# A single sulfatase is required to access colonic mucin by a gut bacterium

Ana S Luis, Chunsheng Jin, Gabriel Vasconcelos Pereira, Robert W P Glowacki, Sadie R Gugel, Shaleni Singh, Dominic P Byrne, Nicholas A Pudlo, James A London, Arnaud Baslé, et al.

# A single sulfatase is required for metabolism of colonic mucin *O*-glycans and intestinal colonization by a symbiotic human gut bacterium

*[1,2]Ana S. Luis, [2]Chunsheng Jin, [1]Gabriel Pereira, [1]Robert Glowacki, [1]Sadie Gugel, [1]Shaleni Singh, [3]Dominic P. Byrne, [1]Nicholas Pudlo, [3]James London, [4]Arnaud Baslé, [5]Mark Reihill, [5]Stefan Oscarson, [2]Niclas Karlsson, [4]Patrick Eyers, [6]Mirjam, Czjzek, [6]Gurvan Michel, [6]Tristan Barbeyron, [3]Edwin A Yates, [2]Gunnar C. Hansson, *[,3]Alan Cartmell, *[1]Eric C. Martens

[1]*Department of Microbiology and Immunology, University of Michigan, Ann Arbor, MI 48109, USA*

[2]*Department of Medical Biochemistry, University of Gothenburg, Box 440, 405 30 Gothenburg, Sweden*

[3]*Institute of Systems, Molecular and Integrative Biology, University of Liverpool, Liverpool L69 3BX, United Kingdom*

[4]*Institute for Cell and Molecular Biosciences, Newcastle University, Newcastle upon Tyne, United Kingdom*

[5]*Centre for synthesis and Chemical Biology, University College Dublin, Belfield, Dublin 4, Ireland.*

[6]*Sorbonne Universités, UPMC Univ Paris 06, CNRS, UMR 8227, Integrative Biology of Marine Models, Station Biologique de Roscoff, CS 90074, Roscoff, Bretagne, France.*

*Correspondence to:

emartens@umich.edu

Alan.Cartmell@liverpool.ac.uk

ana.luis@medkem.gu.se

## Summary

Humans have co-evolved with a dense community of microbial symbionts that inhabit the lower intestine. In the colon, secreted mucus creates a physical barrier that separates these microbes from the intestinal epithelium[1]. Some gut bacteria are able to utilize mucin glycoproteins, the main mucus component, as a nutrient source. However, it remains unclear which enzymes initiate the degradation of the highly complex *O*-glycans found in mucins. In the colon, these glycans are heavily sulfated, but sulfatases active on colonic mucins have not been identified. Here, we show that sulfatases are essential to the utilization of colonic mucin *O*-linked glycans by the human gut symbiont *Bacteroides thetaiotaomicron*. We characterized the activity of 12 different sulfatases encoded by this species, showing that these enzymes collectively are active on all of the known sulfate linkages in colonic *O*-glycans and even possess the ability to cleave additional linkages not yet known to occur in host glycans. Crystal structures of 3 enzymes provide mechanistic insight into the molecular basis of substrate-specificity. Unexpectedly, we found that a single sulfatase is essential for utilization of sulfated *O*-glycans *in vitro* and also plays a major role *in vivo*. Our results provide insight into the mechanisms of mucin degradation by a prominent group of gut bacteria, an important process for both normal microbial gut colonization[2] and diseases such as inflammatory bowel disease[3].


## Introduction

The human gut microbiota (HGM) significantly impacts several aspects of intestinal health and disease, including inflammatory bowel disease (IBD)[1] and colorectal cancer (CRC)[2]. In the colon, secreted mucus creates a physical barrier that separates gut microbes from the intestinal epithelium[3] preventing close contact that leads to inflammation and eventual CRC when this barrier is either experimentally eliminated[4,5] or has reduced glycosylation[6-8]. A major component of the colonic mucus is mucin 2 (Muc2), a glycoprotein that contains up to 80% glycans by weight and more than 100 different glycan structures that are *O*-linked to serine or threonine residues[9]. Mucin glycosylation is variable along the gastrointestinal (GI) tract with a marked increase in sulfation in the

colon[10]. In mucins, *O*-linked sulfate may be attached to the 6-hydroxyl of *N*-acetyl-D-glucosamine (6S-GlcNAc) and non-reducing end D-galactose (Gal) sugars at hydroxyl positions 3-, 4- or 6- (3S-, 4S- and 6S-Gal, respectively)[10,11] (**Fig. 1a**). To degrade and utilise colonic mucin *O*-glycans, members of the HGM need to express appropriate carbohydrate sulfatases to remove these modifications, which often occur as terminal capping sulfates that block enzymatic degradation of these sulfated glycans. *Bacteroides thetaiotaomicron* (*Bt*) is a dominant member of the human gut microbiota that is able to utilize *O*-glycans as a sole nutrient source[12]. Underscoring the importance of sulfatases, *Bt* requires active sulfatases for competitive colonization of the wild-type mouse gut[13] and to induce inflammation in genetically susceptible mice[14]. However, the specific sulfatases that mediate these effects remain unknown. Indeed, despite the critical roles of sulfatases in many biological processes, including several human diseases[15], there is a significant knowledge gap regarding the biochemical, structural and functional roles of these enzymes.

We hypothesized that specific *Bt* sulfatases play key roles in initiation of *O*-glycan degradation. To test this, we measured the activities of 23 putative sulfatases, determining that 12 enzymes are active on either model glycan substrates or purified colonic mucin *O*-glycans. Together with defining the positional activities of these enzymes, we determined the corresponding structures for 3 sulfatases, revealing the basis of substrate specificity. Using molecular genetics, we determined the contributions of these sulfatases to *Bt* fitness *in vitro* and *in vivo,* unexpectedly revealing that a single enzyme is essential for utilisation of sulfated mucin *O*-glycans and for competitive gut colonization. Identifying specific bacterial sulfatases that are critical for intestinal mucin degradation will provide potential targets to inhibit with the goal of blocking progression of diseases like IBD and possibly other disorders that result from bacterial disruption of the mucus barrier.

***Utilization of colonic mucins by HGM species***

Several studies have identified HGM members that are able to utilize porcine gastric mucin *O*-glycans (gMO) as a sole carbon source[12,16,17]. However, this substrate does not adequately reflect the structural complexity of mucin *O*-glycans found in the

colon, especially those with increased sulfation[10]. To identify HGM species that utilize sulfated colonic mucins, we measured the growth of 19 *Bacteroides* type strains, plus *Akkermansia muciniphil*a, on highly sulfated porcine colonic mucin oligosaccharides (cMO). We identified six *Bacteroides* strains that utilize cMO (**Fig. 1b, Extended Data Fig. 1**). Interestingly, two known mucin-degraders, *A. muciniphila*[18] and *B. massiliensis*[19], grew robustly on gMO but failed to utilize sulfated cMO to any detectable level (**Extended Data Fig. 1**), highlighting the importance of using colonic mucins as substrates to draw more complete and potentially relevant conclusions about the metabolic potential of these organisms. *Bt*, the bacterium with the highest number of sulfatases (28), was one of the strains with the best growth on cMO (**Fig. 1b**), suggesting that some of these enzymes might play key roles in promoting this ability. Therefore, to understand the role of sulfatases in colonic mucin utilization by HGM bacteria we focused on characterizing *Bt* enzymes. See **Supplementary Discussion 1** for additional details of GI mucins and bacterial growth on them.

***Substrate Specificity of B.thetaiotaomicron sulfatases***

Sulfatases are classified into four main families (S1 to S4) in the SulfAtlas database according to sequence similarity, catalytic mechanism and fold[20]. Family S1, currently divided into 72 subfamilies (designated S1_X), comprises the formylglycine sulfatases which operate via a hydrolytic mechanism that utilises a non-coded formylglycine amino acid as a catalytic residue. In *Bt* and other anaerobic bacteria, this residue is introduced co-translationally by the anaerobic sulfatase maturating enzyme (anSME)[13], which converts a serine or cysteine, within the consensus sequence **C/S**-X-P/A-S/X-R, to formylglycine that then serves as the catalytic nucleophile[21]. The *Bt* genome encodes 28 S1 sulfatases classified into twelve different subfamilies (**Supplementary Table 1**). Four *Bt* sulfatases have been previously characterized and all act on glycosaminoglycans (GAG) that are components of extracellular matrix and glycocalyx (**Fig. 1c**)[22,23]. Interestingly, several of the uncharacterized S1 sulfatases are encoded within polysaccharide utilization loci (PULs) that are known to be upregulated *in vivo* or during growth on gMO[12] and encode other glycoside hydrolases enzymes potentially involved in degrading mucin *O*-glycans (**Extended Data Fig. 2**).

In order to understand the role of sulfatases in mucin metabolism, we cloned and expressed in soluble form 23 of the remaining 24 uncharacterized sulfatases. The recombinant proteins were tested for activity against a panel of commercially available sulfated saccharides (**Supplementary Table 2**). In this initial screen, we identified activities for twelve sulfatases (**Fig. 1c, Extended Data Figs. 3, 4** and **Supplementary Table 3**). Among these, 5 of the enzymes represent the first activities reported for their respective subfamilies: two S1_20 members (BT1636 and BT1622) were determined to target 3S-Gal, with BT1622 also preferentially cleaving 3S-*N*-acetyl-D-galactosamine (3S-GalNAc), two S1_16 enzymes (BT3796 and BT3057) cleave 4S-Gal/4S-GalNAc and one S1_46 enzyme (BT1918) cleaves 3S-GlcNAc, using the *N*-acetyl group as an absolute specificity determinant. This represents the first reports of a bacterial sulfatase active on 3S-GalNAc, indicating that this sulfation could exist as a yet unidentified modification of host glycans. Herein, we refer to these enzymes by their gene/locus tag number with the corresponding activity in superscript (*e.g.*, BT1636$^{3S\text{-}Gal}$).

In addition to discovering new catalytic activities associated with three subfamilies that previously lacked defined members, we also identified sulfatases displaying novel activities inside other subfamilies. These include three S1_15 enzymes (BT1624$^{6S\text{-}Gal/GalNAc}$, BT3109$^{6S\text{-}Gal/GalNAc}$ and BT4631$^{6S\text{-}Gal/GalNAc}$) that extend this family, previously only known to include 6S-GalNAc sulfatases, to those cleaving 6S-Gal. Two members of S1_4 were active on 3S-Gal (BT4683$^{3S\text{-}Gal}$) or 6S-Gal (BT3487$^{6S\text{-}Gal}$) representing novel activities inside this arylsulfatase subfamily. Finally, consistent with the activity previously described for S1_11 members, two enzymes were 6S-GlcNAc sulfatases (BT1628$^{6S\text{-}GlcNAc}$ and BT3177$^{6S\text{-}GlcNAc}$) (**Fig. 1c** and **Extended Data Fig. 3**).

