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Glycan Synthesis

Identification of a chondroitin synthase from an unexpected source, the green sulfur bacterium *Chlorobium phaeobacteroides*

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Abstract

Glycosaminoglycans (GAGs) are known to be present in all animals as well as some pathogenic microbes. Chondroitin sulfate is the most abundant GAG in mammals where it has various structural and adhesion roles. The Gram-negative bacteria *Pasteurella multocida* Type F and *Escherichia coli* K4 produce extracellular capsules composed of unsulfated chondroitin or a fructosylated chondroitin, respectively. Such polysaccharides that are structurally related to host molecules do not generally provoke a strong antibody response thus are thought to be employed as molecular camouflage during infection. We observed a sequence from the photosynthetic green sulfur bacteria, *Chlorobium phaeobacteroides* DSM 266, which was very similar (~62% identical) to the open reading frames of the known bifunctional chondroitin synthases (PmCS and KfoC); some segments are strikingly conserved amongst the three proteins. Recombinant *E. coli*-derived *Chlorobium* enzyme preparations were found to possess *bona fide* chondroitin synthase activity in vitro. This new catalyst, CpCS, however, has a more promiscuous acceptor usage than the prototypical PmCS, which may be of utility in novel chimeric GAG syntheses. The finding of such a similar chondroitin synthase enzyme in *C. phaeobacteroides* is unexpected for several reasons including (a) a free-living nonpathogenic organism should not "need" an animal self molecule for protection, (b) the Proteobacteria and the green sulfur bacterial lineages diverged ~2.5–3 billion years ago and (c) the ecological niches of these bacteria are not thought to overlap substantially to facilitate horizontal gene transfer. CpCS provides insight into the structure/function relationship of this class of enzymes.

Key words: chondroitin, glycosaminoglycan, glycosyltransferase, synthase

Introduction

Glycosaminoglycans (GAGs), long linear polysaccharides consisting of disaccharide repeats that contain an amino sugar, are found in animals as well as certain microbes (DeAngelis 2002a; Hascall et al. 2009). Chondroitin [-4-GlcA-β1-3-GalNAc-β1-]$_n$, heparin/heparan [-4-GlcA-β1-4-GlcNAc-α1-]$_n$ and hyaluronan (HA) [-4-GlcA-β1-3-GlcNAc-β1-]$_n$ are the three most prevalent GAG backbones found in humans. Chondroitin sulfate and heparin/heparan sulfate are synthesized as glycoproteins in the Golgi and are sulfated at various positions in vertebrates. Many primitive animals possess some of these same GAGs or very similar molecules. GAGs play both structural and recognition roles on the cell surface and in the extracellular matrix where a plethora of proteins bind selectively to one or more of the GAGs.

Many pathogenic bacteria produce an extracellular polysaccharide coating, called a capsule, which serves as a virulence factor (Roberts 1996). In a few cases, the capsule is composed of GAG or GAG-like polymers (DeAngelis 2002b). As the microbial polysaccharide is identical or very similar to the host GAG, the antibody response is either very limited or nonexistent to "self" structures (Cress et al. 2014). In contrast, the nonself carbohydrates of many other bacteria are highly immunogenic and thus repeat infections with the same capsular type are not often seen. The capsule is thought to assist in the evasion of host defenses such as phagocytosis and complement. Examples of this clever strategy of molecular camouflage are the production of authentic HA polysaccharide by Gram-negative *Pasteurella multocida* Type A and Gram-positive Group A and C *Streptococcus* as well as unsulfated chondroitin in Type F *P. multocida* (DeAngelis et al. 2002; DeAngelis 2002b). The *Escherichia coli* K4 polymer is an unsulfated chondroitin backbone decorated with fructose side-branches on the C3 position of the GlcA residues (Rodriguez et al. 1988). *E. coli* K5 and *P. multocida* Type D form capsules composed of unsulfated heparosan, the sugar backbone precursor of heparan sulfate/heparin (Vann et al. 1981; DeAngelis et al. 2002). The three different serotypes of *Avibacterium*, a *Pasteurella*-like pathogen, also have been reported to produce either HA or an unsulfated chondroitin or heparosan (Wu et al. 2010). The GAG capsule of pathogenic microbes can increase virulence by 10^2- to 10^3-fold as measured by LD50 values, the number of colony forming units that will kill 50% of the test animals after bacterial challenge (Wessels et al. 1991; Harmon et al. 1992).

