Identification and Molecular Cloning of a Chondroitin Synthase from Pasteurella multocida Type F*

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Pasteurella multocida Type F, the minor fowl cholera pathogen, produces an extracellular polysaccharide capsule that is a putative virulence factor. It was reported that the capsule was removed by treating microbes with chondroitin AC lyase. We found by acid hydrolysis that the polysaccharide contained galactosamine and glucuronic acid. We molecularly cloned a Type F polysaccharide synthase and characterized its enzymatic activity. The 965-residue enzyme, called P. multocida chondroitin synthase (pmCS), is 87% identical at the nucleotide and the amino acid level to the hyaluronan synthase, pmHAS, from P. multocida Type A. A recombinant Escherichia coli-derived truncated, soluble version of pmCS (residues 1–704) was shown to catalyze the repetitive addition of sugars from UDP-GalNAc and UDP-GlcUA to chondroitin oligosaccharide acceptors in vitro. Other structurally related sugar nucleotide precursors did not substitute in the elongation reaction. Polymer molecules composed of $\sim 10^3$ sugar residues were produced, as measured by gel filtration chromatography. The polysaccharide synthesized in vitro was sensitive to the action of chondroitin AC lyase but resistant to the action of hyaluronan lyase. This is the first report identifying a glycosyltransferase that forms a polysaccharide composed of chondroitin disaccharide repeats, $[\beta(1,4)\text{GlcUA-}\beta(1,3)\text{GalNAc}]_n$. In analogy to known hyaluronan synthases, a single polypeptide species, pmCS, possesses both transferase activities.

Glycosaminoglycans (GAGs), long linear polysaccharides consisting of disaccharide repeats that contain an amino sugar, are found in most animals (1–4). Chondroitin ($\beta(1,4)\text{GlcUA-}\beta(1,3)\text{GalNAc}]_n$, heparin/heparan ($\alpha(1,4)\text{GlcUA-}\beta(1,4)\text{GlcNAc}]_n$, and hyaluronan ($\beta(1,4)\text{GlcUA-}\beta(1,3)\text{GlcNAc}]_n$) are the three most prevalent GAGs found in humans. In the former two polymers, usually $n = 20$ to 100, whereas in the case of HA, $n = 10^4$–$10^5$. Chondroitin and heparin/heparan, but not HA, are synthesized as glycoproteins and are sulfated at various positions in vertebrates. A substantial fraction of the GlcUA residues of heparin are epimerized to form iduronic acid. Many lower animals possess these same GAGs or very similar molecules (5). GAGs play both structural and recognition roles on the cell surface and in the extracellular matrix. By virtue of their physical characteristics, namely a high negative charge density and a multitude of polar hydroxyl groups, GAGs help hydrate and expand tissues. A plethora of proteins bind selectively to one or more of the GAGs (6, 7). Thus the proteins found on cell surfaces or the associated extracellular matrices (e.g. CD44, collagen, fibronectin) of different cell types may adhere or interact via a GAG intermediate. Also GAGs may sequester or bind certain proteins (e.g. growth or coagulation factors) to cell surfaces.

Certain pathogenic bacteria produce an extracellular polysaccharide coating called a capsule that serves as a virulence factor (8). In a few cases, the capsule is composed of GAG or GAG-like polymers. As the microbial polysaccharide is identical or very similar to the host GAG, the antibody response is either very limited or non-existent. 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 Type A Pasteurella multocida (9) and Gram-positive Group A and C Streptococcus (10). The HA capsule of these microbes increases virulence by $10^2$–$10^3$-fold as measured by LD$_{50}$ values, the number of colony-forming units that will kill 50% of the test animals after bacterial challenge (11, 12). The invasiveness and pathogenicity of certain Escherichia coli strains has also been attributed to their polysaccharide capsule (8). Two E. coli capsular types, K4 and K5, make polymers composed of GAG-like polymers. The E. coli K4 polymer is an unsulfated chondroitin backbone decorated with fructose side branches on the C3 position of the GlcUA residues (13). The K5 capsular material is a polysaccharide, called heparosan, identical to mammalian heparin, except that the bacterial polymer is unsulfated, and there is no epimerization of GlcUA to iduronic acid (14).

