The human gut microbe *Bacteroides thetaiotaomicron* encodes the founding member of a novel glycosaminoglycan-degrading polysaccharide lyase family PL29

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Running title: A novel glycosaminoglycan-degrading enzyme family (PL29)

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Abstract

Glycosaminoglycans (GAGs) and GAG-degrading enzymes have wide-ranging applications in the medical and biotechnological industries. The former are also an important nutrient source for select species of the human gut microbiota (HGM), a key player in host-microbial interactions. How GAGs are metabolized by the HGM is therefore of interest and has been extensively investigated in the model human gut microbe *Bacteroides thetaiotaomicron*. The presence of yet uncharacterized GAG-inducible genes in its genome and that of related species however, is testament to our incomplete understanding of this process. Nevertheless, it presents a potential opportunity for the discovery of additional GAG-degrading enzymes. Here, we investigated a gene of unknown function (BT_3328) from the chondroitin sulfate (CS) utilization locus of *B. thetaiotaomicron*. NMR and UV spectroscopic assays revealed that it encodes a novel polysaccharide lyase (PL), hereafter referred to as BtCDH, reflecting its source (*B. thetaiotaomicron* or *Bt*) and ability to degrade the GAGs CS, dermatan sulfate (DS) and hyaluronic acid (HA). When incubated with HA, BtCDH generated a series of unsaturated HA sugars including Δ⁴⁵UA-GlcNAc, Δ⁴⁵UA-GlcNAc-GlcA-GlcNac, Δ⁴⁵UA-[GlcNAC-GlcA]₂-GlcNac and Δ⁴⁵UA-[GlcNAC-GlcA]₃-GlcNac as end products and hence was classed as endo-acting. A combination of genetic and biochemical assays revealed that BtCDH localizes to the cell surface of *B. thetaiotaomicron* where it enables extracellular GAG degradation. BtCDH homologues were also detected in several other HGM species and we therefore propose that it represents the founding member of a new polysaccharide lyase family (PL29). The current discovery also contributes new insights into CS metabolism by the HGM.

Introduction

Mammalian glycosaminoglycans (GAGs) are complex carbohydrates consisting of repeating disaccharide units of uronic acids or galactose linked to a hexosamine sugar (1, 2). These include chondroitin sulfate (CS) made up of glucuronic acid (GlcA) and N-
acetylgalactosamine (GalNAc), dermatan sulfate (DS) or chondroitin sulfate B (CSB) made up of iduronic (IdoA) acid and GalNAc, hyaluronic acid (HA) made up of GlcA and N-acetylglucosamine (GlcNAc), heparin/heparan sulfate (HS) made up of a mixture of GlcA and IdoA linked to GlcNAc and keratan sulfate (KS) made up of galactose (Gal) linked to GlcNAc and keratan sulfate (KS) made up of galactose (Gal) linked to GlcNAc. GAGs such as CS, DS and HS are also characterised by highly diverse sulfation patterns (introduced during biosynthesis by specific sulfotransferase enzymes) which define various molecular subtypes of each glycan (3, 4). CS for example can be sulfated on GalNAc at different positions including C4, C6 or both to yield major CS subtypes namely CSA, CSC and CSE respectively (5, 6) or at both C2 of GlcA and C6 of GalNAc yielding CSD (7). In contrast, HA is not sulfated and hence less structurally complex compared to the other GAG types.

GAGs are ubiquitously distributed in the human body and implicated in several important physiological processes such as cell signalling, inflammation, neuronal development, adhesion and tumour progression (8-11). Dietary GAGs are also a nutrient source for the human gut microbiota (HGM) which is known to impact greatly on human health and disease status (12-17). Indeed recent evidence suggests that CS is a priority nutrient for prominent gut microbes such as B. thetaiotaomicron and B. ovatus and that only a subset of HGM species are capable of metabolising the GAG (13, 15, 17). Understanding how GAGs are metabolised could therefore inform strategies including nutrient-based approaches to manipulate the HGM and hence improve human health. In line with this, several studies (reviewed in Ndeh et al., 2018 (18)) have examined the metabolism of dietary GAGs by the HGM. The results demonstrate that GAG degradation is largely orchestrated by a combination of three major enzyme types including polysaccharide lyases (PLs, EC 4.2.2.-), sulfatases (EC 3.1.6.-) and glycoside hydrolases (EC 3.2.1.-). In the model human gut microbe B. thetaiotaomicron as well as other gut Bacteroidetes, the genes encoding these enzymes are typically located within polysaccharide utilization loci (PULs)(14, 19-21). These genetic loci are diverse and encode a variety of cell-envelop associated multiprotein systems involved in complex glycan metabolism. Although the mechanisms of various GAG PULs in these organisms have been extensively investigated, knowledge of their functioning is incomplete, evident by the presence of yet uncharacterised GAG-inducible genes within these PULs (21).

In the CS PUL of B. thetaiotaomicron, three of such genes BT_3328, BT_3329 and BT_3330 (all of unknown function) appear in a distinct operon (BT_3328-30) conserved in several HGM Bacteroidetes (14, 20, 21). The latter suggests that they might be an important adaptation for CS metabolism in these microbes. Here, we characterised the largest member of this operon (BT_3328), revealing that it encodes a novel polysaccharide lyase hereafter referred to as BtCDH, reflecting its source (B. thetaiotaomicron or Bt) and ability to degrade the GAGs CS, DS and HA. We also provide evidence suggesting that BtCDH is not only the founding member of a novel enzyme family but also a cell surface GAG degrading enzyme, the first to be reported in B. thetaiotaomicron and related species. Taken together, our findings contribute new insights into our knowledge of CS metabolism by the HGM, a key player in host-microbial interactions.