The studies of GAG biosynthesis have been instrumental in understanding polysaccharide production in general. All known GAG glycosyltransferases utilize UDP-sugar nucleotide substrates to produce large polymers containing dozens to thousands of disaccharide repeats. In all of the microbial cases, except the *E. coli* K5 system, a single polypeptide chain is capable of transferring both sugars of the disaccharide repeat to the growing polymer chain (DeAngelis 2002b; DeAngelis et al. 2013), thus breaking the

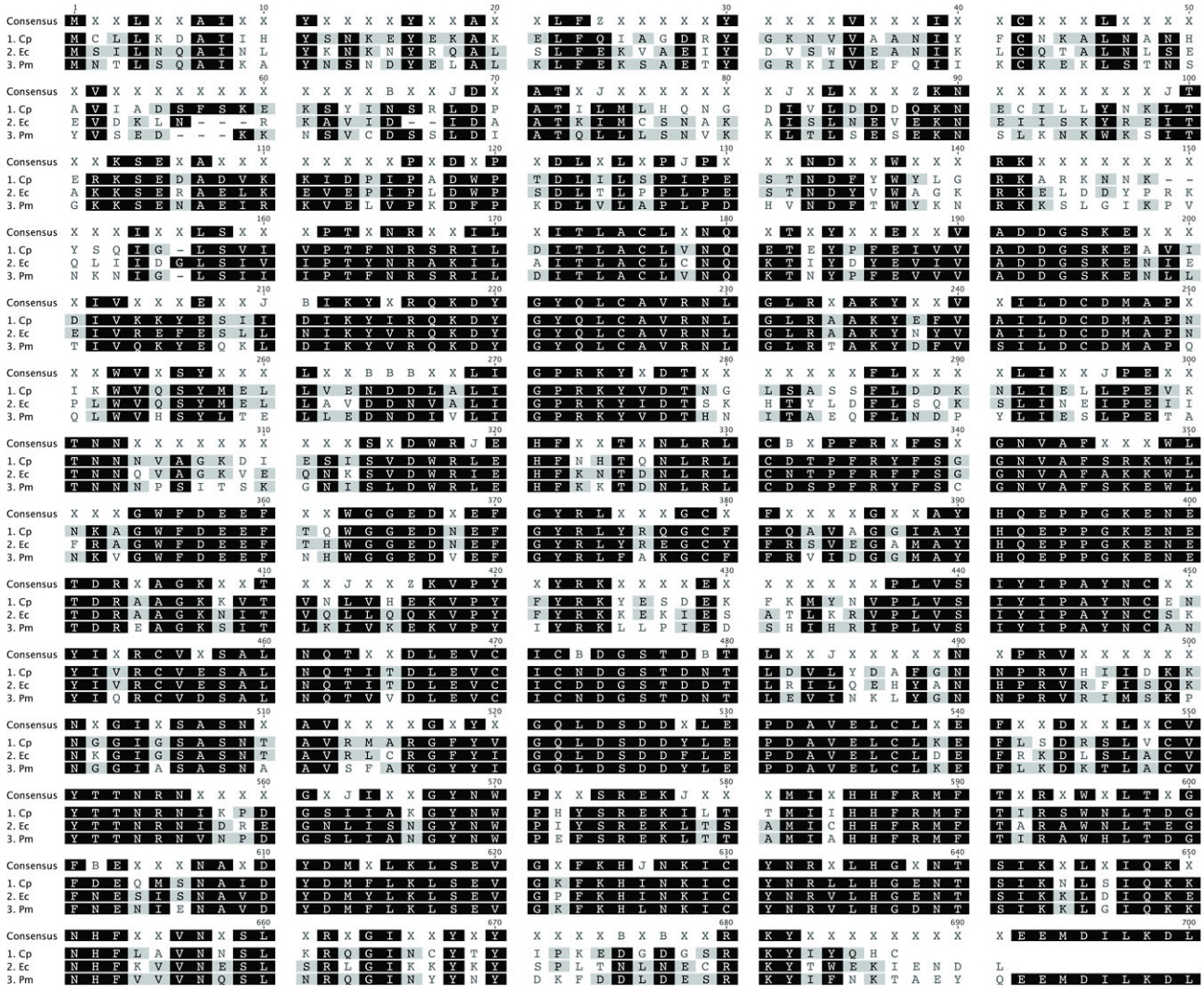

Fig. 1. Protein sequence alignment of the microbial chondroitin synthases, CpCS, PmCS and KfoC. The chondroitin synthases of the three species were aligned with the Clustal W algorithm (Thompson et al. 1994). Substantial similarities (~63% identical overall; majorities highlighted in black) are seen throughout the three enzymes. The two GT2 domains, the GalNAc-transferase and the GlcA-transferase, are found around residues 156–270 and 438–550, respectively, when using the CpCS (ABL66329) sequence for reference. (*Note*: PmCS has a ~250 residue carboxyl terminal extension in comparison to the other two enzymes, but it is not essential for activity and not shown here).

classical glycobiology dogma of "one linkage, one enzyme." In general, these bifunctional enzymes are composed of two fused, but relatively independent, glycosyltransferase activities each containing a donor-binding and an acceptor-binding active site (Jing and DeAngelis 2003; Kane et al. 2006). Potential exceptions are the less understood streptococcal HA synthases, with an integral membrane architecture that is rather recalcitrant to study, that may possess just a single glycosyltransferase domain that alternatively transfers the two sugars of the repeat (Weigel and DeAngelis 2007).