The studies of GAG biosynthesis have been instrumental in understanding polysaccharide production in general. The HA synthases were the first GAG glycosyltransferases to be identified at the molecular level (15–18). These enzymes utilize UDP-sugar nucleotide substrates to produce large polymers containing thousands of disaccharide repeats. The genes encoding bacterial, vertebrate, and viral HAS enzymes have been cloned. In all these cases, expression studies demonstrated that transformation with DNA encoding a single HAS polypeptide conferred the ability of foreign hosts to synthesize HA. Except for the most recent HAS to be identified, P. multocida pmHAS (17), these proteins have similar amino acid sequences and predicted topology in the membrane (18). Two classes of HASs

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF195517.

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have been proposed to exist based on these structural differences as well as potential differences in reaction mechanism (18, 19).

The biochemical study of chondroitin biosynthesis in vertebrates was initiated in the 1960s (20–22). Only recently have the mammalian enzymes for elongation of the polysaccharide backbone of chondroitin been tentatively identified by biochemical means. An 80-kDa GlcUA-transferase found in vertebrate cartilage and liver was implicated in the biosynthesis of the chondroitin backbone by photoaffinity labeling with an azido-UDP-GlcUA probe (23). A preparation from bovine serum with the appropriate GalNAc- and GlcUA-transferase activities in vitro was obtained by conventional chromatography, but several bands on SDS-polyacrylamide gels (including a few migrating 80 kDa) were observed (24). The situation will probably be unclear in vertebrates until a chondroitin synthase has been sequenced at the gene level and functionally expressed.

With respect to related microbial GAG synthases other than the HASs, only the E. coli K5 glycosyltransferase, KfIC, that synthesizes heparosan has been identified by genetic and biochemical means (25). In analogy to the HASs, it appears that KfIC is capable of transferring both sugars of the disaccharide repeat to the growing polymer chain. The chondroitin backbone-synthesizing enzyme of E. coli K4 has been enzymatically characterized (26), but the genes encoding the relevant glycosyltransferases have not yet been identified.

Many P. multocida isolates produce GAG or GAG-like molecules as assessed by enzymatic degradation and removal of the capsule of living bacterial cells (27, 28). Type A P. multocida, the major fowl cholema pathogen, makes a capsule that is sensitive to hyaluronidase. Subsequent NMR structural studies of capsular extracts confirmed that HA was the major polysaccharide present (29). A specific HA-binding protein, aggrekan, also interacts with HA from Type A P. multocida (17). Two other distinct P. multocida types, a swine pathogen, Type D, and a minor fowl cholema pathogen, Type F, produce polymers that are chondroitin or chondroitin-like based on the observation that their capsules are degraded by Flavobacterium chondroitin AC lyase (27, 28). After enzymatic removal of the capsule, both types were more readily phagocytosed by macrophages (17). To obtain the homologous region from the Type F chromosomally encoded chondroitin synthase of P. multocida, Rimler (United States Department of Agriculture, Ames, IA (30)).

The anionic polysaccharide was purified by a combination of treatments. The GAG extract was precipitated by the addition of cetylpyridinium chloride (1% w/v final concentration). After standing for 10 min, the precipitate was collected by high speed centrifugation and redissolved in 2.5 M NaCl. The mixture was clarified by high speed centrifugation, and the supernatant was precipitated with 3 volumes of ethanol. The precipitate was washed twice with 70% ethanol, dried slightly, and resuspended in 2.5 M NaCl. The ethanol precipitation procedure was repeated, and the pellet was redissolved in water. Another round of ethanol precipitation (2 volumes) was performed. The final GAG pellet was dissolved in water. The yield (0.6 mg of uronic acid) was determined with the carbazole assay for uronic acid using a glucuronolactone standard (32).