Results

In silico data on native BtCDH lyase

The gene encoding native BtCDH lyase (BT_3328 from B. thetaiotaomicron VPI-5482) is 2607 bp in length and has a GC content of 51%. BtCDH is a large protein of 868 amino acid residues, a molecular mass of 96 kDa and an isoelectric pl of 5.33. A majority of the top hits (>50% identity) from a BLASTp search with native BtCDH are hypothetical/putative uncharacterised proteins mainly annotated as DUF4955 domain-containing. The DUF4955 domain whose function is unknown is present at the C-terminal of the protein while another
domain DUF4988 is located directly after the putative lipoprotein signal peptide sequence at the N-terminus of the protein (Fig. 1A). Top BtCDH lyase homologues were mostly detected in species from the Bacteroides, Alistipes and Prevotella genera, all highly represented in the HGM. They were also prominent in species from marine environments including Saccharophagus degradans, Zobellia galactinovorans and Vibrio harveyi or Coraliomargarita akajimensis. A phylogenetic tree of selected homologs of BtCDH is presented in Fig. 1B.

Activity of BtCDH

Purified recombinant BtCDH was initially assayed spectrophotometrically using CS from bovine trachea (which is mainly CS-4 sulfate or CSA (22)) (Fig. 2A) as substrate. BtCDH degraded CSA generating unsaturated products which were detected by thin layer chromatography (TLC) and high performance anion exchange chromatography (HPAEC) using a UV detector at 235 nm (A235) (Fig. 2B and 2C respectively). Limit products of the reaction were also resolved by size exclusion chromatography (SEC) and analysed by 'H NMR (Fig. 2D, Supplemental Fig.1). Distinct resonance signals were detected at 5.9 and 5.2 ppm in the spectra of the digested products as opposed to undigested CSA. The former is evidence of a H4 proton chemical shift consistent with glycosidic bond cleavage and the generation of C4,5 double bonds (Δ4,5) in the terminal non-reducing end GlcA or uronic acid (UA) residue (Δ4,5-UA) of various products, while the latter peak at 5.2 ppm corresponds to resonance signals of the H1 anomeric proton of the same sugar (Fig. 2D), (23-26).

To explore the substrate specificity in BtCDH in more detail, the enzyme was also tested against a series of commercially available uronic acid-based substrates including CS from shark cartilage (mainly CS-6 sulfate or CSC), DS from porcine intestinal mucosa (mainly DS-4 sulfate), high and low molecular weight HA sodium salt from Streptococcus equi (10,000-30,000 (HA_L) and 90,000-110,000 Da (HA_H) respectively), heparin, alginate, ulvan and polygalacturonic acid. BtCDH was active against CS DS and HA (in the order CS>C>CSA/HA_L>DS>HA_H) but not heparin, alginate, ulvan and polygalacturonic acid (Table 1). Previous studies analysing CSA and CSC have reported major differences in the sulfated disaccharide composition of both substrates with the most prominent being the levels of the ΔUA-GalNAc4S which is higher in CSA and ΔUA-GalNAc6S which is higher in CSC (12, 22, 27, 28). BtCDH activity thus appears to be affected by the type of sulfation in the GAG substrates.

Degradation pattern and mode of BtCDH lyase activity

To determine whether BtCDH was endo or exo-acting, we performed time course experiments with HA_L, a less chemically complex GAG compared to its sulfated counterparts. HA_L was incubated with BtCDH over different times (0, 1, 5, 60 min and ~12 h (ON) after which reactions were stopped by boiling and analysed by TLC. BtCDH degraded HA_L within 10 min generating a fast migrating band (Band A), (Fig. 3A). After 1 h of the reaction, the original HA_L spot at the origin disappeared accompanied by the appearance another major band (Band B) as well as low staining intermittent bands (Fig. 3A). The pattern of products remained unchanged even after overnight digestion and hence the generated sugars represented the final limit products of HA_L digestion. Bands A and B limit products were purified by SEC and analysed by mass spectrometry (HILIC LC-MS). Purified Band A yielded incremental masses of 380.117, 381.120 and 382.123 Da (differing by a mass of 1 Da) equivalent to the mass of singly charged ions of the unsaturated Δ4,5-UA-GlcNAc disaccharide, while band B yielded incremental masses of 759.227, 759.729 and 760.230 Da (differing by a mass of 1/2 Da) etc (Fig. 3B) equivalent to the mass of doubly charged fragments derived from the unsaturated Δ4,5-UA-[GlcNAc-GlcA]7-GlcNAc octasaccharide parent ion. To detect underrepresented sugars, all SEC fractions were pooled in one sample and
analysed. The spectra revealed the presence of new products with incremental masses of 759.226, 760.230 and 761.232 (differing by a mass of 1 Da) and 1138.337, 1139.340 and 1140.344 (differing by a mass of 1 Da) corresponding to singly charged ions of the unsaturated $\Delta^4$UA-GlcNAc-GlcA-GlcNAc tetrasaccharide and $\Delta^4$UA-[GlcNAc-GlcA]$_2$-GlcNAc hexasaccharide sugars respectively (Fig. 3B). The generation of diverse molecular weight limit products thus suggests that BtCDH is an endo-acting enzyme. In addition, the accumulation of the high molecular weight HA octasaccharide suggests that BtCDH preferentially cleaves sugars with a degree of polymerisation (DP) typically greater than or equal to 10 (≥DP10). The requirement for substrates with a DP≥10 was also demonstrated using sulfated CS oligosaccharides as substrates with HPAEC data showing a major shift in the DP10 oligosaccharide profile (as opposed to a minor shift for DP8) after treatment with BtCDH (Fig. 4). The proportion of each HA product generated was also determined by UV with the results showing that over 70% of the generated products were $\Delta^4$UA-GlcNAc disaccharides (Supplemental Fig. 2). Time course experiments with sulfated GAGs CSA and CSC as substrates also yielded fairly similar patterns observed for HA$_L$ (Supplemental Fig. 3). As with HA$_L$, high amounts of the basic disaccharide building block ($\Delta^4$UA-GalNAc) were detected suggesting high processivity in the enzyme.