Our group identified the *P. multocida* Type F PmCS, the first chondroitin synthase to be identified and molecularly cloned from any source (DeAngelis and Padgett-McCue 2000); it is very similar to the *E. coli* K4 enzyme, KfoC, coined as a "chondroitin polymerase" (Ninomiya et al. 2002), but the two enzyme activities are rather synonymous. We describe here another chondroitin synthase from an unusual source, *Chlorobium phaeobacteroides*, a species of green sulfur bacteria, which is highly homologous to these known Proteobacterial enzymes. This photosynthetic anaerobic microbe, isolated in the anoxic zone of a cold freshwater lake, is from a much more primal phyla of microbes. *C. phaeobacteroides* is not thought to be a vertebrate pathogen in analogy to the other known species that make chondroitin backbones. This finding of such similar enzymes in distinct types of microbes that probably diverged at least an estimated 2.5–3 billion years ago (Gupta et al. 1999) suggests that perhaps this *Chlorobium* has somehow acquired the gene via horizontal gene transfer as the probability of such close functional evolutionary convergence seems a less likely alternative.

Results and discussion

Expression of a Functional Recombinant C. phaeobacteroides *Chondroitin Synthase in E. coli*—During bioinformatics database searches with the tBLASTn algorithm (comparison of translated nucleotide database to a protein query; NCBI, Bethesda, MD), we identified an enzyme candidate, Accession # ABL66329, that shared significant amino acid sequence homology with the prototypical *P. multocida* chondroitin synthase, PmCS and the *E. coli* K4 chondroitin polymerase (or synthase), KfoC (~63% or 62% identity, respectively) as shown in the Clustal W alignment in Figure 1 (Thompson et al. 1994) as well as similarity to *P. multocida* Type A HA synthase, PmHAS (which is ~87% identical to PmCS). Unlike the known Proteobacteria cases, this new putative chondroitin synthase gene does not reside in a capsule locus with a UDP-glucose dehydrogenase, a metabolic enzyme that produces the required UDP-GlcA precursor. Rather in *C. phaeobacteroides*, this predicted precursor gene essential for GAG biosynthesis, ABL65556, resides hundreds of kilobases away from the putative synthase. Another predicted precursor pathway gene, the UDP-glucose/GlcNAc C4 epimerase for production of UDP-GalNAc, is present in *C. phaeobacteroides* (ABL66006) as well as *E. coli* and *P. multocida*, but none of these genes are found near the synthases in any species. In summary, *C. phaeobacteroides* is predicted to possess the needed machinery to polymerize the chondroitin backbone, but these biosynthetic genes are not arranged in an operon. The sequence of the only other publicly disclosed strain, *C. phaeobacteroides* BS1, did not possess a readily recognizable gene encoding for any known GAG glycosyltransferase.

We molecularly cloned the hypothetical 684-amino acid residue ABL66329 open reading frame with two predicted GT2 CAZY family glycosyltransferase domains from the genomic DNA of *C. phaeobacteroides* and tested its ability to polymerize chondroitin polymer. Soluble extracts derived from recombinant *E. coli* XJa cells with the plasmid CpCS/pMAL-c4E encoding ABL66329 synthesized a polymer in vitro when supplied with both UDP-GlcA and UDP-GalNAc simultaneously (Table I); negative control samples from cells containing the empty vector did not. No substantial incorporation of radiolabeled [^{3}H]GlcA into polymer was observed if UDP-GalNAc was omitted, although very low incorporation was detected when UDP-GlcNAc, the C4 epimer used as an HA polymer donor, was substituted for UDP-GalNAc. Conversely, in experiments using UDP-[^{3}H]GalNAc, substantial incorporation of radiolabel into polymer was only noted when UDP-GlcA was present. For comparison, extracts derived from *E. coli* XJa cells containing the *Pasteurella* chondroitin synthase (via PmCS/pKK223-3 plasmid) were tested in a similar manner. As seen previously (DeAngelis and Padgett-McCue 2000) and verified in these experiments, the prototypical PmCS was rather selective for the authentic UDP-sugar precursors.