Characterized sulfatases within the same family, with the exception of two S1_4 members, cleaved the same sulfonate linkages (**Fig. 1C**). However, despite these enzymes targeting the same linkages, their optimal activity depends on the surrounding glycan context. The activity of the 3S-Gal sulfatases is dependent on the linkage between Gal and GlcNAc with BT4683$^{3S\text{-}Gal}$ showing a preference for 3'-sulfate-*N*-acetyl-D-lactosamine (3'S-LacNAc, 3'S-D-Gal-β1,4-D-GlcNAc). In contrast, BT1622$^{3S\text{-}Gal/GalNAc}$ demonstrated enhanced activity towards 3'-sulfate-lacto-*N*-biose (3'S-LNB, 3'S-D-Gal-β1,3-D-GlcNAc) (**Extended Data Fig. 4a** and **Supplementary Table 3**). Furthermore,

additional affinity/activity studies revealed that BT1622$^{3S\text{-}Gal/GalNAc}$ preferentially targets GalNAc and not Gal (**Extended data Fig. 4b** and **Supplementary Table 3**), suggesting that this sulfatase evolved to optimally target sulfate *O*3-linked to GalNAc. *Bt* sulfatase activity is also affected by the presence of terminal epitopes such as those that occur in Lewis antigens. Despite BT1636$^{3S\text{-}Gal}$ being equally active on 3'S-LacNAc and 3'S-LNB, this protein has a lower affinity and it is 100-fold less active when L-fucose (Fuc) is linked to GlcNAc (3'S-Lewis$^{a/x}$) (**Extended data Fig. 4a and 4b** and **Supplementary Table 3**). Whilst BT1622$^{3S\text{-}Gal/GalNAc}$ is only weakly active on 3'S-Lewis$^{a}$ antigen and not active at all on 3'S-Lewis$^{x}$; the reciprocal is true for BT4683$^{3S\text{-}Gal}$ (**Supplementary Table 3**). The subfamily S1_15 enzyme BT1624$^{6S\text{-}Gal/GalNAc}$ was only weakly active on 6S-Lewis antigens (**Extended Data Fig. 4a**), suggesting that these enzymes cannot accommodate Fuc linked to GlcNAc and that for both families Fuc needs to be removed prior to sulfate cleavage. Additionally, affinity studies showed that BT3109$^{6S\text{-}Gal/GalNAc}$ has a strong affinity for Gal, whilst the previously characterized GAG sulfatase BT3333$^{6S\text{-}GalNAc}$ showed a preference for GalNAc, suggesting that optimal activity of S1_15 sulfatases likely depends on the glycan context and BT3333$^{6S\text{-}GalNAc}$ evolved to target sulfated linkages in GAGs, a substrate that contains GalNAc but not Gal.

***B.thetaiotaomicron* sulfatase activity on cMO**

We next tested the activity of *Bt* sulfatases on cMO, which we determined to contain at least 131 different oligosaccharides (**Supplementary Table 5)**. Only 4 of the 6 sulfatases tested displayed activity on cMOs (**Fig 2 and** (**Supplementary Table 5)**. BT1628$^{6S\text{-}GlcNAc}$ and BT3177$^{6S\text{-}GlcNAc}$ removed 6-*O*-sulfate from all GlcNAc structures but only when present at the non-reducing end of *O*-glycans confirming an exo-mode of action observed in commercial oligosaccharides (**Fig 2 and Extended Data Fig. 5a**). After incubation with BT1636$^{3S\text{-}Gal}$, we were able to detect 14 new oligosaccharides and an overall increase of non-sulfated structures (**Fig 2, Extended Data Fig. 5c)**. Compared to the non-enzyme treated control, 36 oligosaccharides could no longer be detected after incubation with BT1636$^{3S\text{-}Gal}$ (**Fig 2a and Supplementary Table 5).** We could only determine the structures of 8 of these glycans, however, they all present a terminal 3S-Gal (**Fig. 2)**. This sulfatase was active on 3'S-Gal-β1,3-GalNAc (core 1) and more

complex sulfated structures built around other common core structures (**Fig. 2c)**, indicating that BT1636$^{3S\text{-}Gal}$ evolved to accommodate the various linkages and substitutions found in mucin *O*-glycans. After incubation with BT4683$^{3S\text{-}Gal}$ we failed to detect 9 oligosaccharides, indicating that this enzyme is active on a smaller subset of structures (**Fig. 2a and Supplementary Table 5)**. We were only able to determine the structure of one of those glycans and, unexpectedly, we found that this enzyme is endo-active on sialylated 3S-Gal (**Fig. 2b)**, which is consistent with structural data discussed below. Additionally, the strong activity of BT1636$^{3S\text{-}Gal}$ on higher m/z glycans with unknown structures (**Supplementary Table 5)** reveals that porcine cMOs are highly sulfated at the O3 position of galactose. Although previous studies have reported the presence of 3S-Gal in colonic mucins[24], the analysis of such complex samples is technically challenging and, until now, no precise enzymatic tools were available to probe these linkages. Together, these results represent the first report of HGM sulfatases active on colonic mucin *O*-glycans and highlight the utility of gut bacterial sulfatases as analytical tools in structural characterization of mucin glycans. See **Supplementary Discussion 2** for additional details of sulfatase activity on cMO.

***Structural characterization of 3S-Gal/GalNAc sulfatases***

To further understand the molecular details of carbohydrate recognition by S1 sulfatases, we determined the crystal structures of the three different 3S-Gal sulfatases that belong to two different subfamilies. Consistent with previous structures of S_1 sulfatases, all 3 enzymes display a $\alpha/\beta/\alpha$ topology with a C-terminal sub-domain, and the active site residues interacting with the sulfate group are fully conserved (**Extended data Fig. 6a,b**). The structure of BT1636$^{3S\text{-}Gal}$ in complex with the product LacNAc revealed that His177 coordinates with *O*4 of D-Gal and mutation to alanine ablates enzyme activity, suggesting that this residue is the major specificity determinant for Gal (presenting an axial *O*4) over glucose (equatorial *O*4) (**Fig. 3** and **Supplementary Table 3**). BT1636$^{3S\text{-}Gal}$ also makes strong interactions with O2 via R353 and E334. The essential His177 in BT1636$^{3S\text{-}Gal}$ is highly conserved (92%) within S1_20 sulfatases (**Extended data Figs. 7, 8**). In BT1622$^{3S\text{-}Gal/GalNAc}$, mutation of the corresponding H176 to alanine causes a ~300-fold reduction in activity (**Supplementary Table 3**), further highlighting the importance of

this residue in Gal recognition. In BT1622$^{3S\text{-}Gal/GalNAc}$, R353 and E334 are replaced by C357 and N334, shorter amino acids, that allow the accommodation of a *C*2-linked *N*-acetyl group found in GalNAc (**Fig. 3**). This observation is consistent with the ability of BT1622$^{3S\text{-}Gal/GalNAc}$ to cleave 3S-GalNAc and preferentially bind GalNAc over Gal, whilst BT1636$^{3S\text{-}Gal}$ only recognizes Gal (**Extended Data Fig. 4b,c**). Additionally, consistent with *exo*-activity observed for both enzymes the substrates are buried in a deep pocket and only *O1* is solvent exposed (**Fig. 3**).

For the S1_4 enzyme BT4683$^{3S\text{-}Gal}$, a structure solved in complex with LacNAc did not reveal any interaction between the protein and the O4 of Gal. In BT4683$^{3S\text{-}Gal}$, the interaction with Gal is driven by the residues R72 and E335, spatially equivalent to R353 and E334 in BT1636$^{3S\text{-}Gal}$, that form hydrogen bonds with *O2* of D-Gal (**Fig. 3**) and disruption of either of these residues eliminates activity (**Supplementary Table 3**). However, in BT4683$^{3S\text{-}Gal}$, a sulfatase that does not have any affinity for monosaccharides (**Extended data Fig. 4b**), the active site is located in an open cleft (**Fig. 3**) that allows the accommodation of additional substitutions on Gal (**Extended Data Fig. 6d**), a finding that is consistent with the endo-activity found in cMO. Together, these structures reveal the key specificity determinants in 3S-Gal/GalNAc sulfatases, highlighting that these enzymes have evolved to target sulfate groups in the different contexts in which they are found in complex host glycans. This is especially true for BT1636$^{3S\text{-}Gal}$ which utilises high affinity interactions with both O2 and O4 to drive enhanced activity to remove terminal 3S-Gal linkages in cMOs. See **Supplementary Discussion 3** for additional details of sulfatase structural characterization and phylogeneny.

***Roles of sulfatases in B. thetaiotaomicron O-glycan utilization***

*Bt* is able to utilize cMO as a sole carbon source (**Fig. 1b**), but the key enzymes involved in the degradation of these glycans remain unclear. Deletion of the gene encoding the only anaerobic sulfatase maturating enzyme (*anSME*) eliminates activation of the 28 family S_1 sulfatases[13] and the ability of *Bt* to grow efficiently on cMOs (**Fig. 4a**), highlighting the importance of sulfatases to this symbiont's physiology. Based on this observation we generated a series of compounded gene deletion strains in which one or more groups of sulfatases were eliminated based on their biochemical activity. Deletion

of all 3S-Gal/GalNAc sulfatases (Δ*bt1636*$^{3S\text{-}Gal}$ + Δ*bt1622*$^{3S\text{-}Gal/GalNAc}$ + Δ*bt4683*$^{3S\text{-}Gal}$) resulted in a growth phenotype similar to Δ*anSME* (**Extended Data Fig. 8a**). Interestingly, we observed a similar growth defect when just BT1636$^{3S\text{-}Gal}$ was deleted, but not the other 3S-Gal sulfatases (**Fig. 4a and Extended Data Fig. 8a**), consistent with the prominent activity of the recombinant form of this enzyme on cMOs. In contrast, a strain with compounded deletions of eight other sulfatases (Δ*bt1622*$^{3S\text{-}Gal/GalNAc}$ + Δ*bt4683*$^{3S\text{-}Gal}$ + Δ*bt1624*$^{6S\text{-}Gal/GalNAc}$ + Δ*bt3109*$^{6S\text{-}Gal}$ + Δ*bt4631*$^{6S\text{-}Gal/GalNAc}$ + Δ*bt1628*$^{6S\text{-}GlcNAc}$ + Δ*bt3177*$^{6S\text{-}GlcNAc}$ + Δ*bt3051*$^{putative\_6S\text{-}GlcNAc}$) displayed a growth phenotype similar to wild-type (**Fig. 4a**), indicating that these enzymes are not essential for cMO utilization. However, a Δ*10X sulf* mutant, which included the deletion of BT1636$^{3S\text{-}Gal}$ and the two 4S-Gal/GalNAc sulfatases showed a similar growth defect as Δ*anSME* and Δ*bt1636*. Complementation of this and other loss of function mutants with only *bt1636*$^{3S\text{-}Gal}$ restored growth on cMO to levels similar to wild-type (**Fig. 4a**, **Extended Data Fig. 8a**). Cellular localization experiments revealed that BT1636$^{3S\text{-}Gal}$ is located at the cell surface in *Bt* (**Fig. 4c**) and together, these data suggest that a single 3S-Gal sulfatase, BT1636$^{3S\text{-}Gal}$, is a critical cell surface enzyme for the utilization of sulfated *O*-glycans that are prominent in the colon.