Enzymatic degradation of the GAG extract was determined by acid hydrolysis (2 N HCl, 4 h, 100 °C) and high pH anion exchange chromatography. The hydrolysate was repeatedly diluted in water and dried under vacuum to remove HCl, then mixed with a rhamnose standard and clarified using a 0.2-μm spin filter. Portions of the hydrolysate (5–5 mmol of uronic acid) were injected onto a PA-1 column (Dionex) equilibrated with 12 mm NaOH. After isocratic elution (20 min) to separate the neutral sugars, a gradient of sodium acetate (0 to 0.18 M in 30 min) was utilized to separate the acidic sugars. Eluted compounds were detected by pulsed amperometric detection. In parallel runs, the Type F sample was spiked with known monosaccharide standards or authentic chondroitin sulfate C (derived from shark cartilage) hydrolysate. HA and heparin hydrolysate standards were also run. Retention times relative to the rhamnose internal standard were calculated.

PCR Analysis of Type F Genomic DNA—Preliminary data from Southern blot analysis using pmHAS hybridization probes (18) suggested that the Type A and the Type F microbes were very homologous at the capsule locus. PCR was utilized to verify these findings. Type F chromosomal DNA (0.1 μg) served as a template in PCR reactions (20 μl) utilizing oligonucleotide primers corresponding to various regions of the Type A capsule locus genes. After 40 cycles of PCR (94 °C 30 s; 42 °C 30 s; 72 °C 4 min) with Taq DNA polymerase in the supplied buffer (Fisher), the samples were separated by agarose gel electrophoresis. Many primer pairs, but not all, amplified Type F DNA to yield products of the predicted size assuming that Type A and Type F loci were homologous. Two primers (Pm10, 5’-ACGTGCTAATTTATTGTTAGCC-3’; Pm21, 5’-TTTTTAAAGATAGGCTGTC-3’) were chosen to amplify a 3.6-kilobase portion of the Type F locus predicted to contain the DNA-encoding the carboxyl terminus half of the KfIC homolog and the amino-terminal portion of the putative polysaccharide synthase. The product from a PCR reaction (26 cycles) was cloned into a TA vector (Invitrogen) according to the manufacturer guidelines. The plasmid was amplified in cycle sequencing (ThermoSequenase® system with 35S labeled nucleotides) and sequenced using the manufacturer guidelines for colorimetric development. E. coli XLI-Blue MRF® was co-infected with the purified, individual positive λ clones and ExAssist helper phage to yield phagemids. The resulting phagemids were transfected into E. coli XLORL cells to recover the plasmids. Sequence analysis of the plasmids revealed a novel open reading frame (GenBank® accession number AF195517), which we called pmCS, with high homology to pmHAS.

Expression of Recombinant P. multocida Chondroitin Synthase—In our previous studies with pmHAS (33), we found that a functional, soluble enzyme would be created if a portion of the carboxyl terminus was truncated by molecular genetic means. Therefore, a portion of the pmCS open reading frame (residues 1–704) in the insert of one of the excised λ clones, pPMF4A, was amplified by 20 cycles of PCR (16) with Taq polymerase. The sense primer corresponded to the sequence at the downstream end of the amino terminus of the pmCS open reading frame, whereas the reverse primer encoded the new carboxyl terminus followed by an artificial stop codon. The resulting PCR product was purified and concentrated using GeneClean. This insert was cloned using the pETBlue-1 Acceptor system (Novagen) according to the manufacturer’s instructions. The Taq-generated single A overhang is used to facilitate the cloning of the open reading frame downstream of the T7 promoter and the ribosome bind-
ing site of the vector. The ligated products were transformed into E. coli NovaBlue and plated on LB carbenicillin (50 \(\mu\)g/ml) under conditions for blue/white screening. White or light blue colonies were analyzed by restriction digestion. A clone containing a plasmid with the desired truncated open reading frame, pMmCS1-704, was transformed into E. coli Tuner, the T7 RNA polymerase-containing expression host, and maintained on LB media with carbenicillin and chloramphenicol (34 \(\mu\)g/ml) at 30 °C. Log phase cultures were induced with isopropyl-