Analysis by gel filtration chromatography indicated that recombinant ABL66329 produced chondroitin polysaccharide when supplied both authentic UDP-sugar donors. Radioactivity from reaction

Table I. Sugar nucleotide donor specificity of *Chlorobium* and *Pasteurella* chondroitin synthases

Second sugar nucleotide present	Incorporation of first sugar (%)			
	CpCS		PmCS	
	[^{3}H]GlcA	[^{3}H]GalNAc	[^{3}H]GlcA	[^{3}H]GalNAc
UDP-GlcA	–[a]	100[b]	–	100
UDP-GalA	–	ND[c]	–	~6.5
UDP-GalNAc	100[b]	–	100	–
UDP-GlcNAc	~0.3[d]	–	~1.5	–
UDP-Glc	ND	ND	ND	ND
UDP-Gal	ND	–	ND	–

The relative percentage of incorporation of various UDP-sugar donors in comparison to the assay containing the authentic chondroitin donor precursor (set to 100%) is shown. For the GalNAc-transferase or GlcA-transferase assays, the crude CpCS lysate yielded activities of ~0.002 or 0.0002 pmol/min/μg, respectively; the crude PmCS yielded ~2.4 and 0.2 pmol/min/μg, respectively. The reactions with very low potential signals were also tested by single sugar extension reactions via fluorophore-assisted carbohydrate electrophoresis (FACE) for verification.

[a]– = Not applicable.

[b]Authentic GAG reaction set to 100%.

[c]ND = not detectable above background controls of empty vector lysate.

[d]Verified by FACE gel.

mixtures labeled simultaneously with [^{14}C]GlcA and [^{3}H]GalNAc sugars co-migrated as a ~2–10 kDa polymer (Figure 2, indicated by a bracket). The identity of the polymer as chondroitin was verified by its sensitivity to both *Proteus* chondroitinase ABC and ovine testicular hyaluronidase (which also possesses a potent chondroitinase activity) and its resistance to the action of both *Streptomyces* HA lyase (a very selective HA-degrading enzyme) and *Flavobacterium* heparin lyase III (digests heparosan and heparan sulfate chains). The radiolabel of chondroitinase ABC-digested polymer eluted in the included fractions with the unincorporated UDP-sugars. Therefore, the *Chlorobium* ABL66329 gene product is a *bona fide* chondroitin synthase that we call CpCS based on the nomenclature system employed for other GAG synthases (Genus species GAG Synthase).

The addition of a chondroitin oligosaccharide acceptor increased sugar incorporation catalyzed by recombinant CpCS greater than in parallel reactions using either HA or heparosan oligosaccharide acceptors, indicating an acceptor preference (Table II). No substantial incorporation of radiolabeled sugars into polymer by CpCS was observed during short time points (<2 h) if acceptor was absent (i.e. corresponding to de novo initiation starting polymer from sugar nucleotides alone). For the short time points (~10 min), the addition of the chondroitin oligosaccharide acceptor increased sugar incorporation catalyzed by PmCS to a larger extent in comparison to parallel reactions using HA oligosaccharide acceptor. No substantial incorporation of radiolabeled sugars into polymer by PmCS was observed if heparosan oligosaccharide acceptor was added or if no acceptor was added.

To verify the relaxed acceptor usage demonstrated by CpCS, we employed fluorophore-assisted carbohydrate electrophoresis to independently prove acceptor extension (Figure 3). The recombinant CpCS extended both the HA oligosaccharide and heparosan oligosaccharide acceptors thus creating artificial GAG structures. The products are chondroitin–heparosan or chondroitin–HA chimeric polymers when both UDP-GlcA and UDP-GalNAc were supplied in excess of the noncognate acceptor.

For both acceptors, the PmCS also transferred a single GalNAc to the nonreducing terminal GlcA (Figure 3). As expected, when both UDP-GlcA and UDP-GalNAc were supplied in excess of the ANTS (8-aminonaphthalene-1,3,6-trisulfonic acid)-labeled HA oligosaccharide acceptor, the PmCS successfully extended this

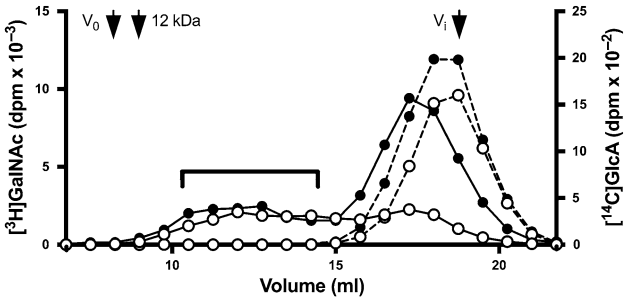

Fig. 2. Gel filtration analysis of radiolabeled polymer synthesized by CpCS in vitro. Chondroitin polymer synthesized chemoenzymatically using the soluble crude CpCS extract was monitored by dual-labeling with [^{3}H]GalNAc (white circles) and [^{14}C]GlcA (black circles). Half of the reaction product was digested using *Proteus* chondroitinase ABC, as described in "Materials and methods" before chromatography. Both the starting reaction mixture (solid line) and digested products (broken line) were analyzed on the Sephadex G-50 column. The bracket identifies the undigested starting polymer. The calibration elution volumes (ml) are denoted with arrows: void volume (V_0), 8.3; 12-kDa dextran, 9.0; totally included volume (V_i), 18.8.

