Heparosan synthase 1 (PmHS1) from Pasteurella multocida
Type D is a dual action glycosyltransferase enzyme that transfers monosaccharide units from uridine diphospho (UDP) sugar precursors to form the polysaccharide heparosan (N-acetylheparosan), which is composed of alternating (\(-\alpha4\text{-GlcNAc} \beta1,4\text{-GlcUA}1-) repeats. We have used molecular genetic means to remove regions nonessential for catalytic activity from the amino- and the carboxy-terminal regions as well as characterized the functional regions involved in GlcUA-transferase activity and in GlcNAc-transferase activity. Mutation of either one of the two regions containing aspartate-X-aspartate (DXD) residue-containing motifs resulted in complete or substantial loss of heparosan polymerizing activity. However, certain mutant proteins retained only GlcUA-transferase activity while some constructs possessed only GlcNAc-transferase activity. Therefore, it appears that the PmHS1 polypeptide is composed of two types of glycosyltransferases in a single polypeptide as was found for the Pasteurella multocida Type A PmHAS, the hyaluronan synthase that makes the alternating (\(-\beta3\text{-GlcNAc} \beta1,4\text{-GlcUA}1-) polymer. However, there is low amino acid similarity between the PmHAS and PmHS1 enzymes, and the relative placement of the GlcUA-transferase and GlcNAc-transferase domains within the two polypeptides is reversed. Even though the monosaccharide compositions of hyaluronan and heparosan are identical, such differences in the sequences of the catalysts are expected because the PmHAS enzyme requires only inverting sugar transfer mechanisms whereas PmHS1 requires both retaining and inverting mechanisms.

The Gram-negative bacterial pathogen Pasteurella multocida Type D is known to cause atrophic rhinitis in swine and pasteurellosis in other domestic animals (1). This microbe produces an extracellular coating of polysaccharide, called a capsule, composed of heparosan (N-acetylheparosan or unsulfated, unepimerized heparin) (2) to enhance infection. It is thought that the capsule confers resistance to nonspecific host immunity and perhaps mediates adhesion to certain host cells. In general, most capsules are antigenic, but glycosaminoglycan polysaccharide capsules are a special case because they closely resemble the host molecules and thus may serve as molecular camouflage (3).

Two heparosan synthase (PmHS) enzymes have been identified from P. multocida that catalyze the formation of the repeating disaccharide (\(-\text{GlcNAc} \alpha1,4\text{-GlcUA}\beta1-) units of the heparosan chain. P. multocida Type D strains encode the 617-residue PmHS1 enzyme in a typical Gram-negative bacterial Type 2 capsule biosynthesis locus (4). Another isoform, the 651-residue PmHS2, is found in many Type A, D, and F strains (5); the genes are \(-73\%\) identical. The gene encoding PmHS2 is found in a different region of the chromosome not associated with typical carbohydrate biosynthesis genes and may be expressed during some stage of infection, but its role is not yet known.

The dual action PmHS1 and PmHS2 are complex enzymes because the UDP sugar precursors are \(\alpha\)-linked and therefore these single polypeptides must employ a retaining mechanism to form the \(\alpha\)-linkages as well as an inverting mechanism to form the \(\beta\)-linkages. For inverting glycosyltransferases, \(S_{N2}\) nucleophilic attack results in \(\beta\)-linkages. For retaining glycosyltransferases, theoretically either a sterically directed \(S_{N2}\) double displacement reaction (with a potential covalent enzyme-substrate intermediate) occurs to form \(\alpha\)-linkages (6).

Escherichia coli K5 is another bacterium known to produce heparosan (7), but this microbe employs multiple proteins to produce the chemically identical polymer. The K5 antigen region 2 encodes four proteins designated KfA to KfD involved in synthesizing the sugar chain (8). KfA was discovered to act as an \(\alpha4\text{-N-acetylglucosaminyltransferase}\) (9) whereas KfC is the \(\beta4\text{-glucuronyltransferase}\) (although initially the latter was erroneously reported to be a dual action enzyme responsible for heparosan polymerization (10)). Therefore, in E. coli the formation of the heparosan polymer occurs by the concerted action of KfA and KfC proteins. In addition, KfB, a protein with a potential scaffolding role, also appears to be required (9). KfD is a UDP glucose dehydrogenase that catalyzes the formation of UDP-GlcUA, a necessary precursor for the K5 polysaccharide.