To further investigate the role of BT1636$^{3S\text{-}Gal}$ in *O*-glycan utilization, we analysed the oligosaccharides present in the culture supernatant of the wild-type and Δ*bt1636*$^{3S\text{-}Gal}$ strains after growth on cMO. Consistent with a robust ability to degrade diverse colonic *O*-glycans, no oligosaccharides were detected in wild-type supernatant (**Supplementary Table 8**). Compared to the control, the supernatant of Δ*bt1636*$^{3S\text{-}Gal}$ showed accumulation of terminal 3S-Gal capped glycans (62% vs 3%, respectively). Indeed, the three most common 3S-Gal structures detected in the Δ*bt1636*$^{3S\text{-}Gal}$ supernatant accounted for 50% of the total oligosaccharides detected in this sample (**Fig. 4b**). These results reveal that deletion of BT1636$^{3S\text{-}Gal}$ results in loss of the ability to utilize 3S-Gal *O*-glycans. Interestingly, 49 of the 72 glycans detected in Δ*bt1636*$^{3S\text{-}Gal}$ supernatant were not present in the control sample (**Supplementary Table 8**), suggesting that although the mutant does not grow well on cMO it is able to modify these oligosaccharides to create new structures (**Supplementary discussion 5**). These data, combined with the cell surface location of BT1636$^{3S\text{-}Gal}$, suggest that this sulfatase works early in *O*-glycan catabolism

likely by cleaving 3S-Gal from *O*-glycans prior to importing them into the periplasm where these oligosaccharides will be sequentially degraded by additional enzymes and also serve as cues for activating transcription of the *O*-glycan PULs (**Extended Data Fig. 2)**. Although *Bt* does encode two additional 3S-Gal/GalNAc sulfatases, the low activity of these additional sulfatases on cMOs (**Fig. 2**) and their likely periplasmic location, suggests why these enzymes cannot compensate for loss of BT1636$^{3S\text{-}Gal}$. Interestingly, all of the *Bacteroides* species able to utilize cMO (**Fig. 2a**) have homologues of BT1636$^{3S\text{-}Gal}$ sulfatase, suggesting that this activity has a key role in mucin utilization by other HGM members (**Supplementary table 10**).

Finally, to investigate the requirement for specific sulfatases *in vivo*, we utilized gnotobiotic mice in which we competed individual mutants against the wild-type strain to evaluate their colonization fitness. It has been reported that mouse colonic Muc2 prominently displays 6S-GlcNAc modifications[25]. However, mutants lacking either just the two active 6S-GlcNAc sulfatases (Δ*6S-GlcNAc* double mutant), or these two enzymes, another putative 6S-GlcNAc sulfatase and all three 6S-Gal/GalNAc sulfatases (Δ*6S-GlcNAc* + Δ*6S-Gal/GalNAc* hexa mutant), competed equally with wild-type (**Extended Data Fig. 8c**), suggesting that neither of these two sulfatase activities are essential determinants *in vivo*. An overall mild defect was observed with a mutant lacking all 3S-Gal/GalNAc sulfatases although this effect was severe in one mouse (**Fig. 4d**). The fitness defect was exacerbated by eliminating 3S-Gal/GalNAc and 6S-GlcNAc sulfatase activities together (Δ*3S-Gal/GalNAc* + Δ*6S-GlcNAc*) (**Fig. 4d**), suggesting that they synergise *in vivo*. Consistent with its prominent role in cMO utilization *in vitro*, a mutant lacking just BT1636$^{Gal\text{-}3Sulf}$ displayed a strong, but variable defect when competed with the wild-type strain (**Fig. 4d**), further suggesting that this enzyme plays an essential role in gut colonization by allowing *Bt* to access 3S-Gal *O*-glycans. See **Supplementary Discussion 4-5** for additional details regarding growth and competition of mutants *in vitro* and *in vivo*.

***Conclusion***

To degrade the complex *O*-glycans found in mucins some HGM bacteria have evolved complex arsenals of degradative enzymes that include sulfatases. Disarming all

of the sulfatases in *Bt* via *anSME* deletion results in drastically reduced competitive colonization (**Extended Data Fig. 8c**)[13] and an inability to elicit colitis in an animal model of IBD[14]. While these findings support a critical role for active sulfatases in both fitness and promoting inflammation, they provide no insight into the complexity of catalytic events carried out by these enzymes. In this study, we reveal that *Bt* has a robust ability to grow on highly sulfated mucin oligosaccharides from colonic tissue and that it has sulfatases to remove sulfate in all of the contexts in which it is known to occur in mucin, and perhaps also unknown linkages. Surprisingly, we found that a single key sulfatase is essential for growth on colonic mucin *O*-glycans. The critical role of $BT1636^{3S\text{-}Gal}$ supports the conclusion that keystone steps exist in the complex pathway of mucin degradation, although, given that mucin glycan structures may vary between mammalian hosts, these critical steps may eventually need to be validated in humans for therapeutic translation. Nevertheless, establishment of these critical steps is a prerequisite to blocking this complex enzyme pathway and potentially inhibiting mucin-degrading activities in bacteria that contribute to diseases such as IBD.

## Methods

### Recombinant Protein Production

Genes were amplified by PCR using the appropriate primers and the amplified DNA cloned in pET28b using NheI/XhoI restriction sites or pETite (Expresso™ T7 cloning and expression system, Lucigen) generating constructs with either N- or C-terminal $His_6$ tags (**Supplementary Table 11**). The catalytic serine was mutated to cysteine since *Escherichia coli* in only able to convert cysteine to formylglycine. Recombinant genes were expressed in *Escherichia coli* strains BL21 (DE3) or TUNER (Novagen), containing the appropriate recombinant plasmid, and cultured to mid-exponential phase before induction with 1 mM (BL21) or 0.2 mM (TUNER) of isopropyl β-D-1-thiogalactopyranoside; cells were cultured for another 16 h at 16 °C and 180 rpm. Recombinant protein were purified to >90% electrophoretic purity by immobilized metal ion affinity chromatography using a cobalt-based matrix (Talon, Clontech) and eluted with imidazole as described previously[22]. For the proteins selected for structural studies,

another step of size exclusion chromatography was performed using a Superdex 16/60 S200 column (GE Healthcare), with 10 mM HEPES, pH 7.5, and 150 mM NaCl as the eluent, and they were judged to be ≥95% pure by SDS-PAGE. Protein concentrations were determined by measuring absorbance at 280 nm using the respective molar extinction coefficient. When necessary, proteins were then concentrated in centrifugal concentrators with a molecular mass cutoff of 30 kDa.

**Site-Directed Mutagenesis**

Site-directed mutagenesis was conducted using the PCR-based QuikChange kit developed by Stratagene and conducted according to the manufacturer's instructions, using the appropriate plasmid as the template, and appropriate primer (**Supplementary Table 12**). The mutations were confirmed by DNA sequencing.

**Sources of Carbohydrates**

All carbohydrates were from Sigma, Carbosynth or Dextra Laboratories. All other chemical reagents were purchased from Sigma. The 3S-GalNAc was chemically synthesized as previously described[26].

**Mucin purification**

Gastric mucin oligosaccharides (gMO) were purified from porcine gastric mucins (type III, Sigma) as previously described. Colonic mucins oligosaccharides (cMO) were purified from pig distal pig colons and rectum. Briefly, the tissue was open and the fecal contents were carefully removed. The mucosa was scrapped off and mucus was extracted by homogenizing the tissue in at least 5 times volume of extraction buffer (6M guanidine chloride, 5mM EDTA, 10mM $NaH_2PO_4$, pH 6.5) and slow stirring at 4 ºC for 16 h. The solution was spun down at 15 000 rpm and 10 ºC for 30 min and supernatant was discharged. The pellets were resuspended in extraction buffer and the process was repeated until the supernatant was clear for at least two extractions. After the extraction the mucins were solubilized by reducing the disulfide bonds. The pellets were resuspended in fresh reduction buffer (6 M guanidine chloride, 0.1M Tris, 5 mM EDTA, pH 8.0) containing 25 mM of 1,4-dithiothreitol and slowly stirred at 37 ºC for 5 h. After this

incubation, 62.5 mM of iodoacetamide were added and the solution was stirred slowly in the dark at room temperature for 16 ºC. The solution was centrifuged at 10 000 rpm at 4 ºC for 30 mim and the supernatant containing the solubilized mucins was extensively dialysed into water. Samples were dissolved into 100 mM Tris-HCl pH 8.0 containing 1 mg/ml of trypsin and incubated slowly stirring at 37 ºC for 16 h. The glycans were β-eliminated by adding 0.1 M NaOH and 1 M $NaBH_4$ and incubate the solution at 65 ºC for 18 h. After cooling the solution to the room temperature the pH was adjusted to 7.0 with concentrated HCl and extensively dialysed in water. The released porcine colonic mucin glycans were recovered by lyophilisation the solution until completely dry and used in further experiments.

**Sulfatase enzymatic assays**

The sulfatase activity screen against commercially available sulfated oligosaccharides (Supplementary Table 2) was performed with 1 μM of recombinant enzyme and 1 mM of substrate in 10 mM MES pH6.5 with 5 mM CaCl2 for 16h at 37 ºC. Sulfated *N*-acetyl-D-lactosamine and lacto-*N*-biose were generated by incubating the respective sulfated Lewis antigens with 1 μM of α-1,3/1,4-fucosidase BT1625[27] in the same conditions. Reactions were analysed by thin layer chromatography (TLC). Briefly, 2 μL of each sample was spotted onto silica plates and resolved in butanol:acetic acid:water (2:1:1) running buffer. The TLC plates were dried, and the sugars were visualized using diphenylamine stain (1 ml of 37.5% HCl, 2 ml of aniline, 10 ml of 85% H3PO3, 100 ml of ethyl acetate and 2 g diphenylamine) and heated at 100°C for 20 minutes. When relevant, the enzymatic activity was confirmed by high-performance anionic exchange chromatography (HPAEC) with pulsed amperometric detection using standard methodology. The sugars (reaction substrate/products) were bound to a Dionex CarboPac P100 column and eluted with an initial isocratic flow of 10 mM NaOH during 20 minutes then a gradient of 10-100 mM of NaOH for 20 minutes at a flow rate of 1.0 ml $min^{-1}$. The reaction products were identified using the appropriated standards. All experiments were performed in triplicate.