**Impact of Temperature, pH and metals on BtCDH**

The effects of temperature, pH and metal ions on BtCDH activity were tested with CSA as substrate. BtCDH preferred higher reaction temperatures with the activity increasing linearly from 20 °C to a maximum rate of about 15 $\mu$M/min at 60 °C (Fig. 5A). After this optimum temperature, the enzyme activity dropped sharply. To determine the optimum pH of BtCDH, enzyme activity was examined against CSA in different buffers over a pH range of 4 to 9. BtCDH activity rose gradually with increase pH between 4 and 6 and then sharply to an optimum pH of 7.0 in 10 mM MES buffer (Fig. 5B). BtCDH activity against CS was also tested in the presence of 5 mM EDTA and metal ions including Mg$^{2+}$, Ca$^{2+}$, Mn$^{2+}$, Co$^{2+}$ and Zn$^{2+}$ and Ni$^{2+}$. Its activity was only slightly affected by metals with the most inhibition (27.42%) observed in the presence of Co$^{2+}$ions (Fig. 5C).

**Growth experiments**

A genetic mutant ΔBtCDH lacking the entire ORF for BtCDH (the BT_3328 gene) was created by counter selectable allelic exchange. Growth experiments with both *B. thetaiotaomicron* wild type (WT) and ΔBtCDH strains were carried out with four different GAG substrates CSA, DS, CSC, HA$_L$ and glucose as sole carbon source for at least 24 h. *B. thetaiotaomicron* WT and ΔBtCDH cells grew at a very similar rates on various substrates except for CSC where a minor defect in late phase growth was observed (Supplemental Fig. 4).

**Polyclonal antibodies and cellular localization assays**

**Polyclonal antibodies to BtCDH detect two forms of the protein:** Rabbit anti-BtCDH polyclonal antibodies where generated using the recombinant BtCDH protein as immunogen. On western blots containing cell free extracts of *B. thetaiotaomicron* grown on CSA, two immunosensitive bands (BtCDH$_L$ and BtCDH$_H$ of high and low molecular weights respectively) were detected (Fig. 6A). Despite its poor enrichment relative to BtCDH$_L$, BtCDH$_H$’s molecular weight was close to 96 kDa corresponding to the mass of native BtCDH while the mass of BtCDH$_L$ was about 30 kDa. Interestingly, when cell extracts of the ΔBtCDH strain lacking BtCDH were tested with the same antibodies, both bands were not detected, suggesting that BtCDH$_L$ is a subcomponent possibly a truncated form of BtCDH (Fig. 6A top). To confirm this, BtCDH was FLAG-tagged (BtCDHFLAG) by genetically introducing a FLAG peptide sequence (DYKDDDK) at the C-terminal of the protein, thus allowing for its
results showed degradation of BtCDH$_H$ was detected by immunoblotting suggesting BtCDH$_L$ was an N-terminal truncated version of BtCDH.

**Proteinase K, immunofluorescence and whole cell assays:** As earlier indicated BtCDH contains an N-terminal lipidation sequence (Fig. 1A) and hence is potentially localized extracellularly at the cell surface of *B. thetaiotaomicron*. To confirm this experimentally, we performed proteinase K (PK) and immunofluorescence assays. *B. thetaiotaomicron* WT cells were grown to exponential phase in 1% CSA, harvested and treated with 0.5 mg/ml PK. Treated and untreated cells (controls) were lysed under reducing conditions and cell extracts analysed by western blotting using anti-BtCDH antibodies. The results showed degradation of BtCDH$_L$ and BtCDH$_H$ in PK treated but not control cells (Fig. 6B). The experiment was also controlled by immunoblotting the same extracts with polyclonal antibodies targeting the constitutively expressed periplasmic heparin lyase BT4657 (16) whose bands remained intact in test and control extracts (Fig. 6B). The data thus suggests that BtCDH is localized to cell surface of *B. thetaiotaomicron*. The cell surface localization of BtCDH was finally confirmed through immunofluorescence assays using anti-BtCDH antibodies (Fig. 6C). We also performed whole cell aerobic assays on *B. thetaiotaomicron* WT and ΔBtCDH following growth on CSA. Under these conditions, transport across the outer membrane is limited and only surface enzyme activity is detected. Cells were harvested at exponential phase and re-incubated with CSA and CSC for 2 h and the reaction examined by TLC. The results showed extensive degradation of both CSA and CSC by the wild-type strain, visible as a smear of products (Fig. 6D). This was significantly reduced in the knockout strain especially for the CSA substrate where just a single band of generated product was observed (Fig. 6D). Supernatants at exponential phase were also analysed for secreted BtCDH by western blotting with polyclonal rabbit anti-BtCDH antibodies but only a faint band of the truncated form BtCDH$_L$ was detected (Supplemental Fig 5). Finally culture supernatants from *B. thetaiotaomicron* WT and ΔBtCDH at exponential phase were tested for secreted activity but the TLC profiles of the treated and untreated CS substrates were very similar, except for a major band initially present in the supernatants (Supplemental Fig. 5B, C, D). We thus conclude that BtCDH activity is mainly present at the cell surface of *B. thetaiotaomicron*.

