteroni* reveals its genetic adaptations to changing environments. *Appl Environ Microbiol*. 75:6812–6819.

Min H, Cowman MK. 1986. Combined Alcian Blue and silver staining of glycosaminoglycans in polyacrylamide gels: Application to electrophoretic analysis of molecular weight distribution. *Anal Biochem*. 155:275–285.

Ninomiya T, Sugiura N, Tawada A, Sugimoto K, Watanabe H, Kimata K. 2002. Molecular cloning and characterization of chondroitin polymerase from *Escherichia coli* strain K4. *J Biol Chem*. 277:21567–21575.

Reddy AK, Murthy SI, Jalali S, Gopinathan U. 2009. Post-operative endophthalmitis due to an unusual pathogen, *Comamonas testosteroni*. *J Med Microbiol*. 58:374–375.

Schleheck D, Knepper TP, Fischer K, Cook AM. 2004. Mineralization of individual congeners of linear alkylbenzenesulfonate by defined pairs of heterotrophic bacteria. *Appl Environ Microbiol*. 70:4053–4063.

Schleheck D, von Netzer F, Fleischmann T, Rentsch D, Huhn T, Cook AM, Kohler H-PE. 2010. The missing link in linear alkylbenzenesulfonate surfactant degradation: 4-sulfoacetophenone as a transient intermediate in the degradation of 3-(4-sulfophenyl)butyrate by *Comamonas testosteroni* KF-1. *Appl Environ Microbiol*. 76:196–202.

Sismey-Ragatz AE, Green DE, Otto NJ, Rejzek M, Field RA, DeAngelis PL. 2007. Chemoenzymatic synthesis with distinct *Pasteurella* heparosan synthases: Monodisperse polymers and unnatural structures. *J Biol Chem*. 282:28321–28327.

Strohalm M, Kavan D, Novak P, Volny M, Havlicek V. 2010. mMass 3: A cross-platform software environment for precise analysis of mass spectrometric data. *Anal Chem*. 82:4648–4651.

van De Rijn I, Kessler RE. 1980. Growth characteristics of group A *streptococci* in a new chemically defined medium. *Infect Immun*. 27:444–448.

Weigel PH, DeAngelis PL. 2007. Hyaluronan synthases: A decade-plus of novel glycosyltransferases. *J Biol Chem*. 282:36777–36781.

Weïwer M, Sherwood T, Green DE, Chen M, DeAngelis PL, Liu J, Linhardt RJ. 2008. Synthesis of uridine 5′-diphosphoiduronic acid: A potential substrate for the chemoenzymatic synthesis of heparin. *J Org Chem*. 73:7631–7637.