A novel bacterial enzyme with D-glucuronyl C5-epimerase activity

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Keywords
D-glucuronyl C5-epimerase, glycosaminoglycans, L-iduronic acid, capsular polysaccharide, Bermanella marisrubri

Background: D-glucuronyl C5-epimerase activity is essential in glycosaminoglycan biosynthesis.

Results: The first demonstration of a bacterial enzyme having D-glucuronyl C5-epimerase activity.

Conclusion: This novel enzyme, probably involved in bacterial capsular polysaccharide biosynthesis, shows catalytic properties similar to murine heparan sulfate D-glucuronyl C5-epimerase.

Significance: Increased insight in bacterial cell wall biosynthesis and a potential biotechnological tool applicable in chemo-enzymatic synthesis of industrially relevant heparin-like glycosaminoglycans.

SUMMARY
Glycosaminoglycans (GAGs) are biologically active polysaccharides that are ubiquitously found in the animal kingdom. The biosynthesis of these complex polysaccharides involves complicated reactions that turn the simple GAG backbone into highly heterogeneous structures. One of the modification reactions is the epimerization of D-glucuronic acid (GlcA) to its C5-epimer L-iduronic acid (IdoA), which is essential for the function of heparan sulfate (HS). Although IdoA residues have been shown to exist in polysaccharides of some prokaryotes, there has been no experimental evidence for the existence of a prokaryotic D-glucuronyl C5-epimerase. This work for the first time reports on the identification of a bacterial enzyme with D-glucuronyl C5-epimerase activity. A gene of the marine bacterium Bermanella marisrubri sp. RED65 encodes a protein (RED65_08024) of 448 amino acids that has an overall 37% homology to the human GlcA C5-epimerase. Alignment of this peptide with the human and mouse sequences revealed a 60% similarity at the carboxyl terminus. The recombinant protein expressed in Escherichia coli showed epimerization activity towards substrates generated from heparin and the E. coli K5 capsular polysaccharide, thereby providing first evidence for bacterial D-glucuronyl C5-epimerase activity. These findings may eventually be used for modification of mammalian GAGs.

INTRODUCTION
Glycosaminoglycans (GAGs) are a group of carbohydrate polymers that are typically composed of disaccharides of an amino sugar and hexuronic acids. GAGs are widely spread in the animal kingdom, having important biological functions. For some time GAGs were believed to exist exclusively in animals; however, today there is an increasing number of examples of GAGs of prokaryotic origin. These prokaryotic GAGs are less complex and lack some modifications as typically seen in animal GAGs. For example,
heparan sulfate (HS) is a ubiquitously expressed GAG in animals, having essential biological functions (1). Similar to the capsular polysaccharide of the bacterium Escherichia coli K5, this complicated animal GAG is initially synthesized as a simple polymer composed of repeating disaccharides of D-glucuronic acid (GlcA) and N-acetyl-D-glucosamine (GlcNAc). This relatively simple polymer is subsequently adjusted by a series of modification reactions, producing distinct structures of the polysaccharide in different tissues (2). One critical modification is the conversion of GlcA to its C5-epimer, L-iduronic acid (IdoA), catalyzed by D-glucuronyl C5-epimerase. It is demonstrated that this modification is crucial for animal development (3). The IdoA residues in the polymers lead to increased flexibility of the GAG chains (4), facilitating binding properties for specific polysaccharide-protein interactions (5). Although IdoA occasionally is found in prokaryotic GAGs (6-9), the enzyme responsible for this C5-epimerization reaction has not been identified in a prokaryote.

In a previous study, we performed an in silico screen on available prokaryotic genomes for putative D-glucuronyl C5-epimerases which revealed multiple candidate genes in both archaea and bacteria (10). In this work we report on the identification, cloning, expression and characterization of a C5-epimerase from the marine bacterium Bermanella marisrubri sp. RED65.

EXPERIMENTAL PROCEDURES
L-iduronic acid standard was purchased from Toronto Research Chemicals Inc. (North York, Canada). Capsular polysaccharide of E. coli K5 (‘K5 polysaccharide’) was ordered from Iduron (Manchester, United Kingdom). The 3H-labeled substrates derived from K5 and heparin are prepared as described before (11). All other chemicals were bought from Sigma-Aldrich. Restriction enzymes, Pfu DNA polymerase and T4 DNA ligase were from Invitrogen and New England Biolabs.

