Prevotella enzymes involved in mucin oligosaccharide degradation and evidence for a small operon of genes expressed during growth on mucin

Damian P. Wright, Douglas I. Rosendale, Anthony M. Roberton *

School of Biological Sciences, The University of Auckland, Private Bag 92019, Auckland, New Zealand

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Abstract

Mucin desulfation is believed to be a rate-limiting step in mucin degradation by colon bacteria. The activities of enzymes hydrolysing nine linkages found in mucin oligosaccharide chains were measured using model substrates, in extracts of two mucin-degrading bacteria, Prevotella strain RS2 and Bacteroides fragilis. Sulfatases desulfating N-acetylglucosamine-6-sulfate, galactose-6-sulfate and galactose-3-sulfate were found. The genomic DNA downstream from the gene encoding the mucin-desulfating sulfatase (N-acetylglucosamine-6-sulfatase) in Prevotella was sequenced, and two putative genes identified which are likely to be coexpressed with this sulfatase, though their activities are unknown. Northern and Western analyses showed that expression of this short operon of three genes is increased during growth on mucin.

Keywords: Mucin degradation; Prevotella; Sulfatase; Sulfomucin

1. Introduction

The colon surface is covered by a layer of mucus, and it is assumed that this mucus barrier helps prevent damage to the underlying epithelium by potentially damaging agents in the lumen, including the bacteria and their enzymes. The relationship between the colon, its mucus layer and its complex bacterial flora is only partly understood [1]. The presence of some bacterial cells in the colon crypts indicates that any barrier function of the mucus cannot be complete.

In normal colon, the major structural components of the mucus, the mucin glycoproteins, are degraded only at a rate at which they can be replenished. However, in the inflammatory disease ulcerative colitis, there is a decrease in the mucus layer thickness [2], which may be due to decreased mucin synthesis and secretion, and to increased degradation by bacteria. The change is paralleled by decreased levels of sulfation in the secreted mucin and increased activity of bacterial mucin-desulfating enzyme(s) in the colon contents [3]. Several research groups have suggested that the step(s) of mucin desulfation rate-limits both the rate and extent of mucin degradation by bacterial enzymes [1,4–6], so information on the mucin-desulfating enzymes would be helpful.

Mucin degradation in the colon is probably a cooperative process involving many bacterial types. Several pure cultures of bacteria have been shown to degrade mucin sufficiently well to use it as a sole growth substrate [7–10]. If the principles which govern the degradation of large polysaccharides by Gram-negative colon anaerobes are applicable also to mucin breakdown, then many of the enzymes involved will be present on the bacterial cell surface or in the periplasm rather than secreted into the intestinal lumen. Duplication of enzymes for many of the reactions will occur, and currently undiscovered binding proteins and transport proteins will be involved [11,12].

In the present work, we have studied mucin-degrading proteins in the bacterium Prevotella strain RS2. Using model substrates, we have measured the activities of many enzymes necessary for breaking glycosidic linkages and sulfate ester bonds found in mucins, and established which activities are inducible by mucin. The levels have been compared to those in Bacteroides fragilis ATCC 25285, which degrades mucin to a lesser extent, but can
use it for growth [13]. We have also looked for other genes located downstream from the previously studied mucin-desulfating sulfatase mdsA gene [14,15] in Prevotella strain RS2, that are coexpressed with the sulfatase under conditions of growth on mucin.

2. Materials and methods

2.1. Bacterial culture and cell extract preparation

*Prevotella* strain RS2 is an isolate from pig colon, chosen for this study because it vigorously degrades mucins [16,17]. *B. fragilis* was chosen for this study because many researchers have shown it degrades mucins, and it is present in high numbers in the human colon. *Prevotella* strain RS2 and *B. fragilis* ATCC 25285 were grown in modified medium 10 [14] with either 0.2% (w/v) galactose or 0.14% (w/v) galactose plus 0.28% (w/v) of a pig gastric mucin (PGM) preparation as carbohydrate energy source [17]. Cells were harvested as they reached stationary phase by centrifugation (17,000×g, 20 min, 4°C) and washed in buffer (25 mM Tris chloride containing 10 mM 2-mercaptoethanol, pH 7.4), then resuspended in 0.1 volumes of buffer and broken by three passes through a French pressure cell. Diisopropyl fluorophosphate (0.002%, w/v) was added, and the broken cells centrifuged (17,000×g, 30 min, 4°C). The extract supernatant was assayed.