Western Blot Analysis of Recombinant P. multocida Chondroitin Synthese—A monospecific polyclonal antibody was generated against a synthetic peptide (acetyl-LSDSYDLYEPDAVCLKEF amide) corre-

Assays for Chondroitin Synthase Activity—Incorporation of radiola-

Size Analysis and Enzymatic Degradation of Labeled Polymers—Gel fil-

RESULTS

Compositional Analysis of Type F P. multocida Polymer—Previous work by others shows that the Type F capsule was removed from bacterial cells by treatment with chondroitin AC lyase (27, 28). We found that a fragment of the specific HA-binding protein, aggrecan, in the HA-TEST assay (Amersham Pharmacia Biotech) did not cross-react with extracts of the Type F polymer but readily detected the HA in parallel extracts from Type A bacteria. (data not shown). Acid hydrolysis and monosaccharide analysis of the Type F polymer showed that it contains the sugars galactosamine and GlcUA (Table I). The ion exchange profile of the chondroitin sulfate C hydrolysate was indistinguishable from the Type F hydrolysate; mixing experiments demonstrated that the component peaks migrated identically (not shown). No other sugars were detected in the Type F polymer including glucosamine, mannose, galactose, glucose, and fucose. Hydrolysates of the HA and heparin stand-

Table I  

| Sugar | C | C/F mix | F | HA | HEP |
|-------|---|---------|---|----|----|

Acid hydrolysis and high pH ion exchange chromatography were utilized to determine the sugar components of the Type F polymer (F). The monosaccharides chondroitin sulfate C (C), hyaluronic (HA), and heparin (HEP), and pure monosaccharides were used as standards. Under these hydrolysis conditions, deacetylation and desulfation as well as the desired fragmentation of glycosidic bonds occur. Retention times relative to the internal standard rhamnose elution time (10.7 min; set to 1) are presented for the relevant hexosamines. Acidic sugars were eluted with a sodium acetate gradient; the retention time of the major uronic acid peak from the start of the gradient is presented. Type F polysaccharide and chondroitin sulfate possess the identical monosaccharide composition, galactosamine and glucuronic acid. ND, not detected.

Molecular Cloning of the Type F P. multocida Capsular Locus—PCR products were obtained utilizing Type F chromo-

104 plaques, and these phage were converted into plasmids. We found that both plasmids contained a novel open reading frame of 965 residues, which we named pmCS, that was highly homologous to the Type A capsule locus. A 3.6-kilobase PCR product that contained large portions of the Type F KfaA homolog (a putative polysaccharide transporter of E. coli) and the putative pmCS gene was used as a hybridization probe to obtain an intact P. multocida capsular locus from a 1 library. Two positively hybridizing plaques were found after screening 7-10 plaques, and these phage were converted into plasmids. We found that both plasmids contained a novel open reading frame of 965 residues, which we named pmCS, that was highly homologous to the Type A HA synthesize, pmHAS (Fig. 1). The level of identity was 87% at both the DNA and protein levels. The differences in amino acid sequence were mainly localized to several regions of the polypeptide in the amino terminus half of the molecules. There is an excellent overall alignment of the enzymes except for a 7-residue insertion in the pmHAS sequence in the position corresponding to residue 53 of the pmCS sequence.