Gene cloning, expression and purification
A synthetic gene was designed based on hypothetical protein RED65_08024, codon optimized for the codon usage bias of Escherichia coli. The gene was cloned in vector pRSF-1b (Novagen) using restriction sites KpnI and BamHI, fusing the gene in frame with a polyhistidine tag at the non-conserved amino-terminus to prevent interference on the activity (12). The resulting vector, named pWUR537, was used to transform E. coli strains DH5α and Rosetta (DE3) containing pRARE (helper plasmid coding for rare tRNAs).

An overnight culture of E. coli Rosetta (DE3) containing pWUR537 was used to inoculate (1% v/v) a shake flask with 1 liter Luria Bertani medium supplemented with 50 μg/ml kanamycin, 50 μg chloramphenicol, and 1 mM MgSO₄. At an optical density A₆₀₀ = 0.5, gene expression was induced by adding 0.05 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). The culture was incubated overnight at 30°C. Cells were harvested by centrifugation (5,000 x g for 15 min), and stored at -20°C until further use. Pelleted E. coli cells were resuspended in 20 mM Tris-HCl buffer (pH 7.5) containing 200 mM NaCl (buffer A) followed by sonication (intermittent cooling on ice). The cell debris was removed by a centrifugation step (16,000 x g for 10 min). The resulting cell lysate was filtered (0.45 μm) and was directly used for activity measurements.

Protein purification was achieved by applying the cell lysate on a Talon Cobalt affinity column (Clontech). After washing with two column volumes of buffer A and one column volume of buffer A containing 10 mM imidazole, RED-C5-epimerase was eluted with one column volume of buffer A containing 10 - 250 mM imidazole in a linear gradient. RED-C5-epimerase containing fractions were pooled and applied onto a HiPrep desalting column to remove imidazole. Enzyme presence and purity was checked by SDS-PAGE analysis. A broad range protein marker (Biorad) was used to estimate the molecular mass. The presence of RED-C5-epimerase was confirmed by trypsin digestion LC-MS analysis as described previously (13).

The oligomeric state of RED-C5-epimerase was determined by size exclusion chromatography on a Superdex 200 HR10/10 column (24 ml) (Amersham Biosciences) equilibrated in 20 mM Tris-HCl (pH 7.5) containing 200 mM NaCl. Approximately 0.1 mg of purified RED-C5-epimerase was loaded onto the column, using a flow rate of 0.5 ml / min. Markers used for calibration were blue dextran...
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2000 (>2000 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa).

Detection of C5-epimerase activity
Cell lysate (10 ml of culture lysed in 200 µl of lysis buffer) of the bacterial clone expressing RED-C5-epimerase was mixed with tritium labeled substrates derived from O-desulfated heparin (5,000 cpm; specific radioactivity 10x10^6 cpm/mg polysaccharide) or N-deacetylated/sulfated K5 capsular polysaccharide (3,000 cpm; specific radioactivity 1x10^6 cpm/mg polysaccharide). The enzyme/substrate mixture was incubated at 30°C overnight. Subsequently, the reaction mixtures were analyzed using a biphasic liquid scintillation procedure to measure tritium release as described previously (14,15).

Murine D-glucuronyl C5-epimerase constructs (full-length and a truncated form) fused to Maltose Binding Protein were used as positive and negative control, respectively.

HPLC-PAD analysis
For detection of the formation of IdoA, an HPLC-PAD (pulse amperometric detection) analysis using a Dionex system was used. Cell lysate or purified RED-C5-epimerase was mixed with N-acetyl-de-O-sulfated heparin (sodium salt; Sigma-Aldrich). The mixture was incubated at 30°C overnight. Subsequently, protein was inactivated by heating at 100°C and removed from the reaction mixture by centrifugation. The reaction mixture was hydrolyzed (90°C for 4 hours) by addition of one volume of hydrochloric acid to an end concentration of 1 M. Hydrochloric acid was removed by evaporation in a speedvac, and hydrolyzed samples were redissolved in Millipore water. Final traces of hydrochloric acid were removed by titration with sodium hydroxide. Samples were analyzed for their hexuronic acid content by anion exchange chromatography on a Dionex system equipped with a CarboPac-100 column. The used effluent was 45 mM sodium hydroxide, with an increasing gradient of sodium acetate (0-960 mM).