2.2. Enzyme activity assays

d-Glucose-6-sulfate, 4-methylumbelliferyl-α-D-N-acetylneuraminic acid and the p-nitro-phenyl (PNP) glycosides of α-D-galactose, β-D-galactose, α-L-fucose, N-acetyl-α-D-galactosamine, and N-acetyl-β-D-glucosamine were purchased from Sigma, St Louis, MO. The PNP glycosides of β-D-galactose-3-sulfate, and β-D-galactose-6-sulfate were synthesised by Industrial Research, Lower Hutt, New Zealand. Enzymic hydrolysis of the PNP glycosides (1 mM) in Tris chloride buffer (20 mM, pH 7.4) was carried out for 20 min at 37°C, followed by adjustment to pH 9.6, and absorbance measurement at 410 nm. In the case of PNP glycosides of the sulfated galactose substrates, an auxiliary enzyme, β-galactosidase which does not hydrolyse the linkage between p-nitrophenol and sulfated sugars, was added. Ascending paper chromatography of the PNP glycosides of sulfated galactoligosaccharides and their enzyme-hydrolysed products (done in the absence of added β-galactosidase) was carried out as described previously to confirm the nature of the reactions [14]. Hydrolysis of 4-methylumbelliferyl-α-D-N-acetylneuraminic acid (0.1 mM) was measured fluorometrically (excitation at 365 nm, emission at 450 nm). Mucin-desulfating sulfatase (MdsA), an enzyme desulfating N-acetylgalactosamine-6-sulfate in mucin oligosaccharide chains, was measured as previously described by d-glucose-6-sulfate desulfation [14].

2.3. Molecular techniques

Genomic DNA (gDNA) from *Prevotella* strain RS2 was used as template for inverse PCR [18] to amplify the gDNA region downstream from the previously described mdsA gene [15]. Successive templates were made by digestion of gDNA with *NcoI*, *PvuII*, and *HindIII*, followed by re-ligation into circular DNA using T4 DNA ligase. Pairs of inverse PCR primers were used to amplify the DNA, followed by sequencing. Primer sequences, and the sequence positions in Fig. 1 to which they anneal, were: TR11(F) 5'-GACGTCGATCTCTGAGCATAA-3' (bases 243–266), TR13(R) 5'-CAATGCTAGATCCTCGTCGATAG -3' (bases 139–118); TR21(F) 5'-CGAGCGCGAGCTTAGACCCCTGCG-3' (bases 2078–2102); TR20(R) 5'-CAAAGGCAATCTGGTACCCTGCGG-3' (bases 1378–1355); DW2(F) CGAGGACGACTGCAATACGCCG-3' (bases 4185–4206); DW3(R) 5'-CCACTTTGTCACATTACAGCCGTGCC-3' (bases 4140–4159). The gene downstream from mdsB, which codes in the opposite direction to mdsA (Fig. 1), is unlikely to be involved in mucin degradation [15], and was not further investigated.

2.4. Design and expression of chimeric glutathione S-transferase fusions proteins

Gene sequences of 300–450 bp from *Prevotella* strain RS2 were chosen for expression as soluble glutathione S-transferase (GST) fusion proteins. Chimeric gene constructs were made by ligating each *Prevotella* open reading frame (ORF) sequence in frame to the 3'-end of *gst* in pGEX-2T plasmid (Amersham Pharmacia Biotech, Uppsala, Sweden), and chimeric proteins were expressed and purified as previously described [15]. Antisera against these fusion proteins were made in New Zealand white rabbits, and the antibodies with affinity for the GST epitopes were removed to increase the specificity for the *Prevotella* peptide sequence [15]. The titre of each antibody was estimated by ELISA, using pET-expressed MdsA, MdsC or MdsD protein (from solubilised inclusion bodies) as antigen (D.P. Wright, unpublished results).

2.5. Northern analysis and gel electrophoresis of RNA

Whole cell RNA was extracted from *Prevotella* strain RS2 grown on galactose or low galactose plus PGM, Cells were grown to upper exponential phase, and harvested by centrifugation under anaerobic conditions (1500×g, 4 min). After removal of medium supernatant, TRIZol reagent (Gibco/BRL, Life Technologies, Gaithersburg, MD) was added. Total RNA was isolated according to the manufacturer’s procedure. RNA molecules were separated by electrophoresis in formaldehyde-denaturing gel using MOPS buffer [19] and capillary blotted onto Hybond N+ membrane (Amersham Pharmacia Biotech).
The membrane was hybridised with random hexamer primer \(^{32}\)P-labelled probe [19], made as detailed in the text.

2.6. Western analysis of protein expression

Proteins were separated by electrophoresis on 8 or 10% SDS–PAGE gels, and blotted onto nitrocellulose membrane. Western analysis using the antisera above was performed using standard procedures [19] with alkaline phosphatase-labelled secondary antibody.