**Liquid Chromatograph-Electrospray Ionization Tandem Mass Spectrometry**

Enzymatic reactions of sulfatases in colonic mucin oligosaccharides and culture supernatant were cleaned up with graphitized. Reactions with sulfated defined saccharides (Lewis antigens and disaccharides) were reduced and desalted. Briefly, reactions were dried in Speed vac, reconstituted in 20 μL of 50 mM NaOH and 500 mM $NaBH_4$ and incubated at 50 ºC for 3 h. Reactions were cool down on ice, neutralised with 1 μL of glacial acetic acid and desalted using a cation exchange column containing AG®50W-X8 resin. All cleaned and desalted reactions were reconstituted in water before analysis by liquid chromatograph-electrospray ionization tandem mass spectrometry (LC-ESI/MS). The oligosaccharides were separated on a column (10 cm × 250 μm) packed in-house with 5 μm porous graphite particles (Hypercarb, Thermo-Hypersil, Runcorn, UK). The oligosaccharides were injected on to the column and eluted with an acetonitrile gradient (Buffer A, 10 mM ammonium bicarbonate; Buffer B, 10 mM ammonium bicarbonate in 80% acetonitrile). The gradient (0-45% Buffer B) was eluted for 46 min, followed by a wash step with 100% Buffer B, and equilibrated with Buffer A in next 24 min. A 40 cm × 50 μm i.d. fused silica capillary was used as transfer line to the ion source. Samples were analyzed in negative ion mode on a LTQ linear ion trap mass spectrometer (Thermo Electron, San José, CA), with an IonMax standard ESI source equipped with a stainless steel needle kept at –3.5 kV. Compressed air was used as nebulizer gas. The heated capillary was kept at 270°C, and the capillary voltage was –50 kV. Full scan (*m/z* 500-1800 for acidic GSLs and *m/z* 380-2000 for neutral GSLs, two microscan, maximum 100 ms, target value of 30,000) was performed, followed by data-dependent $MS^2$ scans (two microscans, maximum 100 ms, target value of 10,000) with normalized collision energy of 35%, isolation window of 2.5 units, activation q=0.25 and activation time 30 ms). The threshold for $MS^2$ was set to 300 counts. Data acquisition and processing were conducted with Xcalibur software (Version 2.0.7). The schematic glycosidic or cross-ring cleavages were assigned according to the Domon and Costello nomenclature[28].

**Microfuidics desulfation assays**

Sulfated carbohydrates were labelled at their reducing end with BODIPY which has a maximal emission absorbance of ~503 nm, which can be detected by the EZ Reader via LED-induced fluorescence. Non-radioactive microfluidic mobility shift carbohydrate

sulfation assays were optimised in solution with a 12-sipper chip coated with CR8 reagent and a PerkinElmer EZ Reader II system using EDTA-based separation buffer and real-time kinetic evaluation of substrate de-sulfation. Pressure and voltage settings were adjusted manually (1.8 psi, upstream voltage:2250 V, downstream voltage: 500 V) to afford optimal separation of the sulfated and unsulfated product with a sample (sip) time of 0.2 s, and total assay times appropriate for the experiment. Individual de-sulfation assays were carried out at 28°C and assembled in a 384-well plate in a volume of 80 µl in the presence of substrate concentrations between 0.5 and 20 µM with 100 mM Bis-Tris-Propane or Tris, as pH appropriate, 150 mM NaCl, 0.02% (v/v) Brij-35 and 5 mM $CaCl_2$. The degree of de-sulfation was directly calculated using the EZ Reader software by measuring the sulfated carbohydrate : unsulfated carbohydrate ratio at each time-point. The activity of sulfatase enzymes was quantified in 'kinetic mode' by monitoring the amount of unsulfated glycan generated over the assay time, relative to control assay with no enzyme; with sulfate loss limited to ~20% to prevent of substrate and to ensure assay linearity. $k_{cat}/K_M$ values, using the equation $V_0=(V_{max}/K_M)/S$, were determined by linear regression analysis with GraphPad Prism software. Substrate concentrations were halved and doubled to check linearity of the rates ensuring substrate concentrations were significantly $<K_M$.

**NMR desulfation assays**

NMR experiments, monitoring the de-sulfation of 6S-D-galactose and 6S-*N*-acetyl-D-galactosamine, were conducted in $D_2O$ with 50 mM sodium phosphate, pH 7.0, supplemented with 150 mM NaCl at 25°C on a 800MHz Bruker Avance III spectrometer equipped with a TCI CryoProbe and a 600MHz Bruker Avance II+ spectrometer, also fitted with a TCI CryoProbe. 1D and 2D proton and TOCSY spectra (mixing time 80 ms) were measured using standard pulse sequences provided by the manufacturer. Spectra were processed and analysed using TopSpin 3.4A and TopSpin 4.0 software (Bruker). Galactose integrals were recorded directly for the C(6)$H_2$-OH peak within the region 3.694 to 3.721ppm, referenced to the combined C(2) peaks of D-galactose and 6S-D-galactose with in the region 3.415 to 3.475ppm. Similarly, 6S-*N*-acetyl-D-galactosamine integrals

were recorded directly for the C(6)$H_2$-OH peak within the region 3.674 to 3.747ppm, referenced to the combined C(4) peaks for N-acetyl D-galactosamine and 6S-N-acetyl D-galactosamine in the region 3.925 to 3.968ppm.

**Differential scanning fluorimetry**

Thermal shift/stability assays (TSAs) were performed using a StepOnePlus Real-Time PCR machine (LifeTechnologies) using SYPRO-Orange dye (emission maximum 570 nm, Invitrogen), with thermal ramping between 20 and 95°C in 0.3°C step intervals per data point to induce denaturation in the presence or absence of various carbohydrates as appropriate to the sulfatase being analysed. The melting temperature (Tm) corresponding to the midpoint for the protein unfolding transition was calculated by fitting the sigmoidal melt curve to the Boltzmann equation using GraphPad Prism, with $R^2$ values of >0.99. Data points after the fluorescence intensity maximum were excluded from fitting. Changes in the unfolding transition temperature compared with the control curve ($\Delta T_m$) were calculated for each ligand. A positive $\Delta T_m$ value indicates that the ligand stabilises the protein from denaturation, and therefore binds the protein. All TSA experiments were conducted using a protein concentration of 5μM in 100 mM Bis-Tris-Propane, pH 7.0, and 150 mM NaCl supplemented with appropriate ligand. When $BT1622^{GalNAc3S}$ and $BT1636^{Gal3S}$ were assessed against 3S-LacNAc and 3S-LNB 100 mM Hepes was used instead of BTP, no difference in the $T_m$ value of the proteins was observed. Three independent assays were performed for each protein and protein ligand combination.

**Glycan labelling**

Sulfated saccharide samples were labelled according to a modification of the method previously described reporting the formation of N-glycosyl amines for 4,6-O-benzilidene protected D-gluopyranose monosaccharides with aromatic amines[29]. Briefly, the lyophilised sugar (1 mg) was dissolved in 0.50 ml anhydrous methanol in a 1.5 ml screw-top PTFE microcentrifuge tube. 0.1 mg, BODIPY-FL hydrazide (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid hydrazide, ThermoFischer, λ ex./em. 493/503, extinction coefficient 80,000 $M^{-1}$ $cm^{-1}$) was added and the mixture vortexed (1 min), then incubated in darkness at 65 ºC for 24. The products were then

cooled and a portion purified by TLC on silica coated aluminium plates developed with methanol or 1:1 v/v ethyl acetate/methanol. The unreacted BODIPY-FL label (orange on the TLC plate) was identified by reference to a lane containing the starting material (BODIPY-FL hydrazide) on the TLC plate, allowing differentiation from the putative labelled product (also orange). This latter band was scraped from the plates and extracted in fresh methanol (2 x 0.5 ml), spun for 3 min at 13,000g, and the supernatant was recovered and dried (rotary evaporator) to recover the fluorescent-coloured product (bright green when dissolved in aqueous solution), which was then employed in subsequent experiments.

**Anaerobically Bacterial Strains Culture and Genetic Manipulation**

All strains were anaerobic grown at 37 °C in a chamber (10% H2, 5% CO2, and 85% N2; Coy Manufacturing, Grass Lake, MI). *Bacteroides* type strains were culture in either tryptone-yeast extract-glucose medium (TYG), brain heart infusion medium or minimal medium (MM) containing an appropriate carbon source. *Bacteroides massilliensis* and *Akkermansia muciniphila* were culture as described before[19,30]. *Bt* strains containing specific gene deletions or inactivated versions of enzymes (BT1636$^{3S\text{-}Gal}$ S77A) were made by counterselectable allelic exchange as previously described[31]. Complemention of deletion strains was performed using pNBU2 vector as previously described[31], containing a constitutive promotor used previously[32]. All primers used to generate the mutants and complementation are listed in **Supplementary table 13**. Growth of the WT and mutants was measured on an automated plate reader by increase in absorbance at 600 nm in 96-well plates containing 200 μl of minimal media mixed with the respective filter-sterilised (monosaccharide and gMO) or autoclave-sterilised cMO as described before[30]. To achieve consistent growth, all carbon sources were used at 5 mg/ml with exception of gMO that was added in a final concentration of 10 mg/ml. All growth curves presented are averages and s.e.m of three technical replicates.

**Crystallization of carbohydrate sulfatases**

After purification, all proteins were carried forward in the same eluent as used for the size exclusion chromatography (see Recombinant Protein Production). Sparse matrix screens were set up in 96-well sitting drop TTP Labtech plates (400-nL drops). Initial hits crystals

for all proteins were obtained between 20 and 35 mg/ml. For BT1622$^{3S\text{-}Gal/GalNAc}$ and BT1636$^{3S\text{-}Gal}$ wildtype *Bt* variants were used, having a Ser at the catalytic formylglycine position, whilst for for BT4683$^{3S\text{-}Gal}$ the S73C mutant was used. BT1622$^{3S\text{-}Gal/GalNAc}$ with 20 mM LNB crystallised in 20 % PEG 3350 and 0.2 M sodium citrate tribasic dihydrate. BT1636$^{3S\text{-}Gal}$ with 20 mM LacNAc crystallised in 40 % MPD and 0.2 M sodium cacodylate pH 6.5. for BT4683$^{3S\text{-}Gal}$ with 20 mM LacNAc crystallised in 20 % PEG 3350, 0.2 M sodium iodide and BTP pH 8.5. All crystals were cryoprotected with the addition of the ligand they were crystallised with plus 20% PEG 400 and 20% glycerol was used as the cryoprotectant for BT4683$^{3S\text{-}Gal}$ and BT1622$^{3S\text{-}Gal/GalNAc}$, respectively. No cryoprotectant was added to BT1636$^{3S\text{-}Gal}$ crystals. Data were collected at Diamond Light Source (Oxford) on beamlines I03, I04, I04-1 and I24 at 100 K. The data were integrated with XDS[33], or Xia2[34] 3di or 3dii and scaled with Aimless[35]. Five percent of observations were randomly selected for the $R_{free}$ set. The phase problem was solved by molecular replacement using the automated molecular replacement server Balbes[36] for all proteins except BT1622$^{3S\text{-}Gal/GalNAc}$. The phase problem for BT1622$^{3S\text{-}Gal/GalNAc}$ was initially solved using Molrep[37] and BT1636$^{3S\text{-}Gal}$ as the search model. This gave a partial solution, which could not be fully solved due to twinning. An acceptable model of BT1622$^{3S\text{-}Gal/GalNAc}$ was constructed to be used to better solve the phase problem and the molecular replacement was re-performed. Models underwent recursive cycles of model building in Coot[38] and refinement cycles in Refmac5[39]. Bespoke ligands were generated using JLigand[40]. The models were validated using Coot[38] and MolProbity[41]. Structural figures were made using Pymol and all other programs used were from the CCP4[42] and CCP4i2 suite[43]. The data processing and refinement statistics are reported in **Supplementary Table 14**.