The central portion of both the pmCS and the pmHAS polypeptides (residues 430–530) is most homologous to bacte-

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contain a sequence that corresponds exactly to the synthetic peptide used to generate the antibody. Extracts derived from E. coli Tuner cells containing the pPmCS1–704 plasmid contained an immunoreactive band of the appropriate size (i.e. predicted to be 80 kDa), but this band was not present in samples from cells with the vector alone control. The use of soluble pmCS1–704 protein provided increased expression levels and facilitated preparation of enzyme in comparison to use of the native-length membrane protein.

Extracts derived from E. coli Tuner cells containing the pPmCS1–704 plasmid, but not samples from cells with the vector alone, synthesized polymer in vitro when supplied with both UDP-GlcUA and UDP-GalNAc simultaneously (Table II). No incorporation of radiolabeled [14C]GlcUA into polymer was observed if UDP-GalNAc was omitted or if UDP-GlcNAc was substituted for UDP-GalNAc. UDP-GalUA or UDP-Glc did not substitute for UDP-GlcUA. No polymerization or transferase activity was detected if the divalent metal ions were chelated with EDTA. The addition of the chondroitin oligosaccharide acceptor increased sugar incorporation catalyzed by pmCS1–704 at least 50–100-fold in comparison to parallel reactions without acceptor (data not shown) in analogy to observations of pmHAS (19).

Analysis by gel filtration chromatography indicated that recombinant pmCS1–704 incorporated only the authentic chondroitin precursors into polysaccharide, ND, not determined.

### Table II

| Second sugar nucleotide present | Incorporation of first sugar | dpm (%) |
|---------------------------------|-----------------------------|---------|
|                                  | [14C]GlcUA | [3H]GalNAc |
| None                            | 60 (0.9)    | 250 (7.5) |
| UDP-GlcUA                       | ND         | 3,310 (100) |
| UDP-GalUA                       | ND         | 315 (9.5) |
| UDP-GalNAc                      | 6,590 (100) | ND      |
| UDP-GlcNAc                      | 85 (1.2)   | ND      |
| UDP-Glc                         | 60 (0.9)   | 370 (11) |

DISCUSSION

We verify that P. multocida Type F produces a chondroitin or chondroitin-like capsule. We have also molecularly cloned the glycosyltransferase responsible for polymerizing the chondroitin backbone component of the capsular polysaccharide. This enzyme seems to be a close homolog of the pmHAS enzyme. Recently, we determined that the pmHAS enzyme contains two active sites in a single polypeptide by generating UDP-[3H]GalNAc, substantial incorporation of radiolabel into polymer was only noted when UDP-GlcUA was also present. UDP-GalUA or UDP-Glc did not substitute for UDP-GlcUA. No polymerization or transferase activity was detected if the divalent metal ions were chelated with EDTA. The addition of the chondroitin oligosaccharide acceptor increased sugar incorporation catalyzed by pmCS1–704 at least 50–100-fold in comparison to parallel reactions without acceptor (data not shown) in analogy to observations of pmHAS (19).
multocida strains has not yet been delineated. Both organisms in vitro analyzed on the PolySep column (calibration elution times in minutes: “Experimental Procedures.” Portions (60%) of the samples were then aliquots and treated with various GAG glycosidases as described under GlcUA, and UDP-[3H]GalNAc (580 μCi each) in a reaction volume of 200 μl for 30 min. The reaction product was split into five aliquots and treated with various GAG glycosidases as described under “Experimental Procedures.” Portions (80%) of the samples were then analyzed on the PolySep column (calibration elution times in minutes: void volume, 12.7; 580-kDa dextran, 15.4; 145-kDa dextran, 16.0; totally included volume, 19.3 min). Radioactivity (solid line, 14C; dotted line, 3H) measured by the in-line detector is presented as disintegrations/s (dps). The double-headed arrow corresponds to a response of 20 dps. A, untreated polymer, peak 15.9 min; B, Flavobacterium chondroitinase AC lyase-treated polymer, peak 19.2 min; C, HA lyase-treated polymer, peak 15.9 min. The polymer peak with a size of ~100 to 400 kDa contained both radiolabeled sugars at a 1:1 ratio and was degraded only by the appropriate enzyme, chondroitin AC lyase.