The PmHS1 enzyme contains sequences that align with both the KfA and the KfC proteins. Site-directed mutagenesis stud-
ies suggest that the enzymatic activity of KfiA is dependent upon the acidic amino acid motif "DDD" that is conserved in certain glycosyltransferases (9). Conservative mutations were made changing certain aspartic acid residues (D) to glutamic acid (E) along with non-conservative mutations substituting aspartic acid with alanine (A). Both conservative and non-conservative mutations abolished N-acetylgalcosaminyltransferase activity. Similarly, mutation of any one of several aspartic acid residues in KfiC resulted in total loss of the relevant GlcUA-transferase activity (10). These data, however, are not definitive because the mutagenized enzymes did not retain any activity, and it is difficult to draw conclusions from any inactive enzyme because of the potential for global misfolding.

The P. multocida Type A hyaluronan synthase (PmHAS) is a dual action glycosyltransferase (11) that forms the hyaluronan polymer composed of (-3GlcNAcβ1→4GlcUAβ1-) repeats. Even though the PmHAS is not very similar at the amino acid sequence level to the PmHS1, both enzymes have the ability to use the same UDP sugar precursors. In this report, we characterize PmHS1 and give evidence for two glycosyltransferase sites in one polypeptide chain.

**EXPERIMENTAL PROCEDURES**

**Materials and PmHS1 Constructs**—Reagents were purchased either from Sigma or Fisher (Pittsburgh, PA) unless otherwise noted. Custom oligosaccharides were from Integrated DNA Technologies (Coralville, IA).

Various PmHS1 truncations (missing 45, 77, 118, 141, or 191 residues from the amino terminus or 50 residues from the carboxyl terminus) were made by PCR using the template (4) and suitable primers with new start or stop codons using the pET Blue-1 AccepTor vector system (Novagen, Madison, WI). The PCR parameters using Taq polymerase were: 1 cycle of 95 °C/2 min; 10 cycles of 94 °C/30 s, 52 °C/30 s, 72 °C/2.5 min; 1 cycle of 72 °C/7 min. The amplicon with A base overhangs was then ligated into the AccepTor vector and transformed into E. coli Top 10 cells (Invitrogen). Transformant colonies selected on ampicillin were screened for directionality by PCR and by restriction digest. The plasmid for producing truncated PmHS1 protein was transformed into E. coli Tuner (Novagen), a host with T7 RNA polymerase, for protein production.

The pBAD/TOPO ThioFusion vector (Invitrogen) was employed to make various mutant forms of PmHS1. The DNA encoding both the full-length (617-amino acid) PmHS1 and the amino-terminal truncation derivatives missing 45 or 77 amino acids were PCR amplified as above and then fused to the thioredoxin gene for increased protein expression levels and higher stability. Site-directed mutagenesis was performed with the QuikChange method (Stratagene, La Jolla, CA) and pairs of custom primers with parameters of 1 cycle 95 °C/30 s, 16 cycles 95 °C/30 s, 55 °C/1 min, 68 °C/13 min. A panel of mutants with a variety of substituting residues was created simultaneously by utilizing primers with mixed bases at desired positions. All constructs were verified by automated sequencing of both DNA strands (Oklahoma Medical Research Foundation DNA sequencing facility).

**Enzyme Preparation**—Protein preparation was performed by a cold lysozyme/sonication method using 0.1 mM mercaptoethanol in the sonication steps (12). Briefly, the frozen E. coli expression host cell pellets were resuspended in a sucrase buffer, treated with ethylenediamine(tetraacetic acid)/lysozyme to weaken the cell walls, and sonicated. MgCl₂ (6 mM) was added, and lysate was DNase/RNase treated. The soluble protein was separated from the membrane portion by ultracentrifugation (200,000 × g, 1 h). The fraction containing soluble protein was collected, and membrane pellets were resuspended. Both fractions were analyzed for the ability to transfer UDP sugar precursors into a heparosan chain.