**Discussion**

In this study, we discovered and characterised the founding member of a novel family of GAG lyases from the prominent gut bacterium *B. thetaiotaomicron*. BtCDH is encoded by a gene from an operon (BT3328-30) of unknown function within the CS PUL of *B. thetaiotaomicron* (14) and hence until now, its function was unknown. We show that BtCDH exhibits polysaccharide lyase activity towards a variety of GAGs substrates including CS, DS, and HA. BtCDH alongside previously characterised CS ABC lyases from *B. thetaiotaomicron* WAL2926 (BactnABC) (22) and *Proteus vulgaris* (ChS ABC lyase I and ChS ABC lyase II) (29), named after their ability to degrade CSA, DS or CSB and CSC are the only few lyases known to exhibit such broad GAG substrate specificity. BtCDH however displays very low catalytic efficiency (based on kcat/Km values) when compared to both enzymes. *B. stercoris*, *Pedobacter saltans*, *Flavobacterium heparinum*, *Vibrio sp.* FC509 CS AC lyases degrade a variety of GAGs but are unable to degrade DS (2, 23, 30, 31) while *B. stercoris* ABC and *F. heparinum* ABC lyases which are capable of degrading DS and CS are unable to degrade HA(2). We also show, using HA as substrate that BtCDH exhibits an endo-mode of activity, preferentially targeting oligosaccharides with DP≥10. It may be possible that BtCDH contains over 10 subsites in its active site, however, further 3D structural studies of the enzyme in complex with the substrate (for which
attempts were made in this study but were unsuccessful) will be required to determine this. The enzyme also showed optimum activity at a very high temperature ~ 60 °C, contrary to most PLs with optimum temperatures between 30 - 40 °C (2, 22, 23, 29, 31). A notable exception is the ScPL8H lyase from Streptomyces coelicolor A3 (a non-pathogenic filamentous soil bacterium) with an optimum temperature of 57 °C (32). BtCDH activity was also only slightly affected by EDTA and the divalent metal ions tested in this study. Although it is possible that a metal ion inaccessible to EDTA chelation maybe present and directly or indirectly involved in enzyme activity, the low sensitivity to diverse metals, broad CS substrate specificity and thermophilic nature of BtCDH make it an attractive target from a biotechnological perspective. It is also worth noting that of the 28 PL families known to date, only 3 contain CS lyases (33) and hence BtCDH and family members make an important addition to this group of enzymes in terms of the diversity of enzymatic options available for CS related investigations. The current family has now been designated polysaccharide lyase family 29 (PL29). Its presence in species from diverse environments including soil and marine bacteria also suggests that it may serve as an important adaptation for CS metabolism in these environments.

Another major finding in our study was the extracellular localization of BtCDH in B. thetaiotaomicron. The significance of this discovery lies in the fact that until now, models of CS metabolism did not include extracellular cell surface degradation of the substrate as no such enzymes had been reported in this organism before. In a recent report by Raghavan et al., 2014 and Raghavan and Groisman, 2015 (14, 20), extracellular CS is bound and directly transported by a SusCD-like complex (BT3331-3332) into the periplasmic space without any initial extracellular cleavage. Hence to date, general consensus has held that enzymatic CS degradation begins in the periplasmic space of B. thetaiotaomicron. We not only present evidence of the extracellular localization of BtCDH but show through whole cell assays that B. thetaiotaomicron is capable, with the aid of BtCDH of degrading CS extracellularly. Although our growth data did not show a significant defect in B. thetaiotaomicron’s ability to utilise most of the GAG substrates during growth, extracellular substrate degradation in a complex polymicrobial setting like the HGM could have significant implications for competition or cooperation between HGM species and hence microbial community structure. We also observed that the absence of BtCDH did not completely prevent extracellular degradation of CS in whole cell assays, suggesting the presence of other unknown surface CS degrading enzymes. This was more pronounced in whole cell assays involving CSC where the mutant strain still showed significant CS degradation compared to the wild type. Nevertheless, based on the evidence thus far for BtCDH, we propose a modification of the current model for CS metabolism in B. thetaiotaomicron to include the extracellular CS degrading activity encoded by BtCDH (Fig. 7). In this model, a complexly sulfated CS substrate such as CSC with a high degree of polymerisation (dp≥10) is initially cleaved on the cell surface generating oligosaccharides which are then bound and transported into the periplasmic space for further degradation by periplasmic enzymes as previously described (12, 14, 34) (also see Fig. 7). In future studies, we will also investigate the possibility that extracellular CS oligosaccharides generated by BtCDH e.g. in the case of CSA are the basis of cross-feeding and inter-bacterial interactions which may influence host health.