mutations that transfer only GlcUA or only GlcNAC (33). Interestingly, mixing the two different mutant proteins reconstituted the HA synthase activity. We hypothesized that one domain, called A1, is responsible for GlcNAC transfer, and the other domain, called A2, is responsible for GlcUA transfer (33). Comparison of the pmHAS and the pmCS sequences reveals that the majority of the sequence differences exist in the vicinity of the Al domain. The pmCS enzyme transfers a different hexosamine, GalNAc; thus, this observation is consistent with our proposed two-domain structure for pmHAS. Future experiments will be directed at identifying which residues of the Al domain are responsible for recognition and/or transfer of GlcNAC or GalNAc sugars.

The pmHAS protein was also hypothesized to interact with a putative polysaccharide transport system or a membrane-bound partner via its carboxyl terminus because deletion of residues 704 to 972 from the native-length enzyme resulted in the formation of a soluble enzyme (33). However, no substantial membrane-associated or hydrophobic regions are predicted to reside in this sequence. Because pmHAS and pmCS are highly homologous in this region, which is not essential for their glycosyltransferase activities, it is quite likely that the carboxyl terminus contains domains or motifs required for interacting with the polysaccharide transport machinery or a membrane-bound partner in vitro.

The evolutionary relationship between Type A and Type F. P. multocida strains has not yet been delineated. Both organisms are widespread causative agents of fowl cholera, but many more isolates from diseased birds in North America are Type A microbes with HA capsules (30). It is likely that the progenitor of the two distinct capsular types had either a chondroitin synthase-like or a HAS-like gene. The specificity of this ancestral enzyme may have changed after a few mutations, resulting in the appearance of another capsular type. Apparently, the sugar transfer specificity is rather selective, since neither recombinant pmCS nor pmHAS misincorporate the inappropriate hexosamine into polymer in vitro. Some Gram-negative bacteria (e.g. E. coli) possess an UDP-GlcNAc/UDP-GalNAc epimerase; therefore, the hexosamine precursor either for HA or for chondroitin could have been available for polysaccharide biosynthesis without the need to gain an auxiliary metabolic enzyme simultaneously. Typically the UDP-glucose dehydrogenase, the enzyme that forms the UDP-GlcUA precursor, is found in Gram-negative bacteria only if the microbe possesses a GlcUA-containing polymer or glycoconjugate. In both Type A and Type F. P. multocida, the UDP-glucose dehydrogenase gene is directly downstream of the GAG synthase (17).

The relationship between the bacterial chondroitin synthase and the putative mammalian counterpart is unclear. No similar vertebrate proteins are deposited in the database as yet. Both bacterial pmCS and the vertebrate chondroitin synthase utilize UDP-sugars to extend acceptor carbohydrates in vitro. In most cases, the mammalian enzyme in cell-free extracts, however, does not produce long chondroitin chains, and only the half-reaction (e.g. adding a single GlcUA to a GalNAc-terminated oligosaccharide or vice versa) is readily observed in vitro (23, 24). In vertebrate tissues, other enzymes modify chondroitin extensively by sulfation and/or epimerization (1). Hopefully, the discovery and the characterization of pmCS will assist the further study of the rather recalcitrant mammalian chondroitin synthase enzymes.

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Note Added in Proof—Recently it was reported (Nadanka, S., Kitagawa, H., Goto, F., Tamura, J., Neumann, K. W., Ogawa, T., and Sugumaran, M. A. (1999) Biochem. J. 340, 353–357) that a partially purified chondroitin synthase preparation from a melanoma cell line catalyzed the repetitive addition of chondroitin disaccharide repeats in vitro to the linkage tetrasaccharide of an α-thrombomodulin acceptor but not to the free tetrasaccharide, suggesting a role for the core protein in mammalian GAG biosynthesis.

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