The concentration of total protein was measured by the Bradford assay (Pierce) using a bovine serum albumin standard. The level of PmHS1 polypeptide in any given preparation was assayed by Western blot (rabbit anti-peptide polyclonal antibody against KDGIFFQDSDVCHHERIER, residues 173–193 of PmHS1 followed by Protein A–alkaline phosphatase conjugate) (5). Basically, a titration of wild-type sequence PmHS1 in a given vector system was employed to normalize the expression levels of the corresponding mutant proteins; the immunoreactivity of the wild-type PmHS1 sequence version was set to 100%.

**Enzyme Assays**—Three types of radiolabeled sugar incorporation assays were employed: (a) dual sugar incorporation for measuring the polymerization of long heparosan chains (Reaction 1), and single sugar addition for measuring either (b) the GlcUA-transferase activity (Reaction 2) or (c) the GlcNACTransferase activity (Reaction 3). The heparosan polysaccharide acceptor (noted as X in Reactions 1–3), which greatly stimulates polymerization activity and is essential for single sugar transferase assays (only polymers greater than ~14 saccharides long remain at the origin of the paper chromatograms), was used in most radiolabeled sugar incorporation activity assays as noted.

\[
\begin{align*}
\text{UDP}-[3\text{H}]\text{GlcNAc} + n \text{ UDP}-[14\text{C}]\text{GlcUA} + X & \rightarrow \\
(\text{[3H]GlcNAc}-[14\text{C}]\text{GlcUA})_nX + 2n \text{ UDP} \\
\text{REACTION 1} \\
\text{UDP}-[14\text{C}]\text{GlcUA} + n X & \rightarrow n ([14\text{C}]\text{GlcA})-X + n \text{ UDP} \\
\text{REACTION 2} \\
\text{UDP}-[3\text{H}]\text{GlcNAc} + n X & \rightarrow n ([3\text{H}]\text{GlcNAc})-X + n \text{ UDP} \\
\text{REACTION 3}
\end{align*}
\]

The 55-kDa heparosan acceptor was obtained by extensive ultrasonication of the Pasteurella Type D polysaccharide (4).

Polymerization reactions (50 μl) typically contained 1.2 μg of heparosan polysaccharide acceptor, 10 mM MgCl₂, 10 mM MnCl₂, 50 mM Tris, pH 7.2, 0.2 mM UDP-(3H)GlcNAc (0.08–0.1 μCi; PerkinElmer Life Sciences), and 0.2 mM UDP-(14C)-GlcUA (0.08–0.1 μCi; PerkinElmer Life Sciences). Reactions were incubated at 30 °C for times ranging from 30 min to 4 h and then stopped by adding SDS (final concentration 2%). The radioactive polymer was separated from the other reaction precursors by descending paper chromatography (Whatman 3 M developed with 65:35 ethanol/1 M ammonium acetate, pH 5.5). After overnight development, the origin of the paper strip (sugars >~14 monosaccharides in length) was cut and eluted with...
Pasteurella Heparosan Synthase

water. The incorporation of radiolabeled sugars into the heparosan chain as measured by liquid scintillation counting using BioSafe II scintillation mixture (Research Products International, Chicago, IL). All assays were performed in duplicate, and the averaged data values are presented. All parameters for the single radiolabeled sugar incorporation activity assay are the same as for the polymerization assay except that only one UDP sugar substrate (i.e. either UDP-GlcNAc or UDP-GlcUA) is added to a particular reaction.

The $K_m$ values of the dual action enzyme thioredoxin-PmHS1 for both of its substrates, UDP-GlcUA and UDP-GlcNAc, were analyzed by titrating one UDP sugar donor and maintaining the other radiolabeled UDP sugar at a saturating, constant value. Reactions were similar to the typical polymerization assay, except the mixtures contained 2.2 μg of heparosan polysaccharide acceptor, 1 mx of the constant UDP sugar with 0.08 – 0.1 μCi of radiolabel, a titration (0–1.5 mx) of the other UDP sugar, and thioredoxin-PmHS1 (120 μg of total protein). The reactions were incubated for 8 min. Less than 5% of the substrates were consumed, and the enzyme concentration was in the linear range. Data were fit to a rectangular hyperbolic curve with SigmaPlot software (Rockware, Golden, CO) as well as analyzed on Hanes-Woolf plots; both methods gave very similar $K_m$ values. The $K_m$ value was the average of three independent determinations.