To conclude, our current findings not only satisfy the growing demand for novel biocatalysts for modern biotechnological and medical research applications but contribute new insights relevant to our understanding of CS or complex glycan metabolism by the HGM, a key player in host-microbial interactions.
Materials and methods

Plasmid Construction and heterologous protein expression

The full length BtCDH gene lacking the N-terminal lipoprotein signal sequence was amplified by PCR from *Bacteroides thetaiotaomicron* VPI-5482 genomic DNA using primers (BtCDH:F:CGCGGGATCCgacgaacgggatgatctg, BtCDH:R:CGCGCTCGAGttatttaagtgcgttgagccat) containing BamHI and XhoI restriction sites respectively. The gene was cloned into pET-28a (+) vector (Novagen) which introduces a C-terminal 6xHis-tag. For this, *E.coli* TOP10 cells (ThermoFisher Scientific) were used. The recombinant construct was sequenced (Eurofins Genomics) and later used to transform *E.coli* BL21 (DE3) expression cells (ThermoFisher Scientific). Cells were cultured in Luria-Bertani (LB) medium containing with 10 µg/ml kanamycin antibiotic to mid-log phase (A600 of 0.6). Protein expression was induced by adding 0.01 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) to cells followed by growth overnight at 16 °C. The next day, cells were harvested by centrifugation (4000 x g) and re-suspended in Talon buffer (20 mM Tris/HCl pH 8.0 plus 100 mM NaCl). Cells were then disrupted and centrifuged (16 000 x g) for 20 min at 4 °C. BtCDH was then purified from the resulting cell free extract by immobilised metal affinity chromatography using fresh Talon resins (Clontech Laboratories Inc.). In the process, the CFE was loaded in a column containing the Talon resin and later washed with Talon buffer. Another wash was performed with Talon buffer containing 10 mM imidazole followed by recombinant protein elution with 100 mM imidazole. Purified proteins were analysed by SDS-PAGE (12.5% gels) under reducing conditions. The analyses revealed a major protein band of about 96 kDa, consistent with the predicted size of BtCDH. Samples were pooled and buffer exchanged into 20 mM NaH₂PO₄ buffer, pH 7.0 or other buffers of choice depending on the intended use.

Enzyme assays and biochemical properties of BtCDH

Activity assays against various GAGs were performed at 37 °C in 20 mM sodium phosphate buffer, pH 7.0. The final reaction volume was 600 µl containing 10 nM of enzyme in all cases except HA (containing 40 nM of enzyme) and varying concentrations of the substrate. Reactions were monitored using a Ultrospec 4000, UV/Visible spectrophotometer at A235. To determine the optimum temperature of BtCDH activity, 40 µM of CSA was digested with 10 nM of BtCDH at different temperatures and the activity monitored. To determine the optimum pH, the same reaction was performed at different pHs with 20 mM of each buffer. For metal dependency assays, various metals (5 mM for Mg²⁺, Ca²⁺, Mn²⁺, Co²⁺ and 1 mM for Zn²⁺ and Ni²⁺) as well as EDTA (5 mM) were included in separate reactions and their effects on enzyme activity tested.

Thin layer chromatography (TLC)

Enzymatic reactions were stopped by heating at 98 °C for 3 mins and later centrifuged at 16 000 x g for 1 min. Each reaction (2 µl drops) was spotted onto a Silicagel 60 TLC plate (Merck) and components resolved in a mobile phase containing butanol/acetic acid/water (2:1:1, v/v). Plates were later dried and developed with orcinol-sulfuric acid reagent (containing 0.1% orcinol and sulfuric acid, ethanol, water in the ratio 3.2:75:21.5 v/v). To visualise sugar bands, plates were gently warmed near a bunsen flame.

Size exclusion chromatography (SEC) of polysaccharides

Samples (5 ml) were resolved using a Bio-Gel® P2 size-exclusion gels packed in Econo-Column® chromatography columns (Biorad). The mobile phase was 50 mM acetic acid and the pump flow rate was set at 0.2 ml min⁻¹. For small scale (200 µl) purifications, elution was achieved by gravity. Fractions were later analysed by TLC.
NMR

Digested products of CSA from bovine trachea were separated by SEC on a Bio-Gel® P2 size-exclusion system (Biorad) and fractions analysed by TLC as described above. Purified fractions were collected and freeze dried at −50 °C using a CHRIST Gefriertrocknung ALPHA 1-2 (Helmholtz-Zentrum) freeze-dryer. Samples were re-dissolved in 0.7 ml deuterium oxide (99.9 atom % D) for NMR analyses. NMR spectra were obtained on a Bruker Avance III HD 700 MHz NMR spectrometer equipped with a TCI cryoprobe. Spectra were acquired at 298K and referenced to Trimethylsilyl propionate (TSP).

High performance anion exchange chromatography (HPAEC)

HPAEC analyses were carried out using an automated Dionex ICS3000 system (Dionex) fitted with Spherisorb®-SAX Columns and linked to an AD25 Absorbance Detector (Dionex). For HA samples, Dionex™ CarboPac™ PA1 columns (Dionex) were used. The mobile phase consisted of 3 M NaCl applied as a 0-100% gradient over 110 min. Data was analysed using the Chromeleon™ chromatography software (Version 6.8) (Dionex).

BtCDH deletion mutant and FLAG-tagging

ΔBtCDH and BtCDHFLAG strains were generated through counter-selectable allelic exchange using the pExchange vector as described in Koropatkin et al., 2008 (35).

Growth of B. thetaiotaomicron WT and mutant strains

B. thetaiotaomicron WT, ΔBtCDH and BtCDHFLAG strains were cultured in 5 ml TYG (tryptone-yeast extract-glucose) medium at 37 °C in an anaerobic cabinet (Whitley A35 Workstation; Don Whitley). The next day 10 μl cells were added to 200 μl of minimal medium (Larsbrink et al., 2014) containing 1% of various GAGs plus 1.2 mg/ml porcine haematin (Sigma-Aldrich). Cell growth was monitored at A600 using a Gen5 v2.0 Microplate Reader (Biotek).