RESULTS
Protein sequence analysis
A BLAST-search (16) with the human heparan sulfate D-glucuronoyl C5-epimerase protein sequence as query confirmed the presence of highly homologous proteins in different mammalian species (17). Although homologous sequences were found in genomes of both bacteria and archaea as well, most of these genes identified have a relatively low homology (10). However, among this group of prokaryotic sequences, the candidate C5-epimerase protein sequence (RED-C5-epimerase) of the marine bacterium *Bermannella marisrubri* sp. RED65 (18) showed significantly higher homology to animal heparan sulfate D-glucuronoyl C5-epimerase than any other prokaryotic sequence. Based on its sequence, RED-C5-epimerase was placed within PFAM family C5-epim_C (PF0662), representing the C-terminal domain of heparan sulfate D-glucuronoyl C5-epimerase (19). The gene coding for RED-C5-epimerase (RED65_08024) contains 1347 base pairs, resulting in a protein of 448 amino acids with a theoretical molecular mass of 51,914 Da.

We constructed a multiple sequence alignment (T-Coffee server (20)), to illustrate the homology between RED-C5-epimerase and the animal heparan sulfate D-glucuronoyl C5-epimerases (Fig. 1). The multiple sequence alignment was visualized using GeneDoc version 2.6 (21). RED-C5-epimerase has 23% identity and 37% similarity with the human heparan sulfate D-glucuronoyl C5-epimerase, mostly based on high similarity at the carboxyl terminus (41% identity, 60% similarity). In contrast with the animal proteins, RED-C5-epimerase has no predicted transmembrane helix at the amino terminus. The overall secondary structure is predicted (PSIPRED Server (22)) to be similar to the animal heparan sulfate D-glucuronoyl C5-epimerase (Fig. 1).

Neighborhood analysis
To have a prediction on the physiological role of RED-C5-epimerase, we performed a gene neighborhood analysis. The genomic context of RED65_08024 comprises genes involved in the biosynthesis of capsular polysaccharides. In bacteria these genes typically are organized in gene clusters. Studies on the organization of similar clusters in *Escherichia coli* (23) or *Pasteurella multocida* (24), show that these clusters consist of three functional regions. Region 1 and 3 are well conserved and the encoded
proteins are involved in capsular polysaccharide export, while proteins encoded by region 2 are involved in the biosynthesis of serotype-specific polysaccharides, and therefore are highly variable (25). RED65_08024 and another eight ORFs, are flanked by these capsular polysaccharide export regions 1 and 3 (Fig. 2).

We compared the genomic context of RED-C5-epimerase with the organization of similar genes in other bacteria. We observed high resemblance with the capsular gene clusters of the aquatic bacteria *Shewanella violacea* DSS12 (26), *Aeromonas hydrophila* PPD134/91 (27) and *Burkholderia ambifaria* IOP40-10. Not only do they all possess the region 1 and 3 genes involved in capsular polysaccharide transport, they also have several genes in common of the type specific region 2. However, the presence of a C5-epimerase appears to be restricted to *Bermanella marisrubri* sp. RED65. Most likely this candidate C5-epimerase is involved in a species- or type-specific sugar modification, which is expected for a bacterial D-glucuronyl C5-epimerase, as IdoA presence in prokaryotes seems to be a species- or type-specific constituent (10).

Characterization of the recombinant protein of RED-C5-epimerase

The RED-C5-epimerase gene was successfully expressed in *E. coli*, resulting in high amounts (~20 mg/L cell culture) of protein (Fig. 3). Based on SDS-PAGE analysis, the protein size was estimated slightly smaller than the theoretical size of 52 kDa. However, protein truncation can be excluded, as trypsin digestion LC-MS analysis did confirm the presence of both termini. We could purify the protein using the amino terminal polyhistidine-tag, however we observed a tendency for protein aggregation.

To determine the oligomeric state of the protein, we performed size exclusion chromatography on a Superdex-200 HR 10/10 column (Fig. 4). In freshly prepared protein samples, RED-C5-epimerase is present in a dimeric/tetrameric form, in about a 2:1 ratio, and appears to be in a dynamic equilibrium. Upon storage, RED-C5-epimerase has the tendency to form larger soluble aggregates. Non-detergent sulfobetaines (NDSB) are known to efficiently prevent protein aggregation (28). We tested the effect of NDSB201 (up to 1 M), but did not observe less aggregation at 4°C, although NDSB201 did prevent precipitation of the protein upon freezing. Addition of reducing agents such as Tris (2-carboxyethyl)phosphine (TCEP, 2 mM) prevented aggregation, maintaining the initial dimeric/tetrameric oligomeric state. High levels of reducing agent (e.g. 50 mM β-mercaptoethanol) even partly reversed aggregation. This effect suggests that the formation of intramolecular and intermolecular disulfide bonds between any of the nine cysteine residues in the RED-C5-epimerase peptide causes the observed aggregation.