RESULTS AND DISCUSSION

Characterization Of Native Sequence and Truncated PmHS1— Both the soluble and the membrane-bound portions of the protein preparations from recombinant E. coli containing the overexpressed wild-type sequence PmHS1 were analyzed for polymerizing activity. The highest amount of activity for the full-length recombinant heparosan synthase appeared to be present in the soluble fraction (~80% soluble activity versus ~20% membrane-bound activity). This finding suggests that without the putative polysaccharide transporter machinery (13) most E. coli K12-derived laboratory strains are thought to be missing the original capsular biosynthesis locus (13) the majority of the PmHS1 polypeptide floats free in the cytoplasm or its interaction with the membrane is weak. Therefore, the soluble portion of protein preparations derived from subsequent constructs was tested for the sugar transferase activity.

Recombinant versions of PmHS1 truncated to delete amino acid residues from either the amino or the carboxyl terminus of the protein were constructed. The 617-amino acid protein sequence (4) was first analyzed to avoid the potential disruption of predicted stretches of α helical secondary structures that might globally disrupt the structure and function of the protein (us.expasy.org/, Network Protein Sequence Analysis). Truncations of the amino terminus were designed in which 45, 77, 118, 141, and 191 amino acids were deleted. A truncation was also designed to delete 50 residues of the carboxyl terminus (residues 567 through 617).

The truncated mutants missing 45 or 77 residues of the amino terminus were visualized by Western blot to have protein expression levels of ~20% of the expression levels of the wild-type PmHS1 protein. The PmHS1 truncation mutants missing 118, 141, or 191 amino acids of the amino terminus were analyzed by Western blot and found to have very poor protein expression levels and formed smaller degradation products in addition to the predicted target protein. The carboxyl-terminal-truncated PmHS1 protein was seen to have many protein degradation products and ~10% expression of the intact target protein. Further delineation of the required residues at the carboxyl terminus was hindered by the mutant protein instability.

The catalytic ability of all mutants was normalized for relative specific activity (moles radiolabeled sugar monosaccharide incorporated into the heparosan chain divided by the amount of specific immunoreactivity relative to the wild-type sequence PmHS1 as assayed by Western blot). Many truncated PmHS1 enzymes had reduced levels of polymerization activity as seen by dual radiolabeled sugar incorporation activity assay (Table 1), but nonetheless authentic heparosan chains were formed in these cases. In summary, at least residues 78–567 appear to be absolutely essential for catalytic activity.

Characterization Of Thioredoxin-PmHS1—The fusion of the thioredoxin protein to the amino terminus of PmHS1 resulted in an enzyme with higher protein expression, stability, and sugar transferase activity than the native sequence protein. The PmHS1 enzyme alone loses enzymatic activity after three to four freeze-thaw cycles and over time in cold storage. In contrast, the thioredoxin-PmHS1 protein retains enzymatic activity when stored at ~80 °C for more than 1 year and can withstand multiple (~20) freeze-thaw cycles.

PmHS1 utilizes two UDP sugar substrates for heparosan polymerization. The apparent $K_m$ of thioredoxin-PmHS1 for UDP-GlcUA is 90 ± 20 μM whereas the $K_m$ for UDP-GlcNAc is 270 ± 30 μM (Fig. 1). The same qualitative difference in $K_m$ for the two different UDP sugar substrates is also observed for PmHAS (14, 15). The observed maximal velocity ($V_{max}$) was 13 and 6.6 nmol of monosaccharide transfer/min for the GlcUA-transferase and the GlcNAc-transferase, respectively. The molecular mass of the native capsular polysaccharide of P. multocida Type D is ~250 kDa (2); thus, the enzyme is expected to rapidly produce the heparosan chain if sufficient UDP sugars are present.