**Ligand docking**

The docking of 3-sulfate-*N*-Acetyl-D-galactosamine (3S-GalNAc) into BT1622$^{GalNAc3S}$ was performed using the automated Swissdock server with the default settings[44,45]. A pdb and cif file for the ligand 3S-GalNAc were generated using JLigand[40] and then the pdb file was converted to a mol2 file using openbabel for use with Swissdock. Clustered solutions were inspected using UCSF chimera[46] and then the solutions which clustered appropriately to

the active site pocket were manually converted to individual pdb files and further inspected using PyMol.

**Immunolabelling of BT1636 in *Bt* cell surface**

For the fluorescence microscopy, *Bt* cells (Wild type (Δ*tdk*) and Δ*bt1636*$^{3S\text{-}Gal}$) were grown to early exponential phase ($Abs_{600nm}$ 0.25–0.35) in rich TYG medium. One ml of the cultures was collected, centrifuged at 13,000 RPM, and subsequently washed three times in MM with no carbon source. *Bt* cells incubated with cMO for four hours and fixed in 4.5% formalin overnight at 4 ºC with gentle rocking. Cells were stained with a polyclonal antibody raised in rabbit against purified recombinant BT1636 (BT1636$^{Ab}$, Cocalico Biologicals) and detected with an Alexa Fluor® 488-conjugated goat anti-rabbit IgG secondary antibody (Molecular Probes). Images were taken with Zeiss Apotome using the same exposure time between samples.

**Gnotobiotic Mouse Experiments**

All experiments involving animals, including euthanasia via carbon dioxide asphyxiation, were approved by the University Committee on Use and Care of Animals at the University of Michigan (NIH Office of Laboratory Animal Welfare number A3114-01) and overseen by a veterinarian. Groups of 3 to 5, 6-8 week old germfree Swiss Webster mice were randomly assigned to each experiment. 7 days prior gavage the animals diet was switched to a fiber-free diet (Envigo-Teklad TD 130343) that was maintained through all the experiment. At day 0, mice were gavage with equal amount of *Bt* WT strain and mutant and fecal samples were collected at day 2 and every 5 days until day 42. At the end-point of the experiment distal small intestine and cecca contents were also collected. The bacteria gDNA extraction and quantification by qPCR of the relative abundance of each strain on the various samples was carried out as described previously[30].

**Phylogenetic analysis**

To avoid identical sequences, we selected 800 and 920 representative sequences of subfamily S1_20 (composed of 1356 sequences) and S1_4 (composed of 1895

sequences), respectively. The sequences were aligned by MAFFT v.7[47] using L-INS-i algorithm. The multiple sequence alignment was visualized by Jalview software v.11.0[48] and non-aligned regions were removed. 404 and 364 positions were used for the S1_4 and S1_20 phylogeny, respectively. Phylogeny was made using RAxML v. 8.2.4[49]. The phylogenetic tree was build with the Maximum Likelihood method[50] and the LG matrix as evolutive model[51] using a discrete Gamma distribution to model evolutionary rate differences among sites (4 categories). The rate variation model allowed for some sites to be evolutionarily invariable. The reliability of the trees was tested by bootstrap analysis using 1,000 resamplings of the dataset[52]. All the final global phylogenetic trees were obtained with MEGA v.7[53]. Fifteen S1_0 sequences from the sulfAtlas database were used as outgroups.

**Quantification and Statistical Analysis**

For in vivo competitions, when three or more fecal samples were collected, Student's t tests were performed for each time point in GraphPad Prism version 8.1 with a paired, two-tailed distribution. When necessary, the statistical analysis for remaining samples in stated in the respective figure legend.

**Data availability statement**

All data for the experiments, along with corresponding statistical test values, where appropriate, are provided within the paper and in its Supplementary Information. The crystal structure datasets generated have been deposited in the PDB under the following accession numbers: 7ANB, 7ANA, 7AN1, 7OQD and 7ALL. The MS raw files have been deposited in the GlycoPOST database under the following IDs: GPST000150 and GPST000196. Glycan structural annotations were deposited to the UniCarb database at https://unicarb-dr.glycosmos.org/references/462. There are no restrictions on data or biological resource availability. Data and biological resources can be obtained by contacting the corresponding authors. Source data are provided with this paper.

**Code Availability Statement:** No new codes were developed or compiled in this study

**Competing Interests Statement**

The authors declare no competing interests.

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**Author Contributions.** A.S.L., A.C. and E.C.M. designed experiments and wrote the manuscript. A.S.L. and A.C. cloned, expressed and purified sulfatases and performed the enzymatic assays. A.C., D.P.B., J.A.L. and P.A.E. carried out and analysed the data from kinetic and binding experiments. E.A.Y., M.R. and S.O. performed chemical syntheses. A.C. and A.B. performed structural biology experiments. C.J., A.S.L., G.C.H. and N.G.K. performed and interpreted data from analytical glycobiology experiments. A.S.L., G.V.P., R.W.P.G., S.G., S.S. and N.A.P. performed bacterial growth experiments and analysed in vivo competition data. M.C., G.M. and T.B. performed sulfatase phylogenetic analyses. All authors read and approved the manuscript.

**Additional information**

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Correspondence and requests for materials should be addressed to Ana S. Luis, Alan Cartmell or Eric C. Martens.

**Figure Legends**

**Figure 1. Bacterial growth on colonic mucin and *B. thetaiotaomicron* sulfatases activities.** **a,** Schematic representation of mucin *O*-glycans and relevant terminal epitopes (dashed box). Sugars are shown according to the Symbol Nomenclature for Glycans system[54]. **b**, Growth of *Bacteroides* type strains and A*kkermansia muciniphila* on colonic mucin *O*-glycans (cMO) and number of respective encoded S1 sulfatases. The bars represent the average of two independent experiments with different batches of cMO. Bacteria species able to utilize gastric mucin glycans are highlighted in blue. Maximum absorbance is the difference of the maximum absorbance value ($Abs_{600nm}$) for each culture and the initial absorbance at time 0 ($T_{0h}$). Graphic shows the example of growth curves for *B. fragilis* (Bf), *B. thetaiotaomicron* (Bt), *B. vulgatus* (Bv) and *B. massilliensis* (Bm). **c**, Phylogeny of Bt sulfatases with S1 sulfatases and respective substrate. Enzymes colour coded according the respective subfamilies. * indicates sulfatase activity previously characterized and arrows point the substrate preferentially targeted by the respective enzyme. Sulfatases sharing more than 86% and 39-58% of sequence identity are highlighted in blue and green background, respectively. Data from biological replicates n = 3 to 6 and error bars denote s.e.m..

**Figure 2. Activity of *B. thetaiotaomicron* sulfatases on colonic mucin O-glycans. a,** Representation of *O*-glycans detected my mass spectrometry in cMO (control) and after sulfatase treatment from the lower (top) to the higher m/z (bottom). **b,** Relative abundance and putative structures for the specific m/z shown in panel a. Remaining structures are shown on Extended figure 5a. **c,** Schematic representation of the putative structures that were not detected in reaction after incubation with $BT1636^{3S\text{-}Gal}$.

**Figure 3. Crystal structures of 3S-Gal/GalNAc sulfatases. a,** Schematic representation of the residues interacting with targeted sugars, including the putative catalytic residues (in dark red), the calcium ion (grey sphere) and subsites S, 0 and +1 highlighted in red. $BT1636^{3S\text{-}Gal}$ and $BT4683^{Gal\text{-}3Sulf}$ in complex with LacNAc (D-Gal-β1,4-D-GlcNAc) and $BT1622^{3S\text{-}Gal/GalNAc}$ in complex with GalNAc. **b,** Surface representation of the active pocket. The equivalent Gal/GalNAc specificity residues in $BT1636^{3S\text{-}Gal}$ and $BT4683^{Gal\text{-}3Sulf}$ are highlighted in red and blue. The open active site of $BT4683^{Gal\text{-}3Sulf}$ is

highlighted in purple. In all structures the amino acids and ligands are represented as stick.

**Figure 4. BT1636$^{3S\text{-}Gal}$ activity is required to the utilization of cMO and competitive fitness *in vivo***. **a**, Growth of *Bt* wild-type Δ*tdk* (WT), different sulfatase gene-deletion mutants (named "Δ*btXXXX*") and strains complemented with *bt1636$^{3S\text{-}Gal}$* on colonic or gastric mucin O-glycans (cMO and gMO, respectively) (line represents the average of biological replicates (n = 3) and error bars denote s.e.m.) **b,** Immunoflurescent and differential interference contrast (DIC) microscopy of *Bt* WT and sulfatase mutant staining with polyclonal antibody (Ab) against BT1636$^{3S\text{-}Gal}$ (green) and DNA staining with DAPI (blue). **c,** Relative abundance of different *O*-glycans structures detected by mass spectrometry in Δ*bt1636$^{3S\text{-}Gal}$* culture supernatant or cMO in minimal media without bacteria (control), after 96h in anaerobic conditions. The m/z and putative structure of the 3 more abundant structures in both samples are shown. **d,** *in vivo* competitions in gnotobiotic mice (n = 3-4) fed fiber-free diet and inoculated with WT and mutants. The fecal relative abundance of each strain determined along the experiment time course and in small intestine and cecum at day 42. The relative abundance in each mice is represented in the respective light colour. The error bars denote s.e.m.. Gal, D-Galactose; GalNAc, *N*-acetyl-D-galactosamine; GlcNAc, *N*-acetyl-D-glucosamine.

**Extended Data 1. Growth of Bacteroides type strains and A*kkermansia muciniphila* in different mucin *O*-glycans.** The graphics show the growth of strains able to utilize *O*-glycans in minimal media containing the indicated carbon source (biological replicates n =3, error bars denote the s.e.m.). cMO, colonic mucin *O*-glycans; gMO, gastric mucin *O*-glycans; GlcNAc, *N*-acetyl-D-glucosamine.

**Extended Data 2. Schematic representation of polysaccharide utilization loci (PULs) encoding sulfatases (sulf).** Genes are colour coded according with the predicted function of the respective proteins. Glycoside hydrolases (GH) in known families

are indicated by GHXX or GH*, where XX and * indicates the respective family number or non-classified, respectively.