Enzyme activity analyses

To test for C5-epimerase activity, cell lysate containing recombinant RED-C5-epimerase was incubated with 3H-labeled substrates derived from modified heparin and K5 polysaccharide (11), by the biphasic separation method (14). For both substrates we detected significant D-glucuronyl C5-epimerase activity, although at a substantially lower level in comparison to the murine C5-epimerase (Table 1). Notably, we were unable to detect activity in the absence of TCEP in the incubation buffer. Also the addition of the detergent Tween 20 has a positive effect on the enzymatic activity (Table 1). Incubations were done at 30°C, as 37°C resulted in inactivation of the enzyme.

To confirm the formation of GlcA vs. IdoA residues in polysaccharides, we analyzed the products of de-O-sulfated heparin after incubation with the recombinant RED-C5-epimerase. We performed chemical hydrolysis with HCl, resulting in partial hydrolysis of heparin thereby liberating monomeric hexuronic acids. Only a small fraction can be recovered as monomeric sugar, most sugars remain unreleased in oligosaccharides. Tight control of hydrolysis time, temperature and acid concentration is crucial to prevent substantial formation of side products and to prevent differential release of GlcA and IdoA, due to differences in acid lability of their glycosidic bonds (29). Therefore a control sample has been included in each run to validate the hydrolysis conditions. Monomeric GlcA can be separated from IdoA by high performance anion exchange chromatography (HPAEC) (30,31). We optimized the protocol for analysis of the hydrolyzed reaction mixture on a Dionex system, monitoring hexuronic acids with an electrochemical detector.
using pulsed amperometric detection (PAD) (Fig. 5A).

Using an anion exchange column (CarboPac PA-100) we were able to separate GlcA and IdoA. An important note is to keep salt-levels low in the samples, as the retention time of IdoA decreases at high salt concentrations, resulting in poor separation of both hexuronic acids. Using this HPLC-PAD method we tested C5-epimerase activity of the RED-C5-epimerase on various available D-glucuronyl containing GAGs and GAG precursors, including chondroitin, hyaluronan, UDP-GlcA, N-sulfated K5 polysaccharide and de-O-sulfated heparin. Most of the samples did not give any significant changes in the hexuronic acid content after incubation with RED-C5-epimerase, except for the de-O-sulfated heparin. Incubation of this substrate with both crude cell lysate or purified enzyme resulted in the change of the proportion of GlcA vs. IdoA residues. In heparin the ratio between both hexuronic acids typically is 35% GlcA and 65% IdoA. This is also visible in the control samples. However, overnight incubations of de-O-sulfated heparin with RED-C5-epimerase resulted in a shift in the ratio, where IdoA levels dropped to 45% and GlcA increased to 55%, whereas GlcNAc levels remained unaffected (Fig. 5B and Fig. 6). Control samples were treated identically but have been incubated with cell lysate lacking the RED-C5-epimerase.

Using the same HPLC method, a time series experiment was performed to monitor the enzyme activity over time. Incubations showed a gradually decrease in the conversion rate of the hexuronic acids until an equilibrium was reached with a ratio of about 55% GlcA and 45% IdoA (Fig. 7). Extending the reaction time did not result in a higher net conversion of the hexuronic acids, neither did the addition of higher or additional amounts of enzyme.