### TABLE 1

| PmHS1 construct | GlcNAc-transferase relative specific activity | GlcUA-transferase relative specific activity |
|-----------------|---------------------------------------------|---------------------------------------------|
| Wild-type       | 100                                         | 100                                         |
| Vector          | <1                                          | <1                                          |
| Δ45 AA N-terminus | 35                                         | 50                                          |
| Δ77 AA N-terminus | 10                                         | 20                                          |
| Δ118 AA N-terminus | <1                                         | <1                                          |
| Δ141 AA N-terminus | <1                                         | <1                                          |
| Δ191 AA N-terminus | <1                                         | <1                                          |
| Δ50 AA C-terminus | 4                                          | 20                                          |
Identification Of Specific Amino Acid Residues Of PmHS1 Involved in Transferase Activity—Site-directed mutations of the heparosan synthase were designed to assess the role of residues potentially involved in transferase activity including (i) the putative DXD motifs (16, 17) and (ii) a conserved QS pair with similarity to other glycosyltransferases. X-ray crystallography of the Bacillus SpSA protein-UDP complex suggests that the DXD motif is involved in binding the metal ion coordinated with the β-phosphate and ribose moiety of the UDP sugar (18), but definitive roles for the other residues are not yet known.

The PmHS1 enzyme contains a DXD motif in each of the two distinct regions of the protein corresponding to KfiA and KfiC sequence alignments. As noted previously (4), an amino-terminal region of PmHS1 is ~29% identical to a portion of KfiC whereas a carboxyl-terminal region of PmHS1 is ~27% identical to a portion of KfiA. Therefore, we postulated that mutating only one of the two DXD motifs of the PmHS1 polypeptide would convert a polymerizing dual action enzyme into a single action sugar transferase if the two active sites are relatively independent as in the case of PmHAS, the HA synthase (14, 15).

The mutations for thioredoxin-PmHS1 were designed to introduce aspartic acid at position 118, 119, 445, and 447 of PmHS1, which are conserved amino acids in both KfiA and KfiC proteins. The D residues similarly to KfiA have a general structural role or be involved in both sugar transfer activities. The approximate extent of the putative GlcUA-transferase and GlcNAc-transferase sites based on sequence alignments with E. coli KfiC and KfiA, respectively, and on our mutation evidence is indicated with arrows. B, the relative order of putative domains within the PmHS1 polypeptide chain is reversed compared with the hyaluronan synthase, PmHAS, and the chondroitin synthase, PmCS (14). PmHS2 is predicted to be organized in a similar fashion as PmHS1.

### TABLE 2

| Thio-PmHS1 construct | Single sugar addition assay | GlcNAc-transferase relative specific activity | GlcUA-transferase relative specific activity |
|----------------------|----------------------------|---------------------------------------------|---------------------------------------------|
| Wild-type            | 100                        | 100                                         |                                             |
| Vector               | <1                         | <1                                          |                                             |
| D181N                | 30                         | <1                                          |                                             |
| D181N,D183N          | 60                         | 2                                           |                                             |
| D445N                | 25                         | 40                                          |                                             |
| D445N,D447N          | 1                          | 90                                          |                                             |

### FIGURE 1

**Kinetic analysis of thioredoxin-PmHS1 for UDP sugar substrates.** These representative analyses of heparosan synthase depict the averages of duplicate assays graphed on Hanes-Woolf plots, [S]/v versus [S]. (UDP-GlcUA titration, solid circles; UDP-GlcNAc titration, open circles). The apparent Vₜ₉₀ value (x-axis intercept) is lower for UDP-GlcUA than UDP-GlcNAc. The PmHS1 UDP-GlcUA-transferase activity is also ~2-fold faster than the UDP-GlcNAc-transferase activity based on the observed Vₜ₉₀ (slope = 1/Vₜ₉₀). Qualitatively similar kinetic findings were previously seen for PmHAS, the hyaluronan synthase (15, 19).

### FIGURE 2

**Schematic model of the dual action Pasteurella PmHS1 proteins.** A, the regions of synthases that are not essential for catalytic activity are indicated with cross-hatching. Two DXD motifs involved in the two-component transferase (Tase) activities are found in two separate regions of the PmHS1 protein. The region with the QT (residues 118–119) sequence may have a general structural role or be involved in both sugar transfer activities. The approximate extent of the putative GlcUA-transferase and GlcNAc-transferase sites based on sequence alignments with *E. coli* KfiC and KfiA, respectively, and on our mutation evidence is indicated with arrows. B, the relative order of putative domains within the PmHS1 polypeptide chain is reversed compared with the hyaluronan synthase, PmHAS, and the chondroitin synthase, PmCS (14). PmHS2 is predicted to be organized in a similar fashion as PmHS1.
Pasteurella Heparosan Synthase