Preparation of periplasm and spheroplast fractions from *Prevotella* cells was carried out as previously described [14]. Spheroplasts were lysed osmotically, briefly sonicated, and separated by centrifugation into cell membranes and cytoplasm.

2.7. Nucleotide sequence accession number

The 4954-bp sequence downstream from the end of *mdsA* is available from GenBank accession number AF248951.

3. Results

3.1. Mucin oligosaccharide chain degrading enzyme activities

When *Prevotella* strain RS2 is subcultured from medium containing a simple hexose as energy source into medium with mucin as its main energy source, a growth lag occurs. To determine which enzymes involved in mucin oligosaccharide chain degradation are constitutive and which are partly inducible, the activities of a selection of hydrolytic enzymes were assayed in cell extracts from cells grown on galactose or low galactose plus PGM (Table 1). Activities that were similar for both growth substrates included \(\alpha\)-fucosidase and galactose-3-sulfatase. Activities that increased significantly \((P < 0.05,\) using the Mann–Whitney U-test for comparing medians of two samples) during growth on mucin medium included the \(\beta\)-galactosidase \((40\%), \alpha\)-N-acetylgalectosaminidase \((95\%), \) \(\beta\)-N-acetylglucosaminidase \((91\%),\) glucose-6-sulfatase \((110%)\) (this is the mucin-desulfating sulfatase, MdsA, known to desulfate N-acetylglucosamine-6-sulfate in mucin chains) \([14]\) and neuraminidase \((163\%).\)

For comparison, similar activity measurements were made on *B. fragilis* ATCC 25285, another bacterium that grows on mucin. Some of these activities have been previously reported in cell extracts of another strain of *B. fragilis* grown on galactose or mucin \([20]\). None of the measured activities changed significantly when cells were grown on mucin compared to galactose. Comparing the enzyme activities in mucin-grown *Prevotella* and *Bacteroides* extracts, the latter exhibited considerably higher activities of \(\alpha\)-galactosidase, galactose-6-sulfatase and galactose-3-sulfatase. The activities of the rest were comparable within a factor of four, except for glucose-6-sulfate desulfation which was considerably higher in the *Prevotella*.

To confirm that the sulfatases from *B. fragilis* produced PNP-galactose from PNP glycosides of galactose-3-sulfate and galactose-6-sulfate, paper chromatography was used. This was demonstrated only after partial purification of the sulfatases, because crude extracts contain levels of \(\beta\)-galactosidase that immediately hydrolyse PNP-galactose (D.I. Rosendale, unpublished results).

3.2. Genes located downstream of mucin-desulfating sulfatase in *Prevotella*

Mucin desulfation appears to be one of the rate-limiting steps in degradation of mucin, and the MdsA protein in *Prevotella* is known to remove a portion of the sulfate from \(^{35}\)Smucin \([14]\). To look for other genes that may be coexpressed during growth on mucin, the gDNA sequence downstream of the *mdsA* gene has been examined by inverse PCR (Fig. 1). The 4954 bp of gDNA downstream from the terminus of the sulfatase were sequenced. Two new putative ORFs were found, designated *mdsC* and *mdsD* (bases 75–1160 and 1258–4476 downstream from the *mdsA* stop codon), encoding predicted proteins of 362 and 1073 amino acids, and coding in the same direction as *mdsA*. After the *mdsD* sequence, a putative RNA polymerase stop-motif occurs, which is likely to terminate message transcription.

The sequences of the predicted proteins MdsC and MdsD show some homology to sequences in data banks. Alignment of MdsC with a putative 377 residue protein BAA17692.1 (GenBank accession number D90908) from *Synechocystis* sp. gives 34.3% amino acid identity and 45.7% similarity. The function of the *Synechocystis* protein...
is unknown. It is likely that MdsC will be a cytoplasmic protein, as it has no signal sequence. MdsD shows 20.9% amino acid identity and 29.9% similarity to tricorn protease from *Thermoplasma acidophilum* (GenBank accession number U72850) [21] and 22% identity and 30.7% similarity to a putative protein from *Sulfolobus sulfataricus* (GenBank accession number Y08256). All three proteins are 1069–1073 amino acids in length. MdsD has a 19 amino acid signal sequence and is likely to be transported from the cytoplasm. Detailed analysis of the deduced protein sequence of MdsD reveals several motifs that may be useful indicators for assigning a function to this protein at a later date. At the amino end there are nine repetitive sequences (each 40–43 amino acids in length) with homology to WD repeats [22,23], each likely to form 4-stranded antiparallel sheets which in turn will form β-propeller structures (amino acids 25–499 of the pro-protein). An amphipathic sequence, in which 50% of the amino acids are charged and mainly positive (amino acids 546–617), is likely to form an α-helical structure in the middle region. Another region (amino acids 770–857) shows the characteristics of a PDZ domain [23,24].

