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Structural and mechanistic insights into a *Bacteroides vulgatus* retaining *N*-acetyl- β -galactosaminidase that uses neighboring group participation

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***Bacteroides vulgatus* is a member of the human microbiota whose abundance is increased in patients with Crohn's disease. We show that a *B. vulgatus* glycoside hydrolase from the carbohydrate active enzyme family GH123, BvGH123, is an *N*-acetyl- β -galactosaminidase that acts with retention of stereochemistry, and through a 3-D structure in complex with Gal-thiazoline, provide evidence in support of a neighbouring group participation mechanism.**

The human gastrointestinal tract undergoes a constant process of renewal, leading to production of 5×10^7 cells per minute and the discharge of 250 g of cells per day.¹ Colonocytes derived from the epithelia of the large intestine can be isolated at concentrations of up to 10^7 per gram of wet fecal material,² and are an important nutrient source for the gut microbiota. A wide range of carbohydrates are represented on intestinal epithelial cells, including polysaccharides, glycoproteins and glycolipids. *B. vulgatus* is a widespread member of the human microbiota, and is generally considered beneficial, although it is an occasional opportunistic pathogen. *B. vulgatus* is typically present at levels of around 4% of bacteria in the gut microbiota, but can rise to constitute >40% of the gut microflora in Crohn's disease patients.³ *B. vulgatus*, like other *Bacteroides* spp., possesses specialized polysaccharide utilization loci (PULs) encoding complex enzymatic machineries able to degrade and harness a wide variety of complex dietary polysaccharides along with SusC/SusD orthologues capable of importing oligosaccharide fragments into the periplasm.⁴ In addition, *B.*

vulgatus and other members of the Bacteroidetes have specialised loci for the degradation of intestinal mucosal glycoproteins and the glycocalyx.⁵ However, it appears that the ability of *Bacteroides* spp. to degrade host glycoconjugates is also enabled by enzymes encoded outside of the PULs. *B. vulgatus* encodes a single copy of an enzyme from glycoside hydrolase family GH123;⁹ a poorly characterized family with an emerging ability to degrade host-derived glycoconjugates. In this work we demonstrate that previously uncharacterized protein BvGH123 is a *N*-acetyl- β -galactosaminidase. We experimentally demonstrate for the first time that a member of this family hydrolyses glycosides with retention of anomeric stereochemistry, and provide evidence for a neighbouring group participation mechanism through an X-ray crystal structure in complex with an inhibitor that mimics the proposed intermediate of the reaction mechanism.

Previous studies of GH123 members, initially NagP from *Paenibacillus* sp.,⁶ and CpNga123 from *C. perfringens* (whose structure was recently solved)⁷ have led to the proposal of a two-step neighbouring group participation mechanism for catalysis (Figure 1). According to such a mechanism, the enzyme possesses two key active site residues acting in the role of acid/base and transition state stabilization.⁸ In the first step, nucleophilic attack by the 2-acetamido group oxygen on the anomeric centre, assisted by charge stabilization and orientation by the transition state stabilizing residue, occurs simultaneously with protonation of the glycosidic oxygen by the acid/base residue to assist leaving group departure, resulting in the formation of an oxazolinium ion. In the second step, the acid/base residue acts as a base to assist in deprotonation of a nucleophilic water molecule, while the stabilizing residue facilitates ring-opening of the oxazolinium ion intermediate.⁹ Evidence in support of this mechanism was initially based on the nanomolar inhibition of NagP by Gal-thiazoline;⁶ an inhibitor that mimics the proposed oxazolinium ion intermediate (more formally a closely-related transition-state),¹⁰ along with the proposal of a catalytic diad of adjacent carboxyl residues reminiscent of those seen in β -hexosaminidases of GH20 and GH84, which are known to act through a

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neighbouring group participation mechanism.^{11,12} Recently, the first X-ray structure of a GH123 enzyme, *CpNga123*, in a product complex and a pseudo-Michaelis complex of an inactive mutant with substrate confirmed the identity of the catalytic diad, and revealed binding orientations of the 2-acetamido group that while not oriented directly below C1, was positioned in a manner consistent with that needed for neighbouring group participation.⁷

Fig. 1 Neighbouring group participation mechanism by a retaining *N*-acetyl- β -hexosaminidase. Hydroxyl groups have been omitted for clarity.