Proteinase K experiments

Experiments were performed as described in Ndeh et al., 2017 (36) with the following modifications; 1) B. thetaiotaomicron WT cells were grown in 5 ml minimal medium containing 1% CSA from bovine trachea to exponential phase (OD600 of 0.8). Final cell pellets were dissolved in 300 μl Laemmli buffer and samples heated for 3 min. For each sample, 10 μl was analysed by SDS–PAGE (7.5%). Immunoblotting was performed with rabbit anti-BtCDH antibodies at a 1:5000 dilution. For BtCDHFLAG cells, a 1:2000 dilution of rabbit anti-FLAG antibodies was used. In all cases, secondary antibodies were Goat Anti-Rabbit IgG Antibody, (H+L) HRP conjugate (Invitrogen) at a 1:4000 dilution. Signals were developed using Clarity™ Western ECL Substrate (Biorad). To detect the native control protein (BT4657), blots were washed overnight in PBS-tween 20 (1x PBS containing 5% tween 20) and re-probed with rabbit anti-BT4657 antibodies (1: 2000) as primary.

Immunofluorescence assays

B. thetaiotaomicron WT, ΔBtCDH and BtCDHFLAG cells were grown to exponential phase (A600 of 0.8) in 5 ml minimal medium containing 1% CSA from bovine trachea. Cells were fixed by adding an equal volume of cell culture to a 1 x PBS solution containing 3.7% formalin and rocking overnight at 4 °C. Cells were harvested by centrifugation at 4000 x g for 5 min and washed in 5 ml PBS three times. Cells (1 ml) was later incubated in blocking solution (2% goat serum, 0.02% NaN3 in 1x PBS) overnight at 4°C while rocking. The next day cells were harvested by centrifugation at 16 000 x g for 1 min and incubated with primary rabbit anti-BtCDH antibodies (1:1000) for 2 h at room temperature. Another PBS wash was performed and secondary Goat anti-Rabbit IgG (H+L) Cross-Adsorbed, Alexa Fluor 488 antibodies (ThermoFisher Scientific (cat. R37116)) added
according to the suppliers instructions. Cells were rocked for 30 min at room temperature and later washed two times in 1x PBS. The final pellet was re-suspended in 100 µl of PBS alongside 20 µM Hoechst 33342 nuclear stain (ThermoFisher Scientific (cat 62249). Samples were mounted on agarose beds (1% w/v in PBS) in Gene Frames (ThermoFisher Scientific (cat. AB0576) and visualised on a Zeiss Axios Observer Zi, with LSM800 confocal scan head. Images were taken using a 63x (N.A. 1.4) oil immersion lens with 5x scan zoom applied. Excitation of the Alexa 488 was performed using the 488 nm laser line, and the reflected light collected on a GaAsp detector between 510-700 nm through the variable beam splitter dichroic block. Excitation of the Hoechst was performed using the 405 nm laser line with emission detected between 400-510 nm. A z-section of 25 images was recorded with an interval of 0.1µm using Zeiss Zen 2.3 software with identical settings for all images. Deconvolution was carried out using Huygens Professional (Scientific Volume Imaging, NL) before a maximum intensity projection was carried out within ImageJ.

Whole cell assays and supernatant analyses

*B. thetaiotaomicron* WT, ΔBtCDH and BtCDHFLAG cells were cultured to exponential phase (A₆₀₀ of 0.8) in 5 ml minimal medium. Cells (1 ml) were harvested at 4000 x g for 5 min and washed once in 1 x PBS by centrifuging again at 4000 x g for 5 min. Cells were then re-suspended in 100 µl of PBS containing 4% of the GAG substrate. Samples were incubated overnight for 2 h followed by centrifugation 4000 x g for 5 min. The supernatants (2 µl) were then analysed by TLC. To test for BtCDH secretion, 10 µl of the *B. thetaiotaomicron* WT supernatant was analysed by western blotting using polyclonal anti-BtCDH antibodies. For this, filtered and unfiltered aliquots including a ten times concentrated aliquot of the former were analysed. Whole cells (10 µl) prepared by resuspending centrifuged cells in 1 ml of PBS were also analysed alongside as controls. To confirm extracellular CS degradation during growth, cells were harvested at exponential phase (16 000 x g 1 min) and supernatant (8 µl) analysed by TLC. Supernatants were also analysed for secreted BtCDH activity. For this, supernatants were collected at exponential and centrifuged at 4000 x g for 5 min to remove cells. This was repeated once and the supernatants finally filtered through a 0.2 µm syringe cap filter (Pall Life Sciences). For each reaction, 20 µl of the supernatant was incubated overnight with 20 µl of 40 mg/ml CS substrate. The next day 4 µl of each sample was analysed by TLC.

Mass spectrometry

After purification of HA oligosaccharides, 200 µl fractions were freeze dried and dissolved in distilled water. Samples were diluted 1:10 (v/v) with buffer B (80% acetonitrile / 20% 50 mM ammonium formate in water, pH 4.7) and 2.0 µL was injected onto an LC-MS instrument configuration comprising a NanoAcquity HPLC system (Waters) and QToF mass spectrometer (Impact II, Bruker). Analysis was performed via gradient elution from a ZIC-HILIC (SeQuant®, 3.5 µm, 200Å, 150 X 0.3 mm, Merck) capillary column heated to 35oC and flowing at 5 µL/min. The elution gradient was as follows; 100% buffer B for 5 min, followed by a gradient to 0% B / 100% buffer A (50 mM ammonium formate in water, pH 4.7) over 40 min. 10 column volumes of buffer B equilibration was performed between injections. MS data was collected in positive ion mode, 50 – 2000 m/z, with capillary voltage and temperature settings of 2800 V and 200 °C respectively, together with a drying gas flow of 6 L/min and nebulizer pressure of 0.4 Bar. The resulting MS data was analysed using Compass DataAnalysis software (Bruker).