acceptor by multiple sugars to make a chimeric chondroitin-HA polymer as seen before (Tracy et al. 2007). The PmCS, however, even if supplied both donors was unable to further extend the ANTS-labeled heparosan oligosaccharide acceptor into polysaccharide indicating a more selective acceptor usage than CpCS. In fact, in the presence of both UDP-sugars, the first sugar transfer was actually inhibited as observed by FACE (Figure 3, lane 2 vs. 3) as well as by mass spectroscopy (data not shown). KfoC was reported to be unable to add chondroitin chains onto heparin acceptors (Ninomiya et al. 2002), but these are sulfated polymers that may have masked the groups critical for enzyme recognition or binding. More recently, KfoC was shown to extend a heparosan trisaccharide by a single GalNAc (Xue et al. 2016) in a fashion similar to PmCS. However, their data for repeated sugar addition and polymerization of a chondroitin chain onto the same acceptor was not definitive; polymers were observed in the reaction mixture only by size exclusion chromatography-light scattering analysis. Neither further proof of the identity of the construct (e.g. detection of the acceptor moiety in the product) nor the use of a control reaction to account for any de novo initiation (i.e. polymerization without a priming acceptor group that could mimic the observed polydisperse profiles) was presented for KfoC.

CpCS is the first glycoengineering catalyst demonstrated to possess the ability to produce chimeric chondroitin-heparosan block polysaccharides. After undergoing sulfation, perhaps these novel chimeric molecules will possess bioactivity due to their rudimentary structural similarity to certain proteoglycans, such as syndecan, containing both GAG types on a single macromolecule.

Analysis of the concentration dependence of Mg^{2+} and Mn^{2+} ions on the chondroitin synthase activity of recombinant CpCS indicated the importance of these metals for the synthase's activity as observed for other microbial GAG synthases (DeAngelis 2002b). No appreciable sugar incorporation of the radiolabeled [^{3}H]GlcA into polymer was detected when the divalent metal cations were chelated (using EDTA) in the reactions. Transferase activity of CpCS was increased ~3- to 18-fold at a concentration of 1 mM when Mn^{2+} was supplied instead of Mg^{2+}. The addition of 10 mM Mg^{2+} achieved a similar effect to that observed with 1 mM Mn^{2+}.

CpCS, the first GAG synthase to be discovered and characterized from the Chlorobi phylum, shares many functional characteristics with the *Pasteurella* chondroitin synthase that we previously discovered, but has some distinctions. Both enzymes demonstrate rather restricted donor UDP-sugar specificity in vitro, but CpCS appears to be more tolerant of noncognate acceptors than the PmCS enzyme, as evidenced by our tests using the HA or the heparosan oligosaccharide acceptors. The CpCS elongates the HA oligosaccharide acceptor more quickly than its heparosan counterpart, suggesting that this enzyme is more tolerant of a nonauthentic epimer sugar in its acceptor-binding region than if the nonauthentic acceptor also possesses different glycosidic linkages. While both CpCS and PmCS extend acceptors to produce chondroitin polymers, the polymers observed in our gel filtration studies of CpCS were <12 kDa, as opposed to the longer ~100–400 kDa PmCS polymers reported in the past (DeAngelis and Padgett-McCue 2000). The CpCS reaction was not extensively optimized; the production of only short polymer products in vitro could be due to factors including (a) the CpCS's relatively slower transferase activity when compared to the PmCS, (b) potential lower affinity for the nascent GAG chain and/or (c) to its potential thermolability (i.e. leading to rapid loss of catalyst). The latter feature may be due to the environment from which *C. phaeobacteroides* was isolated, namely deep in a cold Scandinavian lake.

Table II. Oligosaccharide acceptor specificity of CpCS and PmCS chondroitin synthases

Oligosaccharide acceptor present	[^{3}H]GlcA Transferred (%)	
	CpCS	PmCS
None (de novo initiation)	ND[a]	ND
Hep4/6	6.7	ND
Chon4	100[b]	100[b]
HA4	67	14

The relative percentage of UDP-sugar incorporation in comparison to the assay containing the authentic cognate chondroitin tetrasaccharide acceptor, Chon4 (set to 100%; the crude lysates of CpCS and PmCS yielded activities of ~0.003 or ~9.2 pmol/min/μg, respectively), to noncognate hyaluronan (HA4 tetrasaccharide) or heparosan (Hep4/6 tetrasaccharide and hexasaccharide mixture) acceptors is shown.

[a]ND = not detectable.

[b]Authentic cognate GAG acceptor, Chon4, reaction set to 100%.