**Extended Data 3. Enzymatic screen of *B. thetaiotaomicron* sulfatases against sulfated monosaccharides.** Recombinant enzymes (1 μM) were incubated with 1 mM of substrate in 10 mM MES pH6.5 with 5 mM $CaCl_2$ for 16h at 37 ºC. Reactions were analised by thin layer chromatography (left hand side) or HPAEC with pulsed amperometric detection (right hand side). Control reactions without sulfatases were carried in the same conditions. The standards in TLC and HPAEC are labelled on the left hand side and top, respectively. The different panel represent activities found for sulfatases targeting: **(a)** 4S-Gal/GalNAc; **(b)** 6S-Gal/GalNAc; **(c)** 6S-GlcNAc and; **(d)** 3S-GlcNAc. The data shown is a representative from biological replicates (n = 3).

**Extended Data 4. Activity and affinity of sulfatases to targeted substrates. a,** Recombinant enzymes (1 μM) were incubated with 1 mM of substrate in 10 mM MES pH6.5 with 5 mM $CaCl_2$ for 16h at 37 ºC. Sulfated disaccharides were generated by adding 1 μM of an characterized α1,3/1,4-fucosidase (BT1625) in the enzymatic reaction. Control reactions without sulfatases were carried in the same conditions. Samples were analysed by mass spectrometry and the intensity of the substrate and reaction products was used to determine the relative abundance of these sugars after incubation with the respective enzymes. **b,** Affinity studies looking at the effect of ligand binding on the melting temperature of 3S and 6S-Gal sulfatases. All reactions were performed in 100 mM BTP, pH 7.0 with 150 mM NaCl. **c,** Activity of 3S-Gal/GalNAc sulfatases (10 μM) against 3S-GalNAc (10 mM). Reactions were performed in 10 mM Hepes, pH 7.0, with 150 mM NaCl and 5 mM $CaCl_2$. The data shown is a representative from biological replicates (n = 3).

**Extended Data 5. Activity of *B. thetaiotaomicron* sulfatases against colonic mucin *O*-glycans (cMO) detected by mass spectrometry. a,** Relative abundance of defined oligosaccharides after incubation of cMO with different sulfatases or no enzyme (control). The putative structure for the different m/z is shown of the right hand side of the graphic.

The reactions were performed with 1 μM of enzyme and 0.5% cMO in 10 mM MES pH6.5 with 5 mM $CaCl_2$ for 16h at 37 ºC. **b,** Relative abundance of structures detected in different samples organized by sulfate-linkage (top panel) or presence of one or several sugar substitution such as sulfate, sialic acid and fucose (bottom panel). The colour-coded bars represent the relative abundance and the numbers the total number of the structures containing the specific linkage/substitution. The complete data is shown in Supplementary Table 5.

**Extended Data 6. Schematic representation of 3S-Gal/GalNAc sulfatases. a,** Cartoon representation colour ramped from blue (α/β/α N-terminal domain) to red (b-sheet C-terminal domain). **b**, Overlay of the active site S residues of $BT1636^{3S\text{-}Gal}$ (green) $BT1622^{3S\text{-}Gal/GalNAc}$ (blue) and $BT4683^{3S\text{-}Gal}$ (pink). The putative catalytic residues are shown in bold. The calcium ion is represented as a grey sphere and its polar interactions indicated as bashed lines. **c,** Ligand density of maps for LacNAc in $BT1636^{Gal\text{-}3sulf}$ and $BT4683^{Gal\text{-}3sulf}$ , and GalNAc in $BT1622^{Gal\text{-}3sulf}$, contoured at 1σ (0.33 e/$A^3$, 0.37 e/$A^3$ and 0.18e/$A^3$, respectively); **d,** Docking of putative structures *O*-glycans targeted by $BT4683^{3S\text{-}Gal}$ using the LacNAc as reference point showing that this structure can accommodate a sialic acid in -1 subsite and additional sugars in positive subsites (left hand side). The docking sugars are schematic shown as sticks (middle panel) and schematic represented inside the dashed box (right hand side). Using the LacNAc product as an 'anchor' additional sugars were built in manually with Coot 0.9 and regularised to low energy conformations.

**Extended Data 7. Phylogenetic tree of S1_20 and S1_4 sulfatases.** The radial tree were constructed using the branched trees shown in Supplementary figures 2 and 3. For clarity all labels and sequence accession codes have been omitted. Red filled circles designate sequences from *B. thetaiotaomicron* sulfatases. The residue is written in black without any attributes if present in the sequence, in grey and italics if the residue is mutated to any type in that sequence, or to a specific residue type if given in brackets right after. **a,** Radial representation of the phylogenetic tree constructed with representative sequences of the sulfatase S1_4 subfamily. The colour code is given as a

pattern of presence or absence of the residues R72, E335 and W505, which are crucial in substrate recognition by BT4683 (acc-code Q89YP8, coloured red). A grey X in italics specifically designates that the residue W505 is absent in that sequence, and no obvious orthologous residue can be found from the alignment. **b,** Radial representation of the phylogenetic tree constructed with representative sequences of the sulfatase S1_20 subfamily. The colour code is given as a pattern of presence or absence of the residues E100, Q173 H177, E334, R353, which are crucial in substrate recognition by BT1636 (acc-code Q8A789, coloured red). A grey X in italics specifically designates that the residue E100 is absent in that sequence, and no obvious ortologous residue can be found from the alignment.

**Extended Data 8. Sulfatase activity is required to growth in cMO and *in vivo* fitness.** **a,** Growth curves of Bt wild-type Δ*tdk* (WT), different sulfatase mutants (Δ*btXXX*) and complemented strains on glucose, colonic or gastric mucin O-glycans (cMO and gMO, respectively). The curves represent the average of biological replicates (n = 3) and the error bars denote s.e.m.. **b,** Relative abundance of oligosaccharides detected by mass spectrometry in culture supernatant of WT and Δ*bt1636*$^{3S\text{-}Gal}$ after growth in cMO for 96h at anaerobic conditions. The control correspond to cMO incubated in the same conditions without bacterium. The colours represent the relative abundance of structures grouped according the presence of epitopes (sulfate, fucose and sialic acid) and the numbers represent the total number of structures that contain the respective substitution. **c**, Colonization of gnotobiotic mice fed with fiber-free diet by *Bt* WT and mutants lacking the full (Δ*anSME*) or specific sulfatase activity (Δ*6S-GlcNAc* and Δ*6S-GlcNAc*+Δ*6S-Gal/GalNAc*). The fecal relative abundance of each strain was determined in regular intervals until day 42. The relative abundance of time 0 represents the abundance in gavaged inoculum. At the experimental endpoint the relative abundance was also determined in small intestine and cecum. The graphics represent the average of n =3 and the error bars denote the s.e.m.. The relative abundance in each animal is represented in a lighter colour in each of the respective graphics.

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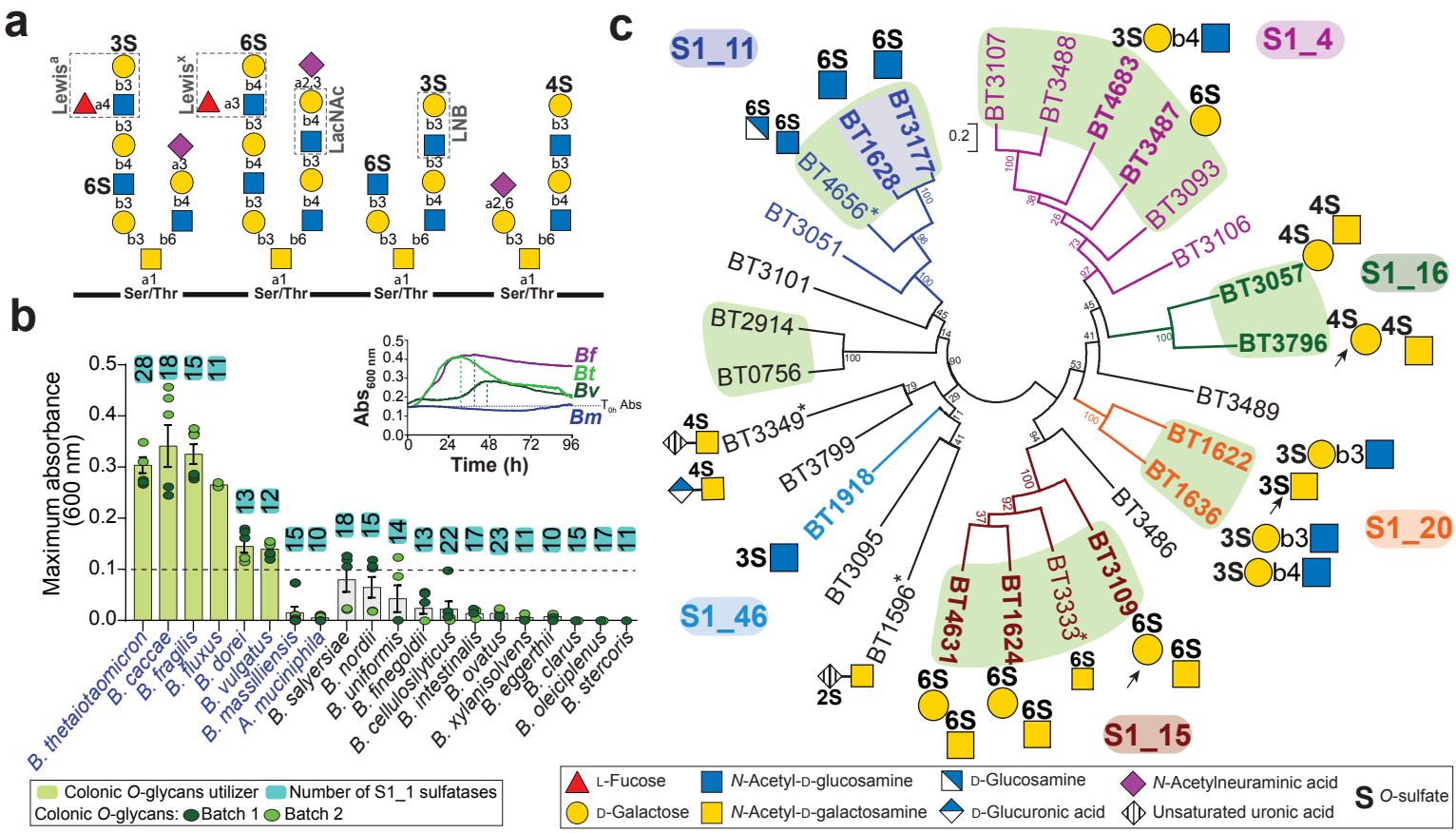

a
Lewis^a
Lewis^x
LacNAc
LNB
Ser/Thr
b
Maximum absorbance (600 nm)
Abs 600 nm
Time (h)
Bf
Bt
Bv
Bm
B. thetaiotaomicron
B. caccae
B. fragilis
B. fluxus
B. dorei
B. vulgatus
B. massilliensis
A. muciniphila
B. salyersiae
B. nordii
B. uniformis
B. finegoldii
B. cellulosilyticus
B. intestinalis
B. ovatus
B. xylanisolvens
B. eggerthii
B. clarus
B. oleiciplenus
B. stercoris
Colonic O-glycans utilizer
Number of S1_1 sulfatases
Colonic O-glycans: Batch 1 Batch 2
c
S1_11
S1_4
S1_16
S1_20
S1_46
S1_15
BT3177
BT1628
BT4656*
BT3051
BT3101
BT2914
BT0756
BT3349*
BT3799
BT1918
BT3095
BT1596*
BT4631
BT1624
BT3333*
BT3109
BT3486
BT3489
BT1622
BT1636
BT3057
BT3796
BT3106
BT3093
BT3487
BT4683
BT3488
BT3107
L-Fucose
D-Galactose
N-Acetyl-D-glucosamine
N-Acetyl-D-galactosamine
D-Glucosamine
D-Glucuronic acid
N-Acetylneuraminic acid
Unsaturated uronic acid
S O-sulfate

Fig 1.