DISCUSSION
IdoA is a common and essential sugar residue in several animal GAGs. However, it has been shown that IdoA is not only typical for eukaryotic GAGs, but occasionally also can be found as a constituent in sero-type specific prokaryotic cell wall polysaccharides (6-10). From the animal GAG biosynthesis we know that IdoA is synthesized via a C5-epimerization of GlcA (review:(32)). In prokaryotes IdoA is likely to be synthesized in a similar way, which implies the involvement of a prokaryotic D-glucuronyl C5-epimerase. We selected a hypothetical gene from the marine bacterium Bermanella marisrubri sp. RED65 that has a relative high homology to animal heparan sulfate D-glucuronyl C5-epimerases. This similarity is mostly based on the conserved C-terminal domain of the protein, but also based on a very similar predicted secondary structure. Phylogenetical analysis (10) indicates that prokaryotic C5-epimerase sequences do not cluster to the clade of animal heparan sulfate D-glucuronyl C5-epimerases, making any (recent) horizontal gene transfer from animal to prokaryote unlikely. The presence of this enzyme in ‘higher animals’ (e.g. humans) as well as in ‘lower animals’ (e.g. C. elegans) may imply an evolutionary scenario with an initial development of this enzyme in bacteria or archaea with a horizontal gene transfer event to a common ancestor of animals, after which molecular complexity (N-terminal domain) increased. We cloned and expressed the gene coding for the candidate C5-epimerase in E. coli and tested gene product RED-C5-epimerase for heparan sulfate D-glucuronyl C5-epimerase activity. We have been able to detect significant levels of tritium release from 3H-labeled substrates derived from modified heparin and K5 polysaccharides, indicating C5-epimerase activity. This enzymatic conversion of hexuronic acids in de-O-sulfated heparin has been confirmed using an HPLC-PAD method (Fig. 6 and Fig. 7).

An epimerization reaction typically ends up in an equilibrium between the two specific epimers. For animal heparan sulfate D-glucuronyl C5-epimerases it has been postulated that the epimerization equilibrium is slightly towards GlcA over IdoA (11). Thus, it is tempting to speculate that RED-C5-epimerase has similar catalytic properties and results in similar conversion ratios as the animal heparan sulfate D-glucuronyl C5-epimerase. The data show that an equilibrium of GlcA and IdoA in de-O-sulfated heparin was reached upon incubation with RED-C5-epimerase, in a similar pattern to the product of murine heparan sulfate D-glucuronyl C5-epimerase (11). This equilibrial state is clearly shown in Figure 7. No further conversion was observed after prolonged incubation or after addition of more
enzyme. The observed GlcA conversion must be an effect of the RED-C5-epimerase, as control-samples did not show a shift in the hexuronic acid ratio. We have been unable to prove significant IdoA levels for the RED-C5-epimerase / K5 polysaccharide incubations using HPLC-PAD analysis, although the enzyme did release tritium from $^{3}$H-labeled modified K5 polysaccharide. Likely, the K5 polysaccharide is a poor substrate, which is also the case for the murine heparan sulfate C5-epimerase (Table 1). Whether this is due to a differential $N$-sulfation pattern between the K5 polysaccharide and de-O-sulfated heparin should be further verified.

As far as we know, this is the first time that experimental evidence is provided on a prokaryotic enzyme with heparan sulfate D-glucuronyl C5-epimerase activity. Obviously, the bacterial enzyme has distinct features compared to the murine enzyme. Apart from the substantial lower activity towards the substrates tested, the bacterial enzyme functions in a different buffer system requiring the reducing agent TCEP as additive. TCEP prevents the formation of aggregates that results in inactivation of the enzyme; apparently only the dimeric/tetrameric enzyme fraction is active. The dimeric and tetrameric fractions appear to be in a dynamic equilibrium and therefore we were unable to conclude which of the two fractions is active.

Based on the effect of TCEP, we conclude that the observed protein aggregation is most likely caused by inappropriate disulfide bond formation. Compared to the murine heparan sulfate D-glucuronyl C5-epimerase (6 cysteines; of which 3 in the signal peptide), the RED-C5-epimerase is relatively cysteine-rich (9 cysteines, no signal peptide), which increases the potential to form intra-disulfide bonds. This aggregation may have led to depletion of the competent enzyme molecules, resulting low enzymatic activity. The inappropriate formation of disulfide bonds should be a key consideration for potential exploitation of RED-C5-epimerase for potential applications. Considering that the activity of the RED-C5-epimerase is relatively low, it may indicate that the physiological substrate of this enzyme in the bacterium could differ from heparin and HS. Analysis of the Bermanella marisrubri sp. RED65 cell wall polysaccharides could be a first step in providing more insight in both the physiological role and substrate specificity of RED-C5-epimerase. Moreover, the identification of novel bacterial C5-epimerases not only increases our insight in bacterial cell wall polysaccharide biosynthesis but eventually may provide potential new biotechnological tools applicable in the controlled chemo-enzymatic synthesis of industrially relevant IdoA containing GAGs like heparin, HS or variants.
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FOOTNOTES
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The abbreviations used are: GlcA, D-glucuronic acid; HS, heparan sulfate; IdoA, L-iduronic acid; GAG, glycosaminoglycan; GlcNAc, N-acetyl-D-glucosamine; IPTG, isopropyl-1-thio-β-D-galactopyranoside; NDSB, Non-detergent sulfobetaines; TCEP, Tris (2-carboxyethyl)phosphine; PAD, pulse amperometric detection; HPAEC, high performance anion exchange chromatography.