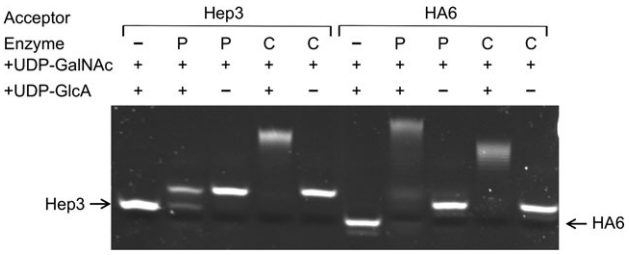

Fig. 3. FACE gel analysis of noncognate acceptor usage by CpCS and PmCS enzymes. Fluorescently tagged heparosan (*Hep3* = GlcA-GlcNAc-GlcA-ANTS) or hyaluronan (*HA6* = [GlcA-GlcNAc]$_3$-ANTS) oligosaccharides (starting bands marked with *arrows*) were tested as acceptors for both chondroitin synthases. The reactions were incubated with either UDP-GalNAc (50-fold molar excess over acceptor) alone or in combination with UDP-GlcA (also 50-fold molar excess) and then run on a PAGE gel with detection by ultraviolet excitation. The *Chlorobium* CpCS enzyme (*C*) readily extended HA or heparosan with longer chondroitin chains, but the *Pasteurella* PmCS (*P*) only efficiently elongated HA acceptors. (*note*: the dark band migrating near the HA6 position in all lanes is the shadow from the loading buffer dye).

The UDP-sugar binding pockets of KfoC have previously been identified via X-ray crystallography of the enzyme/substrate complexes (Osawa et al. 2009), but there are no similar clues to the nature of acceptor-binding pockets of either GT2 domain. Perhaps the CpCS sequence and its relative differences in extension activity will assist in identifying and understanding this critical feature of these GAG chain-elongating enzymes. The sequence data from multiple CSs should help guide future mutagenesis studies of this interesting class of carbohydrate-active proteins.

The potential role of CpCS in the *C. phaeobacteroides* life cycle is not known at this point. Theoretically, the organism has the ability to perform the chondroitin polymerization reaction because in addition to the synthase gene, the putative gene candidates for the two pathways essential for production of the required UDP-sugars, UDP-GlcA and UDP-GalNAc, appear to exist. An extracellular capsule has not been reported in this *Chlorobium* species, but the microbe may need certain environmental cues to induce exopolysaccharide production that are not present under typical artificial culture conditions. The appearance of mucoid colonies, encapsulated cells via microscopy or viscous spent media, the hallmarks of abundant exopolysaccharide production, was not reported for *C. phaeobacteroides*. Thin or small diameter capsules, however, are not readily detectable except with the use of an immunological reagent; for chondroitin, such specific antibody probes are not available due to its similarity to animal GAGs.

It was not predictable that *C. phaeobacteroides* would make a GAG because a free-living, photosynthetic, nonpathogenic, noncommensal organism should not need an animal "self" molecule for protection. As usual, Nature always holds surprises, especially in the field of glycobiology; the past discovery of the CvHAS HA synthase of the *Chlorella* virus PBCV-1 that infects protozoan green algae with similarities to the streptococcal and vertebrate enzymes is another case in point (DeAngelis et al. 1997).

Another puzzling aspect of this study is that the chondroitin synthases of Proteobacteria (*Pasteurella* and *Escherichia*) and Chlorobi, two separate phyla, are so alike at the amino acid level even though these microbes diverged ~2.5–3 billion years ago. Some *Chlorobium* species, but not *C. phaeobacteroides*, have been reported to be transformable in the laboratory (Frigaard and Bryant 2001), but the fact that the two phyla of microbes probably do not co-exist in high concentrations in the same ecological niche suggests that the putative ancestral *cpCS* gene capture may have been a very rare event. In analyses comparing the codons comprising very similar or identical amino acid stretches of *cpCS*/ABL66329 to either the *pmCS* or the *kfoC* DNA sequences with the BLAST algorithm, some *cpCS* segments more closely resembled *pmCS* while an equal amount appeared to be more *kfoC*-like (not shown). Therefore, the original source organism is still difficult to pinpoint at this time. When more genomic and biochemical data from other green sulfur bacteria is accumulated, it may become apparent if GAG synthases are a normal or unusual attribute.

Materials and methods

Reagents

Unless otherwise noted, all chemicals used were from Sigma (St. Louis, MO) or Fisher (Pittsburgh, PA), and all molecular biology reagents were obtained from Promega (Madison, WI). Custom synthetic DNA oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). The genomic DNA of wild-type *C. phaeobacteroides* strain DSM 266 was generously provided by Dr. Jorg Overmann (Ludwig Maximilians University, Munich, Germany).