3.3. Northern analysis of mRNA encoding mucin-desulfating sulfatase

Whole cell RNA was isolated from *Prevotella* grown on mucin and on galactose. Following electrophoresis in an agarose gel, the mRNAs were hybridised with a 637-bp probe [15] from the *mdsA* sulfatase (Fig. 2A) or with a 554-bp probe of *mdsD* (bases 2349–2902 of the *mdsD* sequence) (Fig. 2B). In Fig. 2A, the hybridised mRNA band was too polydisperse to measure the exact size of the message, but the size range (6–10 kb) was clearly too large to encode only the sulfatase, and could encompass the sequences encoding MdsA, MdsC and MdsD. The hybridisation intensity of mRNA was much greater in the mucin-grown cells than in galactose-grown cells, consistent with the data in Table 1 indicating that sulfatase activity was greater in extracts of mucin-grown cells. In Fig. 2B, where the mRNAs were hybridised with *mdsD* probe, similar results were obtained, consistent with the message coding for the three genes.

3.4. Western analysis of cell fractions containing the mucin-desulfating sulfatase (MdsA) and the protein product MdsD

An antibody recognising MdsA was previously made using a GST-MdsA epitope fusion protein [15]. Fig. 3A shows Western analysis of cell fractions from *Prevotella* grown on mucin and galactose, using this antibody. Lanes

Table 1
Activities of mucin oligosaccharide-degrading enzymes in extracts of *Prevotella* strain RS2 and *Bacteroides fragilis*

<table>
<thead>
<tr>
<th>Enzyme</th>
<th><em>Prevotella</em> strain RS2</th>
<th><em>Bacteroides fragilis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Activity in galactose-</td>
<td>Activity in galactose-</td>
</tr>
<tr>
<td></td>
<td>grown cells*</td>
<td>grown cells*</td>
</tr>
<tr>
<td>α-Galactosidase</td>
<td>0.26 ± 0.13</td>
<td>4.57 ± 0.58</td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>0.03 ± 0.14</td>
<td>33.46 ± 3.91</td>
</tr>
<tr>
<td>α-Fucosidase</td>
<td>5.26 ± 0.97</td>
<td>4.48 ± 1.30</td>
</tr>
<tr>
<td>α-N-Acetylgalactosaminidase</td>
<td>0.44 ± 0.16b</td>
<td>0.86 ± 0.33b</td>
</tr>
<tr>
<td>β-N-Acetylgalactosaminidase</td>
<td>15.16 ± 2.58b</td>
<td>28.94 ± 1.65b</td>
</tr>
<tr>
<td>Glucose-6-sulfatase</td>
<td>2.59 ± 0.30b</td>
<td>5.43 ± 0.88b</td>
</tr>
<tr>
<td>(mucin-desulfating sulfatase)</td>
<td>0.06 ± 0.10</td>
<td>0.25 ± 0.17</td>
</tr>
<tr>
<td>β-N-Galactose-6-sulfatase</td>
<td>0.06 ± 0.10</td>
<td>5.07 ± 1.54</td>
</tr>
<tr>
<td>β-N-Galactose-3-sulfatase</td>
<td>0.62 ± 0.29</td>
<td>6.78 ± 1.43</td>
</tr>
<tr>
<td>N-Acetyleneuraminidase</td>
<td>2.09 ± 0.36b</td>
<td>5.49 ± 0.40b</td>
</tr>
</tbody>
</table>

*a*nmol product min⁻¹ (mg protein)⁻¹. Mean ± S.D. from three measurements.

b *P* < 0.05, using the Mann-Whitney *U*-test for comparing medians of two samples.
2 and 3 demonstrate the increased sulfatase in the periplasm of mucin-grown cells compared to galactose-grown cells. Lanes 4 and 5 show absence of sulfatase in spheroplasts and culture supernatant.

Attempts to raise antibody in rabbits against MdsC, using a chimeric recombinant protein containing a MdsC domain attached to GST, were not successful. However, a good antibody recognising MdsD was raised using as antigen a chimeric recombinant protein containing GST with an internal highly hydrophilic MdsD domain attached (amino acids 499–608), and the antibodies recognising GST were subsequently removed by affinity chromatography.