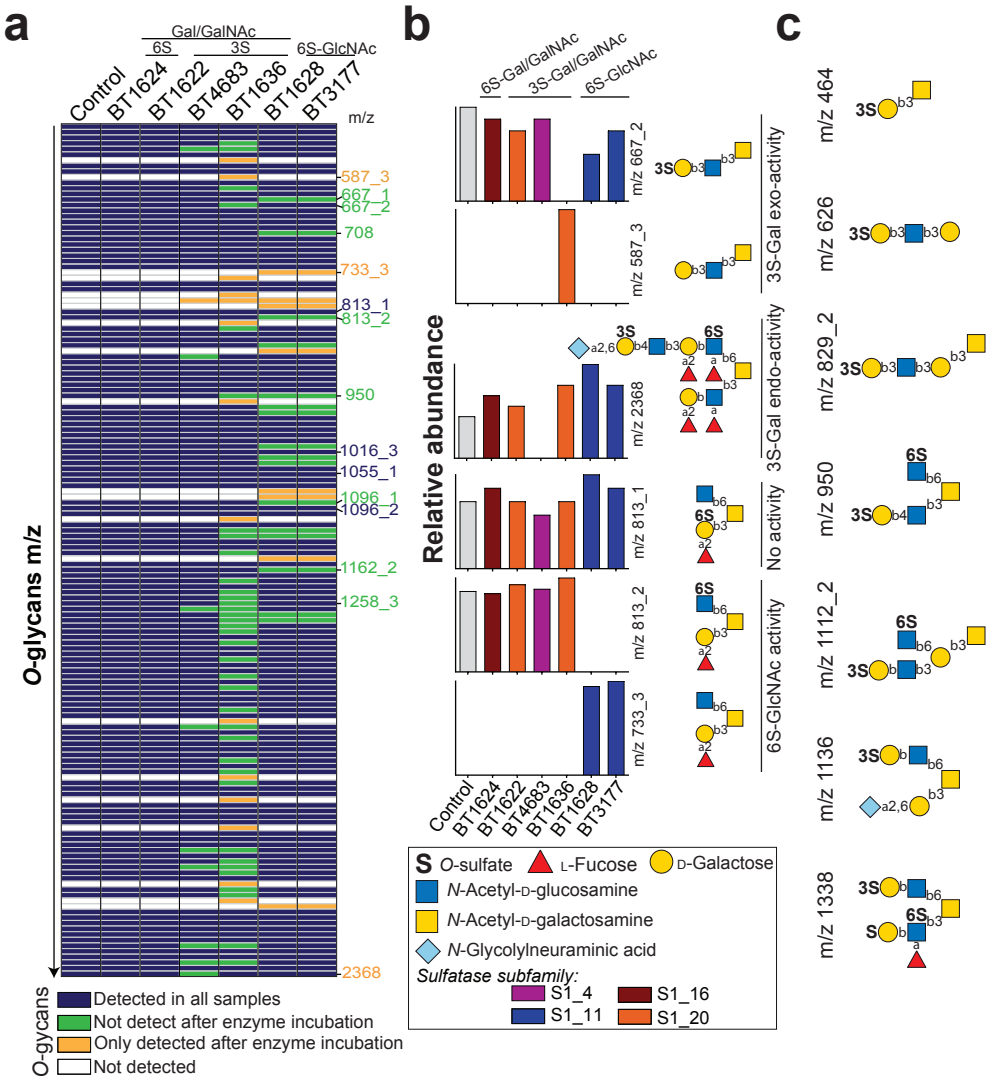

a
Gal/GalNAc
6S
3S
6S-GlcNAc
Control
BT1624
BT1622
BT4683
BT1636
BT1628
BT3177
m/z
587_3
667_1
667_2
708
733_3
813_1
813_2
950
1016_3
1055_1
1096_1
1096_2
1162_2
1258_3
2368
O-glycans m/z
O-glycans
Detected in all samples
Not detect after enzyme incubation
Only detected after enzyme incubation
Not detected
b
6S-Gal/GalNAc
3S-Gal/GalNAc
6S-GlcNAc
Relative abundance
m/z 667_2
m/z 587_3
m/z 2368
m/z 813_1
m/z 813_2
m/z 733_3
3S-Gal exo-activity
3S-Gal endo-activity
No activity
6S-GlcNAc activity
S O-sulfate
L-Fucose
D-Galactose
N-Acetyl-D-glucosamine
N-Acetyl-D-galactosamine
N-Glycolylneuraminic acid
Sulfatase subfamily:
S1_4
S1_11
S1_16
S1_20
c
m/z 464
m/z 626
m/z 829_2
m/z 950
m/z 1112_2
m/z 1136
m/z 1338

Fig 2.

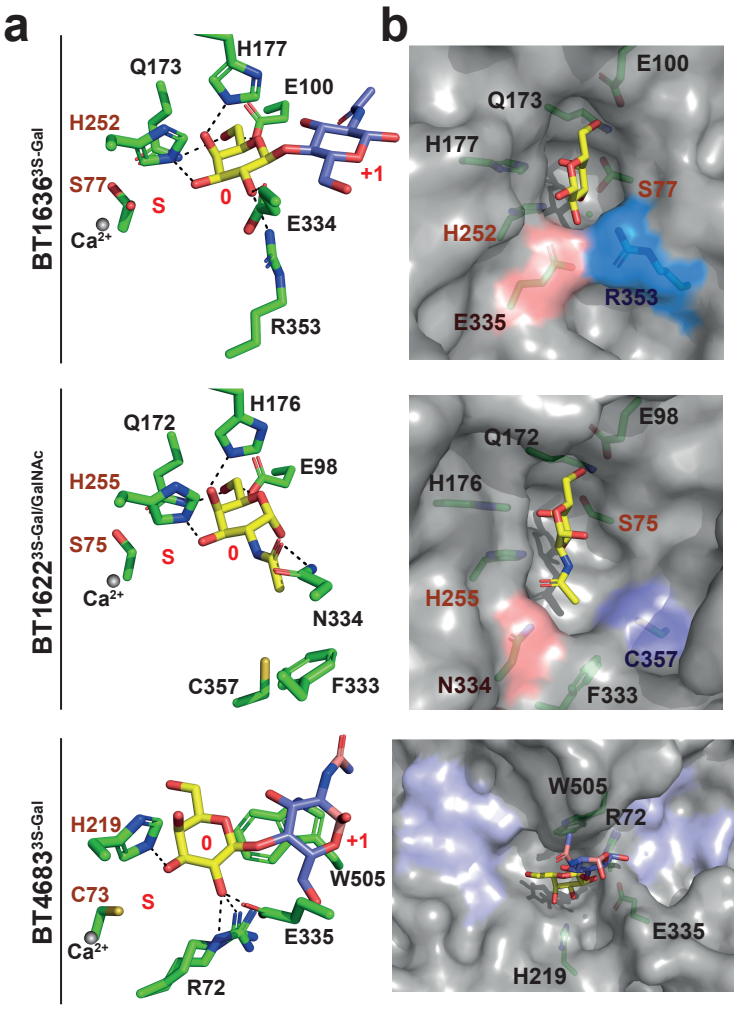

a
b
BT1636 3S-Gal
Q173
H177
E100
H252
S77
S
0
+1
Ca2+
E334
R353
E100
Q173
H177
S77
H252
R353
E335
BT1622 3S-Gal/GalNAc
Q172
H176
E98
H255
S75
S
0
Ca2+
N334
C357
F333
E98
Q172
H176
S75
H255
C357
N334
F333
BT4683 3S-Gal
H219
0
+1
W505
C73
S
Ca2+
E335
R72
W505
R72
E335
H219

Fig 3.