Sequence analysis of *C. phaeobacteroides* and other chondroitin synthases

The DNA sequences encoding regions (~10–23 amino acids stretches) of very high similarity or identity between all three GAG synthases (*C. phaeobacteroides* ABL66329, *P. multocida* Type F chondroitin synthase, PmCS and the *E. coli* K4 KfoC) were compared by standard nucleotide BLASTn (NCBI) for simple evolutionary analysis. The number of identical codons and substituted codons including first or third/wobble positions were compared among the three genes. Neighboring genes upstream and downstream of the synthases were also evaluated by BLAST searches for similarity with known related genes and with the longer 3′ end of the *pmCS* sequence.

Construction of recombinant maltose-binding chondroitin synthase fusion construct

ABL66329, the CpCS, was expressed as a carboxyl-terminal fusion to maltose-binding protein (MBP) using the pMAL-c4E vector (New England Biolabs, Ipswich, MA). Polymerase chain reaction using *Pfu*Ultra Hotstart DNA polymerase (Stratagene, La Jolla, CA) was used to amplify and clone the open reading frame (684-amino acid residues) from isolated genomic DNA into a cloning vector. The codon for the third amino acid of the protein (leucine) was optimized for *E. coli* via a silent mutation (CTT to CTG, a more highly used codon) introduced with the forward PCR primer. The resulting fragment was then further amplified with the same polymerase and unique flanking restriction sites (*Pst*I and *Hin*dIII) were added with the next pair of primers used for amplification. The resulting PCR product was purified from primers and nucleotides using the QIAquick® PCR purification kit (Qiagen, Valencia, CA), and restriction-digested with both appropriate enzymes, purified again and ligated to similarly double-digested and gel-purified pKK223-3 plasmid (Pharmacia, Uppsala, Sweden). This construct was then used as a template for further PCR amplification using the aforementioned polymerase and the same unique restriction sites were added with the primers used for amplification as before. The resulting PCR product was subcloned as above into the final expression vector, pMAL-c4E. *E. coli* One Shot® TOP10 (Invitrogen, Carlsbad, CA) was used for the initial transformation on LB ampicillin (100 μg/ml) plates and grown at 30°C. The construct was confirmed by DNA sequencing (OMRF Sequencing Facility, Oklahoma City, OK). Plasmid DNA extracted from the sequenced verified clone containing the tentative *Chlorobium* chondroitin synthase was then ethanol precipitated and transformed into *E. coli* XJa (Zymo Research, Irvine, CA) for purposes of protein expression.

Expression of *C. phaeobacteroides* chondroitin synthase

Cultures of *E. coli* XJa were grown in Superior Broth (AthenaES, Baltimore, MD) with ampicillin (100 μg/ml) and L-arabinose (3.25 mM) at 25°C. Log phase cultures were induced with isopropyl-β-D-1-thiogalactopyranoside (0.2 mM final) for 22 h. At 1 h post-induction, growth was supplemented with fructose (12.8 mM final). Cells were harvested by centrifugation (3800 × *g*, 30 min at 4°C). The cell pellet was resuspended in 50 mM Tris, pH 7.2, and a broad spectrum protease inhibitor cocktail on ice, and then freeze-thawed from −80°C twice allowing the bacterially-encoded phage lysin to degrade cell walls in a similar fashion to the PmCS. The lysates were clarified by centrifugation (40,000 × *g*, 30 min at 4°C). The crude soluble extract was used as the source of enzyme for in vitro assays; the use of MBP-tag to purify the enzyme via affinity chromatography with amylose resin in several trials did not result in high specific activity preparations (data not shown). The truncated *P. multocida* enzyme (PmCS residues 46–695 expressed with pKK223-3 vector with XJa cells) was produced in a similar manner as the crude extract above (for a more fair comparison of the two enzymes) as well as a purified version (Tracy et al. 2007). Protein content of extracts was measured using the Bradford assay with a bovine serum albumin standard (Pierce, Rockford, IL).

Chondroitin synthase activity assays

Incorporation of radiolabeled monosaccharides from UDP-[^{3}H]GlcA and/or UDP-[^{3}H]GalNAc precursors (Perkin Elmer, Boston, MA) was used to monitor chondroitin synthase activity, including acceptor and donor specificity. Samples of soluble cell lysate were usually assayed in a buffer containing 90 mM Tris, pH 7.2, 5 mM $MnCl_2$, 0–1 mM UDP-GlcUA and 0–1 mM UDP-GalNAc in the presence of an oligosaccharide acceptor, for various times at 25°C; either 0.05 μCi UDP-[^{3}H]GlcA or UDP-[^{3}H]GalNAc was used as the tracer as described in each experiment. The reactions were quenched by addition of sodium dodecyl sulfate (2% final) and the polymer products were separated from substrates by descending paper (Whatman 3 MM) chromatography with ethanol/1 M ammonium acetate, pH 5.5, development solvent (65:35). The origin of the paper strip containing GAG products was cut out, eluted with water, and the incorporation of radioactive sugars into GAG chains was detected by liquid scintillation counting with EconoSafe™ cocktail (Research Products International, Mt. Prospect, IL).