FIGURE LEGENDS

FIGURE 1. Multiple sequence alignment of RED-C5-epimerase. Hypothetical protein RED_08024 from Bermanella marisrubri sp. Red65 (REDC5) compared to characterized animal heparan sulfate D-glucuronyl C5-epimerases (H sapi: Homo sapiens; M mus: Mus musculus; C ele: Caenorhabditis elegans). Secondary structure predictions are indicated for human and B marisrubri (h indicates predicted α-helices, s indicates predicted β-strands).

FIGURE 2. Genomic context of Red_08024 (in red) in comparison with other bacterial capsular polysaccharide biosynthesis gene clusters (23,24,27). The flanking genes in blue (region 1; M = kpsM, T = kpsT, E = kpsE and D = kpsD) and green (region3; C = kpsC and S = kpsS) are likely to be involved in polysaccharide export. The genes in between (region 2) are likely to be involved in serotype specific capsular polysaccharide biosynthesis. The conserved genes are predicted to be a: glycosyl transferase (ORF1, dark-purple), UDP-N-acetyl glucosamine-2-epimerase (ORF2, purple), UDP-N-acetyl-D-mannosaminuronic acid dehydrogenase (ORF3, violet) and glycosyltransferase (ORF4, magenta). The D-glucuronyl C5-epimerase (ORF5, red) is unique to B marisrubri Sp. RED65. The other region 2 genes in B marisrubri are predicted to be (conserved) hypothetical proteins (ORF6, ORF7), glycosyl transferase (ORF8), 2-dehydro-3-deoxyphosphooctonate aldolase (ORF9).

FIGURE 3. Recombinant RED-C5-epimerase production in E. coli. RED-C5-epimerase presence in cell lysate and after purification. The control lysate is made from E coli not harboring the recombinant RED-C5-epimerase containing vector pWUR537. The full length presence of the protein has been confirmed by trypsin digestion LC-MS analysis.

FIGURE 4. Size exclusion chromatography to determine the oligomeric state of RED-C5-epimerase. In freshly prepared samples RED-C5-epimerase is present in a dimeric/tetrameric form occurring in a 2:1 ratio (A). In time the protein has the tendency to form larger aggregates (B). Despite the fact that the non-detergent sulfobetaines NDSB201 does prevent precipitation upon freezing, it has no obvious effect in prevention of aggregation (C). Addition of the reducing agents TCEP does help to prevent aggregation, maintaining the initial oligomeric state (D).

FIGURE 5. Separation of N-acetyl-D-glucosamine (GlcNAc), D-glucuronic acid (GlcA) and L-iduronic acid (IdoA) using HPLC-PAD. (A) Separation of standards (B) GlcNAc and (C) GlcA vs IdoA recovery upon hydrolyses of (de-O-sulfated) heparin; black/dashed line = control sample, gray/solid line = sample incubated with RED-C5-epimerase.
FIGURE 6. Hexuronic acid ratio in heparin after treatment with RED-C5-epimerase. Both incubation with cell lysate and pure protein show partial epimerization of L-iduronic acid (IdoA; white bars) into D-glucuronic acid (GlcA; gray bars), whereas the control sample shows the initial ratio indicating no epimerization.