Fractions obtained from mucin-grown and galactose-grown cells were examined by Western analysis, using purified antibody against the MdsD domain (Fig. 3B). A protein of approximately 119 kDa was detected in the membrane fraction of mucin-grown cells (lane 4), but not galactose-grown cells (lane 3). Periplasmic extracts, spheroplast lysate and culture medium supernatant from mucin-grown cells showed no bands (lanes 1, 2 and 5). Inclusion body, formed during expression of MdsD from a pET17b Escherichia coli expression system, also gave a single band at the same molecular size (lane 6), while the chimeric recombinant protein containing a MdsD domain attached to GST gave the expected band at 41 kDa (lane 7).

Fig. 4 shows the sequences of three mono-sulfated mucin oligosaccharide chains. It indicates the sites at which seven of the nine bacterial enzymic activities, measured in cell extracts during the present research, might act. The interpretation of the data depends on the ability of the artificial substrates used to model the sugars and glycosidic linkages in mucins. The list of possible hydrolytic enzymes is not exhaustive, and the existence or possibility of other enzymes has been reviewed elsewhere [1].

Two of the substrates, PNP-β-d-galactoside-6-sulfate and PNP-β-d-galactoside-3-sulfate, have not been used previously for measuring the predicted desulfation of the 3-sulfated and 6-sulfated galactoses that can occur at the end of certain sulfomucin chains. Galactose-3-sulfatase has not been previously documented in mucin-degrading...
bacteria, though [3H]lactitol-6'-sulfate has been used as a substrate to measure galactose-6-sulfatase [28]. Activities against both sulfated galactoses were found in cell extracts from Prevotella and Bacteroides, though at much higher levels in the latter. Proof that these enzymes specifically desulfate sulfomucin must await their purification and subsequent testing against suitable isolated mucin preparations. Both galactose-3-sulfate and galactose-6-sulfate have been found in mucin from colon cancer cell lines [26,29].

The activities of glycosidases, measured with artificial substrates, cannot be used to determine whether they are rate-limiting, as this will depend on their affinities and maximum activities against physiological substrate (mucin). In addition, Salyers et al. [12] have noted that there is often more than one protein catalysing a single enzymic step during polysaccharide degradation by the predominant Gram-negative bacteria found in the colon. If this is also the case during mucin degradation, then measured activity will be a function of one or more proteins. What can be deduced from the results in Table 1 is that activities are present which potentially represent hydrolytic enzymes involved in mucin degradation. Also, in the case of Prevotella strain RS2, the β-galactosidase, α-N-acetylgalactosaminidase, β-N-acetylgalactosaminidase, glucose-6-sulfatase (N-acetylglucosamine-6-sulfatase) and neuraminidase were significantly increased during growth on mucin compared to galactose. No statistically significant increases in enzyme activities were found for the B. fragilis extracts.

Because the mucin-desulfinating sulfatase MdsA in Prevotella strain RS2 was inducible, we looked for other genes downstream of mdsA that might be coexpressed. Two ORFs coding in the same direction as mdsA were found, followed by a putative RNA polymerase transcription-termination sequence. They were designated mdsC and mdsD. The sequences of the predicted proteins were determined. The codon usage pattern was similar in the three proteins, which is evidence that the sequences were correct.

The putative protein MdsC is probably a cytoplasmic protein, since it had no signal sequence. At present its role is unknown. The putative protein MdsD is likely to be transported as it contains a signal sequence, and was localised by Western analysis to a membrane-associated fraction of the cell. A role for MdsD cannot be assigned presently, though its coexpression with the sulfatase, MdsA, and induction by growth on mucin, indicates a probable function in this area of metabolism. The proposed β-propeller structure, by analogy with similar structures, is likely to coordinate interactions with other proteins or small ligands, but is unlikely to have a catalytic function [22]. Very few β-propeller regions have been found in prokaryotes. The putative PDZ domain suggests interaction of this region of MdsD with a carboxyl terminal peptide or a β-finger region from another protein, or binding to other PDZ domain(s) to form a complex. The nature of this interaction is again not yet determined. Possible roles for MdsD might be transport of mucin degradation products, binding of mucin substrate or product, or a role as a core protein in a multi-enzyme complex, but such speculation based on epitope sequence homology is tentative.

The three genes mdsA, mdsC and mdsD are coexpressed, as judged from sequence data, and Northern and Western analyses. A scheme consistent with the data to date is drawn in Fig. 5, outlining a predicted small operon of genes coexpressed during mucin desulfation. The gene upstream from the sulfatase, mdsB, is expressed from the opposite DNA strand, and is thought to encode a protein catalysing post-translational modification of a group at the active centre of the sulfatase [15]. The sequence containing mdsA, mdsC and mdsD is the first operon of genes associated with mucin degradation to be proposed.

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References