The various enzymes were assayed for activity as in Table II. Thr-119 may be altered, but Gln-118 is more sensitive. Overall, lesions in this site of PmHS1 appear to disable both component sugar transferase activities.

| Thio-PmHS1 construct | Single sugar addition assay | GlcNAc-transferase relative specific activity | GlcUA-transferase relative specific activity |
|----------------------|-----------------------------|---------------------------------------------|---------------------------------------------|
| Wild-type             | 100%                        | 100%                                        |                                             |
| (Gln-118 Thr-119)     | 98%                         | 100%                                        |                                             |
| Vector                | 90%                         | 100%                                        |                                             |
| Q118H                 | 80%                         | 100%                                        |                                             |
| T119S                 | 70%                         | 100%                                        |                                             |
| T119A                 | 60%                         | 100%                                        |                                             |
| Q118K,T119S           | 50%                         | 100%                                        |                                             |
| Q118K,T119A           | 40%                         | 100%                                        |                                             |
| Q118H,T119S           | 30%                         | 100%                                        |                                             |
| Q118N,T119S           | 20%                         | 100%                                        |                                             |

**Table 3** Single sugar activity of Thio-PmHS1 Gln-18 Thr-119 mutants

The data for the thioredoxin-PmHS1 Gln-118 and/or Thr-119 mutants suggest the importance of these residues for the catalytic activity of the heparosan synthase, but in these cases, the mutations did not result in a functional single action thioredoxin-PmHS1 mutant. Potentially these residues, especially the Gln-118, are involved in maintaining structure or are groups important for both sugar transfer reactions. Apparently, the two component activities of PmHS1 are more interdependent than in the case of PmHAS. Preliminary attempts to cleave the PmHS1 enzyme into the two component glycosyltransferase activities were not successful.

**PmHS1 Structural Possibilities**—The GAG synthases add the monosaccharides from UDP sugar precursors onto the nascent polymer chain. Four simple possibilities exist for the arrangement of the UDP sugar donor and the acceptor sites (i.e. corresponding to the nascent polysaccharide chain binding site) within any dual action heparosan synthase enzyme (Fig. 3). These models include: (I) two different acceptor sites (i.e. one each for either a GlcNAC- or a GlcUA-terminated chain) and two different donor sites; (II) one acceptor site, two different donor transfer sites; (III) two different acceptor sites and one donor site (i.e. accommodates both UDP-GlcNAC and UDP-GlcUA); or (IV) one acceptor site for both types of acceptor and one donor site for both UDP-GlcNAC and UDP-GlcUA.

Of the four simple models for the arrangement of donor and acceptor sites within PmHS1, two of the four possibilities are improbable (models III and IV) based on our experimental evidence. It is unlikely that the PmHS1 enzyme would contain only one site for both UDP sugar donor molecules. The results of studies of mutation of the two DXD motifs at positions 181–183 and positions 445–447 suggest that two distinct UDP sugar binding sites do exist within PmHS1, as based on the ability to separate functionally the two active sites by molecular genetic means (Fig. 2). Future experiments focusing on the acceptor site will be required to identify whether model I or II is correct for PmHS1. PmHS2, the isozyme with a highly similar sequence, is expected to possess the same overall architecture as PmHS1. A recent study on PmHAS, the HA synthase, suggests that model I operates; two separate acceptor sites appear to exist based on competition studies (19). However, due to the lack of strong protein sequence similarity between the two putative PmHS1 domains and the relevant regions of the HA synthase (GlcUA-transferase region and GlcUA-transferase region of PmHS1 are ~12% or ~21% identical to PmHAS, respectively), direct extrapolation is not possible. The vertebrate enzymes that catalyze production of the repeating backbone of heparin chains, EXT1 and 2 (20, 21), also do not have similar primary structures as PmHS1.

The P. multocida synthases that form heparan (PmHS1 and 2), hyaluronan (PmHAS), and chondroitin (PmCS) are rather unique in glycobiology because a single polypeptide chain acts as a dual action enzyme that transfers two distinct monosaccharides. This could be a result of evolutionary fusion of two distinct separate enzymes (e.g. combining a KfiA-like and a KfiC-like protein). Alternatively, perhaps the pair of Kfi co-polymerase enzymes resulted from a split of one ancestral dual action synthase.

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