FIGURE 7. Time-series analysis of the hexuronic acid content of heparin as a result of RED-C5-activity. Incubations showed a gradually decrease in the conversion rate of the hexuronic acids until an equilibrium was reached with a ratio of about 55% GlcA and 45% IdoA.
### TABLE I Activity measurement of D-glucuronyl C5-epimerase mediated tritium release.

| Substrate          | Enzyme                        | Buffer                  | $^3$H release (cpm) |
|--------------------|-------------------------------|-------------------------|---------------------|
| Heparin$^1$        | RED-C5-epimerase (5 µl)       | TRIS with TCEP          | 25                  |
| Heparin            | RED-C5-epimerase (10 µl)      | TRIS with TCEP          | 15                  |
| Heparin            | RED-C5-epimerase (50 µl)      | TRIS with TCEP          | 15                  |
| Heparin            | RED-C5-epimerase (5 µl)       | TRIS with TCEP + Tween 20 | 58                  |
| Heparin            | RED-C5-epimerase (10 µl)      | TRIS with TCEP + Tween 20 | 116                 |
| Heparin            | RED-C5-epimerase (50 µl)      | TRIS with TCEP + Tween 20 | 200                 |
| Heparin            | Mouse C5-epimerase (5 µl)     | HEPES                   | 1200                |
| Heparin            | Mouse C5-epimerase (10 µl)    | HEPES                   | 1540                |
| Heparin            | Mouse C5-epimerase (50 µl)    | HEPES                   | 2054                |
| K5 polysaccharide$^2$ | RED-C5-epimerase (5 µl)       | TRIS with TCEP          | 32                  |
| K5 polysaccharide  | RED-C5-epimerase (10 µl)      | TRIS with TCEP          | 84                  |
| K5 polysaccharide  | RED-C5-epimerase (50 µl)      | TRIS with TCEP          | 117                 |
| K5 polysaccharide  | RED-C5-epimerase (5 µl)       | TRIS with TCEP + Tween 20 | 25                  |
| K5 polysaccharide  | RED-C5-epimerase (10 µl)      | TRIS with TCEP + Tween 20 | 32                  |
| K5 polysaccharide  | RED-C5-epimerase (50 µl)      | TRIS with TCEP + Tween 20 | 181                 |
| K5 polysaccharide  | Mouse C5-epimerase (50 µl)    | HEPES                   | 305                 |
| K5 polysaccharide  | Mouse C5-epimerase (50 µl)    | HEPES                   | 320                 |

Note: Substrate production: $^1$De-sulfated and re-N-sulfated heparin was incubated with purified murine C5-epimerase in the presence of $^3$H$_2$O. Tritium was introduced into both IdoA and GlcA residues as described in (33); $^2$E coli K5 was cultured in the presence of C5-$^3$H-glucose to introduce $^3$H-labeled GlcA. The purified capsular polysaccharide was deacetylated and N-sulfated.
Figure 1
Figure 2

| I. Capsule Export | II. Capsule Biosynthesis | III. Capsule Export |
|-------------------|--------------------------|---------------------|
| *Escherichia coli* K10 |
| Pasteurella multocida X-73 (A:1) |
| *Bermanella marisrubri* sp. Red65 |
| *Shewanella violacea* DSS12 |
| *Aeromonas hydrophila* PPD134/91 |
| *Burkholderia ambifaria* JOP40-10 |

- **I. Capsule Export**
  - *Escherichia coli* K10
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  - *Aeromonas hydrophila* PPD134/91
  - *Burkholderia ambifaria* JOP40-10

- **II. Capsule Biosynthesis**
  - *Escherichia coli* K10: serotype specific genes
  - *Pasteurella multocida* X-73 (A:1): HyaA, HyaB, HyaC, HyaD, HyaE
  - *Bermanella marisrubri* sp. Red65: 5 specific genes
  - *Shewanella violacea* DSS12: 2 specific genes
  - *Aeromonas hydrophila* PPD134/91: 6 specific genes
  - *Burkholderia ambifaria* JOP40-10: 2 specific genes
Figure 3

Control lysate  Marker (kDa)  Cell lysate  Purified

150  100  75  50
Figure 4

A. Bacterial D-glucuronol C5-epimerase activity

B. Absorbance at 280 nm (mAU)

C. Aggregation

D. Elution volume (ml)

E. Kan = (Ve-Vo)/(Vt-Vo)

y = -0.3042x + 1.0016

R² = 0.9906
Figure 5

A

Value (µC)

GlcNAc

GlcA

IdoA

Time (min)

B

Value (µC)

Time (min)

C
Figure 6

Bacterial D-glucuronyl C5-epimerase activity

Relative amount of hexuronic acid composition (%)
Figure 7

![Graph showing bacterial D-glucuronyl C5-epimerase activity](http://www.jbc.org/Downloaded from)
A novel bacterial enzyme with D-glucuronyl C5-epimerase activity
John Raedts, Magnus Lundgren, Servé W. M. Kengen, Jin-ping Li and John van der Oost

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