To further investigate the mechanism of the GH123 galactosaminidases, residues 20–582 of ABR39589.1, encoding BVU_2198, without its signal peptide (hereafter *BvGH123*) was expressed in *E. coli* (Figure S1). Size exclusion chromatography–multi-angle laser light scattering revealed *BvGH123* to be a dimer in solution. The enzyme displays a pH optimum of 5 using 4-nitrophenyl *N*-acetyl- β -D-galactosaminide (PNPGalNAc, **1**) as a substrate (Figure S2a). The kinetic preference for β -galactosaminides versus β -glucosaminides was determined using PNPGalNAc and 4-nitrophenyl *N*-acetyl- β -D-glucosaminide (PNPGlcNAc). For PNPGalNAc, $k_{\text{cat}}/K_M = 8.0 \pm 0.4 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ and for PNPGlcNAc, $k_{\text{cat}}/K_M = 3.8 \pm 0.6 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$; saturation could not be achieved, preventing the accurate determination of k_{cat} and K_M values. These data indicate a 21-fold preference for β -galactosaminides, consistent with previous reports on NagP⁶ and *CpNga123*,⁷ providing further evidence that the enzymes of this family are dedicated *N*-acetyl- β -galactosaminidases.

Previous studies have only speculated on the stereochemical outcome of substrate hydrolysis, which is of obvious importance for proposal of a feasible enzymatic mechanism.¹³ We used ¹H NMR spectroscopy to monitor the stereochemical outcome of the enzyme-catalyzed hydrolysis of PNPGalNAc (**1**) (Figure 2). Upon addition of enzyme, rapid consumption of substrate led to the production of a new product, with an anomeric signal at δ 4.63 ppm that displayed a large ($J = 8 \text{ Hz}$) coupling constant. Over time, mutarotation led to the formation of a mixture of the first compound, and a new compound with an anomeric signal at δ 5.22 ppm characterized by a small ($J = 4 \text{ Hz}$) coupling constant. These experimental data are consistent

with the initial formation of β -GalNAc (**2 β**), revealing that *BvGH123*, and by inference all GH123 members, act with a net retention of anomeric configuration. Using isothermal titration calorimetry, Gal-thiazoline¹⁴ was shown to bind *BvGH123* with $K_D = 9.6 \text{ nM}$ (Figure S2b). Collectively, these results support previous suggestions that enzymes of family GH123 use a neighbouring group participation mechanism involving the 2-acetamido group.

Fig. 2 Stacked ¹H NMR spectra showing time course of *BvGH123* hydrolysis of PNPGalNAc. Substrate hydrolysis results in initial release of β -GalNAc with retention of anomeric configuration, which then undergoes mutarotation.

Table 1. 3-D structure data for *BvGH123*.

	Native (apo)	<i>BvGH123</i> –GlcNAc	<i>BvGH123</i> –Gal-thiazoline
Space group	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁
Resolution limit	1.85 Å	2.1 Å	2.3 Å
R_{merge}	0.141(1.930)	0.104(1.530)	0.063(1.552)
$I/\sigma I$	9.9(1.2)	12.0(1.2)	15.6(1.1)
Completeness (%)	99.9(100)	100(100)	93.4(63.6)
R.m.s.d bond lengths (Å)	0.016	0.014	0.013
R.m.s.d bond angles (°)	1.70	1.59	1.63

At the time of our structure solution, no GH123 structure had been reported and thus the three-dimensional structure of *BvGH123* was solved by experimental phasing using single anomalous diffraction from a selenomethionine-substituted derivative and subsequently refined against a native dataset to a resolution of 1.85 Å (Table 1, S1). The overall structure reveals a two domain structure with an N-terminal β -sheet domain (residues 1–195) followed by a catalytic (β/α)₈-barrel domain (residues 196–563) (Fig 3a). The latter has three insertions forming a 4-stranded β -wing domain and a C-terminal α -helical cap, as observed recently in the *CpGH123* structure.⁷ Structures of *BvGH123* and *CpNGA123* overlap with an r.m.s.d. of approximately 1.7 Å over 500 residues. Differences include a longer N-terminus for *BvGH123*, and a C-terminal β -strand insertion followed by a longer C-terminal helix for *CpNG123*. Additionally a disulfide bond joining Cys117 and Cys430 is observed only in *BvGH123*, connecting the loop comprising

conformation in which the catalytic residues engage in close contacts with the inhibitor. As the inhibitor is a mimic of the oxazolinium intermediate (or possibly the transition state),¹⁵ these interactions appear to represent those of a catalytically competent form of the enzyme.