For donor specificity, reactions were incubated for 10–180 min. To test the acceptor specificity of CpCS, the incorporation of the two authentic sugars with various oligosaccharide acceptors (either HA4 (DeAngelis et al. 2003) or mixtures of Hep4/6 (Sismey-Ragatz et al. 2007)) were compared to the authentic cognate chondroitin tetrasaccharide, Chon4, in 10–120 min assays. For these assays, a "negative control" lysate of XJa with empty plasmid was also analyzed; this low background was subtracted from the values obtained in the parallel assays with the CS enzymes. To test the donor specificity of the *Chlorobium* enzyme, various UDP-sugars (UDP-GlcNAc, UDP-galactose, UDP-GalA or UDP-Glc) were substituted for the authentic chondroitin UDP-sugar precursors in the presence of HA4. For these assays, a "no second sugar" control was also analyzed to determine the background value of the reaction mixture itself without one of the essential chondroitin precursors present; this value was subtracted from the value obtained in the parallel assays with two UDP-sugars (a uronic acid and a hexosamine form the repeating unit). All reactions were run in at least duplicate and the signals were averaged.

GAG synthase metal specificity

Incorporation of radiolabeled monosaccharide from UDP-[^{3}H]GlcA precursor was used to compare the activity of CpCS in the presence of either $MnCl_2$ or $MgCl_2$ or EDTA (= "no metal"). Samples of soluble cell lysate were assayed in a buffer that also contained 90 mM Tris, pH 7.2, 50 μM UDP-[^{3}H]GlcA (0.05 μCi), and 1 mM UDP-GalNAc in the presence of HA4, for 1–2 h at 25°C. The reaction products were separated from substrates by descending paper chromatography as mentioned above, and detected by liquid scintillation counting.

Size analysis and enzymatic degradation of labeled polymers

Gel filtration chromatography was used to analyze the size distribution of chondroitin produced by CpCS in vitro. Polymers were dual-labeled with both [^{3}H]GalNAc and [^{14}C]GlcA in a pulse-chase format. Basically, the CpCS lysate (944 μg total protein) was incubated with HA4 acceptor (1 μg = 1.3 nM) in 90 mM Tris, pH 7.2, 5 mM $MnCl_2$, 5 mM $MgCl_2$ buffer and the pulse of radioactive UDP-sugars (50 μM each with 0.04 μCi [^{3}H] and 0.02 μCi [^{14}C] tracers) for 2 h, followed by the addition of the chase of higher amounts of unlabeled donors (2 mM each final; 100 μl total reaction volume) for 16 h. Separations were performed with a Sephadex G-50 Fine column (270 × 10 mm) eluted with 0.15 M sodium nitrate. Radioactivity of the collected fractions was

monitored using liquid scintillation counting with EconoSafe™ cocktail. The column was standardized with 2000 kDa blue dextran (void marker), 12 kDa fluorescein-labeled dextran and vitamin B_{12} (included marker; 1.4 kDa).

To confirm the identity of the radiolabeled polymers, portions of the reactions were digested with one of the various selective GAG-degrading enzymes (10 milliunits *Proteus* chondroitinase ABC, 5 units *Streptomyces* HA lyase, 5 units ovine testicular hyaluronidase or 0.1 units *Flavobacterium* heparin lyase III) for ~21 h at 37°C and analyzed by gel filtration as above or by paper chromatography.

Fluorophore-assisted carbohydrate electrophoresis

HA (pentamer or hexamer) and heparosan (trimer) oligosaccharides were labeled with ANTS to facilitate fluorescent visualization of the extended oligosaccharides and polysaccharides by gel electrophoresis (Oonuki et al. 2005). For acceptor usage trials, samples of soluble CpCS or PmCS as cell lysates were reacted in a buffer containing 60 mM Tris, pH 7.2 and 5 mM $MnCl_2$, with the ANTS-labeled oligosaccharide and either UDP-GalNAc alone or with UDP-GalNAc and UDP-GlcA for 18 h at 25°C. For UDP-sugar specificity verification reactions, only a single type of donor (UDP-GlcNAc, UDP-GalA or UDP-Glc vs. positive controls of an authentic chondroitin donor) was added to the acceptor and incubated under similar conditions before FACE.

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Conflict of interest statement

None declared.

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Abbreviations

ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; CpCS, *Chlorobium phaeobacteroides* chondroitin synthase; CS, chondroitin synthase; FACE, fluorophore-assisted carbohydrate electrophoresis; GAG, glycosaminoglycan; GlcA, glucuronic acid; GalNAc, *N*-acetylgalactosamine; HA, hyaluronan; PAGE, polyacrylamide gel electrophoresis.

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