Phylogenetic analyses

Sequences were retrieved from the NCBI database (cut-off ≥ 35% identity) from a BlastP search with BtCDH (residues 308-708) as query. After trimming the N and C-terminal DUFs, sequences were submitted to the Phylogeny.fr
server (37) in “one click mode” to generate a phylogenetic tree.

Acknowledgements

This work was supported by a grant from European Union’s Seventh Framework Program (FP/2007/2013)/European Research Council (ERC) Grant Agreement 322820. We would also like to thank Professor Harry Gilbert for the guidance provided during the preparation of this manuscript.

Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

References


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Table 1. Activity of BtCDH lyase against various uronate substrates
Figure 1. Modular architecture of native BtCDH and phylogenetic analyses. A) BtCDH (GenBank ID: AA078434) contains an N-terminal lipoprotein signal peptide sequence (SP) and a large central region sandwiched by two domains of unknown function (DUF4988 and DUF4955). B) Phylogenetic tree showing the distribution of BtCDH homologues in selected species from various environments.
Figure 2. Analyses of chondroitin 4-sulfate (CSA) degradation by BtCDH lyase. A) Chemical structure of intact CSA. B) TLC of CSA before and after digestion by BtCDH lyase. For the reaction, 13.3 mg/ml of CSA from bovine trachea was treated with 0.3 µM of BtCDH lyase overnight. The reaction was stopped by boiling and 53.4 µg of digested CSA analysed by TLC. Lane 1; CSA, Lane 2; CSA treated with BtCDH lyase. C) HPAEC chromatogram of CSA digestion. Half the amount (26.7 µg) of sample in A was analysed by HPAEC. D) 1H-NMR results of CSA digestion by BtCDH lyase. Digested CSA products were purified by SEC and later analysed by NMR. D1) NMR spectrum of undigested CSA. D2) Spectra of species from all SEC fractions combined (fractions 1-43 in supplemental Figure 1). D3) Spectra of species from selected homogenous fractions (pooled fractions 37-39 in in supplemental Figure 1). Both spectra show resonance peaks for H1 and H4 (red and black star) at 5.1 and 5.9 ppm respectively, consistent with the formation of terminal unsaturated CSA oligosaccharides. D4) A typical chemical rearrangement of components of the non-reducing end sugar in CSA after lyase digestion. H4 and H1 are starred red and black respectively.
Figure 3. Evidence of BtCDH endo-lyase activity. A) TLC showing progressive degradation of hyaluronanic acid (HA_L, 10-20 kDa) by BtCDH lyase over time. For the reaction, 32 mg/ml of HA_L was digested with 2 µM of enzyme. The reaction was stopped at various time points indicated and 64 µg of each sample analysed by TLC. B) Mass spectrometry data of purified HA_L oligosaccharide species. B1: Spectrum for Δ^{4,5}UA-disaccharide (Δ^{4,5}UA-Di or [Band A]), B2: Spectrum for Δ^{4,5}UA-octasaccharide (Δ^{4,5}UA-Octa or [Band B]), B3: Spectrum for Δ^{4,5}UA-tetrasaccharide (Δ^{4,5}UA-Tetra), B4: Spectrum for Δ^{4,5}UA-hexasaccharide (Δ^{4,5}UA-Hexa) C) Structural representation of various species detected in B.
Figure 4. Activity of BtCDH against various chondroitin sulfate oligosaccharides. Substrates (1 mg/ml each) were treated with 0.5 µM of BtCDH (shown as DPx +, with x being the number of constituent monosaccharides) overnight or diluted in equivalent volume of buffer as control (DPx). HPAEC chromatograms show major shifts in the signals of sulfated DP10 and DP12 oligosaccharides following treatment, accompanied by the appearance of a strong signals for the Δ⁴,⁵UA-GalNAc disaccharide in each case.
Figure 5. Effect of temperature and pH and metals on BtCDH lyase activity. A) Effect of temperature. Enzymatic activity was measured using 40 µM of CSA as substrate in 20 mM NaH$_2$PO$_4$ at pH 7.0 over different temperatures. B) Effect of pH. BtCDH activity was measured at different pHs using 20 mM of various buffers at 37°C. C) Effect of various metals. Reactions were carried out in 20 mM NaH$_2$PO$_4$ buffer containing 5 mM EDTA, Mg$^{2+}$, Ca$^{2+}$, Mn$^{2+}$, Co$^{2+}$ and 1 mM Zn$^{2+}$ and Ni$^{2+}$ in separate experiments.