Fig. 3 Structures of *Bacteroides vulgatus* GH123 and complexes with Gal-thiazoline and GalNAc. (a) Overview of the *Bv*GH123 structure in complex with Gal-thiazoline, rainbow-coloured from the N- to C-terminus. Gal-thiazoline is shown as space filled van der Waals surface. Views of the active site of *Bv*GH123 in complex with (b) GalNAc and (c) Gal-thiazoline, highlighting the active site residues. The $2mF_o - Df_c$ map for GalNAc in blue is contoured at $0.22 \text{ e}/\text{\AA}^3$ (1σ r.m.s.d.) and $0.25 \text{ e}/\text{\AA}^3$ (1.5σ r.m.s.d.) for Gal-thiazoline. The C1-O interaction of the *N*-acetyl group in the GalNAc complex is highlighted with a red dotted line. (d) Interaction plot of Gal-thiazoline within the active site.

The X-ray structures of the inhibitor and product complexes of *Bv*GH123 provide an intimate view of the geometry of the active site. In the *Bv*GH123–Gal-thiazoline complex, which is in a fully closed state, the adjacent carboxyl residues D361 and E362 adopt positions consistent with roles as acid/base and transition state stabilizer (Figure 3c). The *Bv*GH123–GalNAc product complex revealed only a partially closed conformation with W306 nestled above the pyranose and D361 engaging with the acetamido group of GalNAc (Figure 3b). This complex contrasts with that reported for GalNAc bound to *Cp*Nga123, which is in an open conformation (Figure S4).⁷ The pyranose ring of Gal-thiazoline adopts a 4C_1 conformation consistent with an expected ${}^1S_3 \rightarrow {}^4H_3 \rightarrow {}^4C_1$ conformational itinerary for the glycosylation half-reaction.¹⁷ Complexes of *Cp*Nga123–D344N/E345Q with substrate (ganglioside glycans GA2 or Gb4) were observed in a 4C_1 conformation, which is inconsistent with the substrate distortion expected with a pseudo-axial orientation of the anomeric group, as anticipated for stereoelectronic reasons.⁷ Combined with the location of the 2-acetamido group away from C1, the Michaelis complexes obtained with *Cp*Nga123 have been suggested to represent non-catalytically-competent complexes in which the open conformation prevents the active centre residues from fully engaging with the substrate.

Fig. 5 Overlay of BvGH123–Gal-thiazoline active site residues (wheat) with members of other neighbouring group representative enzyme families (GH18, 20, 25, 84, 85). (a) Overlay of family representatives with adjacent catalytic residues: GH20 *Paenibacillus* sp. β -HexNAcase (purple, PDB: 3SUR),¹⁸ and GH84 *Bacteroides thetaioamicron* O-GlcNAcase (light green, PDB: 2CHN),¹¹

which contain adjacent. (b) Overlay of family representatives with non-adjacent catalytic residues: GH18 *Serratia marcescens* chitinase A (grey, PDB: 2WK2),¹⁹ GH25 *Aspergillus fumigatus* lysozyme (steel blue, PDB: 2X8R),²⁰ and GH85 *Streptococcus pneumoniae* endo-D (plum, PDB: 2W92).²¹

A comparison of the active sites of GH families that are known to utilise a substrate-assisted retaining mechanism, which includes GH18, 20, 25, 84 and 85, reveal a remarkable spatial conservation of the catalytic residues located on the fourth β -strand of the respective $(\beta/\alpha)_8$ -barrel (Figure 4). Families GH20 and 84, like GH123, have the catalytic acid/base and stabilizer adjacent in their protein chains, and have essentially identical arrangements of these groups and the linking peptide backbone. Families GH18, 25 and 85 have a one residue insertion between the catalytic residues, yet still adopt a remarkably similar spatial arrangement. Likewise the active site closure motion now observed for two GH123 enzymes is also seen for a GH20 hexoaminidase and a GH84 O-GlcNAcase.^{7,12,22} These hexosaminidase families have little or no sequence conservation yet display similar folds and arrangement of catalytic residues and use a neighbouring group participation mechanism, suggesting a common solution to the chemical problem of hydrolysis of *N*-acetyl- β -hexosaminides.²³

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Notes and references

Electronic supplementary information (ESI) available: SI Figures, cloning, expression, biochemistry, and crystallography methods. See DOI: xxxxxxxxxx

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