Figure 6. Localization of BtCDH and evidence of extracellular chondroitin lyase activity. A) Western blots showing the detection of full and truncated versions of BtCDH (BtCDH$_H$ and BtCDH$_L$ respectively) in B. thetaiotaomicron WT, ΔBtCDH and BtCDHFLAG cell extracts using polyclonal rabbit anti-BtCDH (top) and anti-FLAG Tag antibodies (bottom). B) Western blot analyses of proteinase K treated and control cells using anti-BtCDH antibodies. Top results show loss of BtCDH$_H$ and BtCDH$_L$ after the treatment of whole cells with proteinase K. Bottom results show detection of an intact periplasmic control protein, the BT4657 heparinase of B. thetaiotaomicron (16). C) Immunofluorescence detection of BtCDH in B. thetaiotaomicron WT and ΔBtCDH cells. Top pane shows green fluorescence signals of BtCDH. The middle pane shows blue signals from stained nuclei. Bottom pane is a merger of both signals. D) Evidence of extracellular CS degradation in B. thetaiotaomicron by TLC. Top gel: TLC analyses of CS degradation when whole cells of B. thetaiotaomicron WT, ΔBtCDH and BtCDHFLAG are incubated with CSA. Bottom gel: TLC
analyses of CS degradation when whole cells of *B. thetaiotaomicron* WT, ΔBtCDH and BtCDHFLAG are incubated with CSC

**Figure 7.** Proposed model of chondroitin sulfate (CS) metabolism showing the context of BtCDH activity. BtCDH orchestrates extracellular degradation of sulfated CS (≥dp10) to generate oligosaccharide products which are imported through an outer membrane protein complex (encoded by BT_3331 and BT_3332 genes) into the periplasmic space. These are further degraded by periplasmic sulfatases, lyases and unsaturated glycoside hydrolase enzymes yielding end products 5-keto, 4-deoxyuronate (kdu) and GalNAc which can be metabolised to provide energy for cell growth. Disaccharide intermediates generated during the process serve as signalling molecules activating expression of locus genes through binding to a hybrid two-component sensor protein (encoded by BT_3334) when CS is present.
The human gut microbe *Bacteroides thetaiotaomicron* encodes the founding member of a novel glycosaminoglycan-degrading polysaccharide lyase family PL29

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The human gut microbe *Bacteroides thetaiotaomicron* encodes the founding member of a novel glycosaminoglycan-degrading polysaccharide lyase family PL29

Didier Ndeh\(^1\), Jose Munoz Munoz\(^2\), Alan Cartmell\(^1\), David Bulmer\(^1, 4\), Corinne Wills\(^3\), Bernard Henrissat\(^5, 6, 7\), Joseph Gray\(^1\)

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Supplemental figure 1. Purification and TLC analyses of end products of chondroitin 4-sulfate (CSA) digestion with BtCDH. CSA was digested overnight and end-products separated by size exclusion chromatography followed by TLC analyses. Aliquots from fractions 1-43 and 37-39 were separately pooled and used for NMR experiments.
Supplemental figure 2. HPAEC analyses of HA degradation by BtCDH. A) HPAEC chromatogram of time course samples. HA (32 mg/ml) was incubated with 2 µM of BtCDH at 37 °C. Reactions were stopped at various time points indicated and 80 µg of digested HA analysed by HPAEC linked to a UV detector. B) Scatter plot showing the percentage of each product generated from the complete digestion of HA with BtCDH. Data was obtained by measuring the peak area for each product at the end of the reaction and expressing it as a percentage of the total. $\Delta^{4,5}$UA-Di: $\Delta^{4,5}$UA-disaccharide, $\Delta^{4,5}$UA-Tetra: $\Delta^{4,5}$UA-tetrasaccharide, $\Delta^{4,5}$UA-Hexa: $\Delta^{4,5}$UA-hexasaccharide, $\Delta^{4,5}$UA-Octa: $\Delta^{4,5}$UA-octasaccharide
Supplemental figure 3. Time course degradation of chondroitin sulfate by BtCDH lyase. For each sample, 13.3 mg/ml of substrate was treated with 0.3 µM of BtCDH. The reaction was stopped at various time points indicated and 53.4 ug of digested substrate analysed by TLC. UAG: Δ4,5 UA-GalNAc disaccharide. Ctrl : Substrate without enzyme.
Supplemental figure 4. Growth curves of *B. thetaiotaomicron* WT and ΔBtCDH strains on various GAG substrates. Cells were grown in minimal medium containing 1% final concentration of each GAG substrate as sole sources of carbon. Bt-wt; *B. thetaiotaomicron* WT, BtΔBtCDH; *B. thetaiotaomicron* ΔBtCDH mutant, CSA; chondroitin sulfate from bovine trachea, DS; dermatan sulfate from porcine intestinal mucosa, CSC; chondroitin sulfate from shark cartilage and HA_L; Hyaluronic acid sodium salt from *Streptococcus equi*.
Supplemental figure 5: Analyses of culture supernatants of *B. thetaiotaomicron* WT, ΔBtCDH and BtCDHFLAG for secreted enzyme activity  

A) Analyses of supernatants by western blotting. Culture media at exponential phase growth in CSA were collected and centrifuged. The resulting supernatant (Sup1) was filtered to remove any remaining cells (Sup2) and later analysed by Western blotting using polyclonal rabbit anti-BtCDH antibodies. Lysates from whole cells (WC) and Sup2 samples concentrated ten times (10x Sup2) were also analysed.  

B) Comparison of growth supernatants from cultures of *B. thetaiotaomicron* WT (Sup_WT), ΔBtCDH (Sup_ΔBtCDH) and BtCDHFLAG (Sup_BtCDHFLAG) in CSA (10 mg/ml). Each supernatant (8 ul) at exponential phase alongside a control CSA medium (ME) which was not inoculated with any cells was analysed by TLC.  

C) Examination of growth supernatants for secreted enzyme activity. Supernatants (20 µl) obtained from cultures of *B. thetaiotaomicron* WT and ΔBtCDH in CSA were incubated overnight with 20 µl of 40 mg/ml CS substrate. The next day 4 µl of each sample was analysed by TLC.