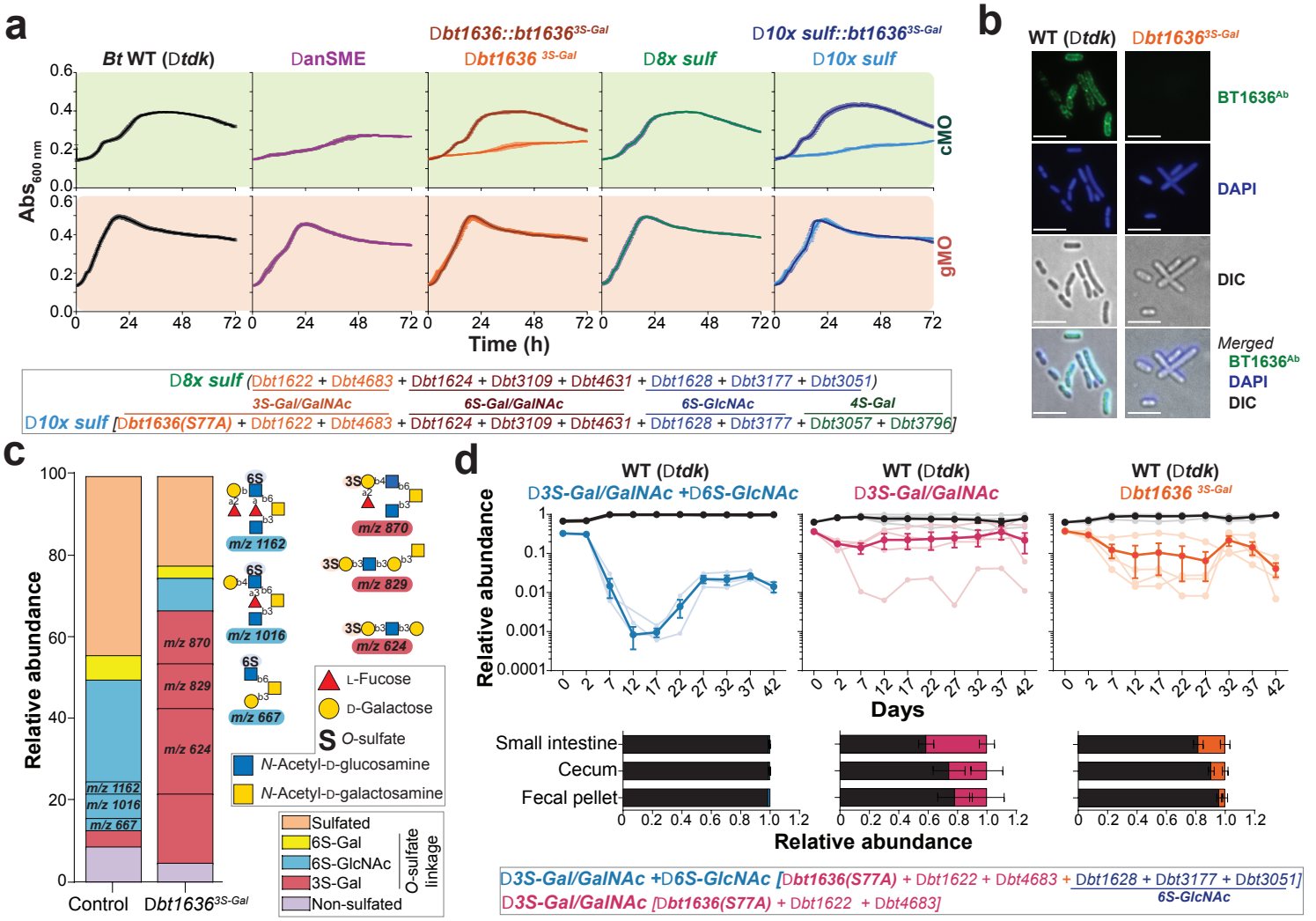
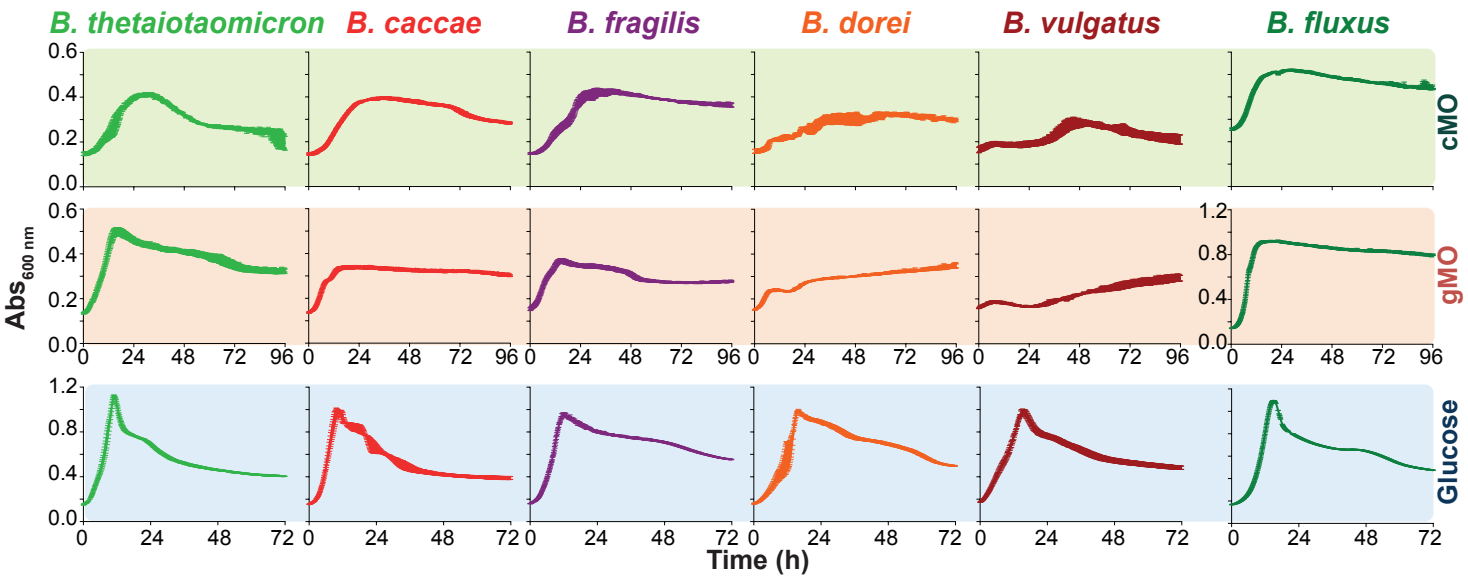
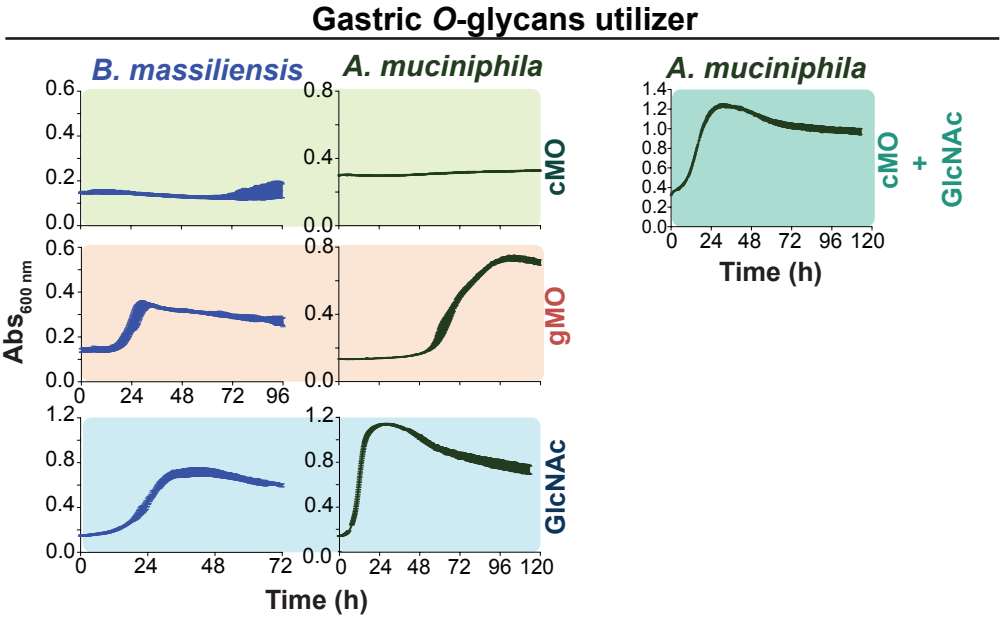
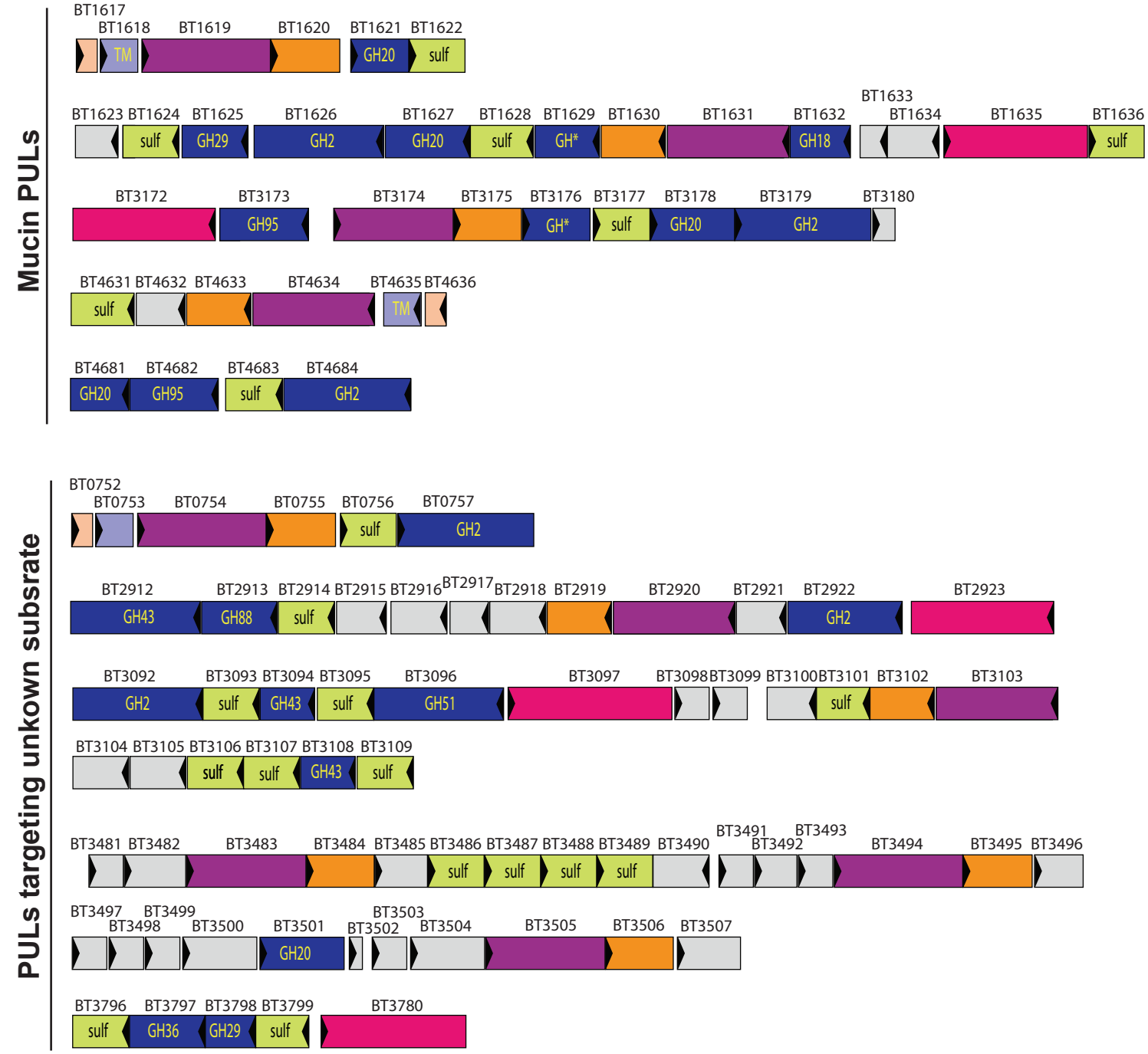

a
Bt WT (Dtdk)
DanSME
Dbt1636::bt1636 3S-Gal
Dbt1636 3S-Gal
D8x sulf
D10x sulf::bt1636 3S-Gal
D10x sulf
cMO
gMO
Abs 600 nm
Time (h)
D8x sulf (Dbt1622 + Dbt4683 + Dbt1624 + Dbt3109 + Dbt4631 + Dbt1628 + Dbt3177 + Dbt3051)
3S-Gal/GalNAc
6S-Gal/GalNAc
6S-GlcNAc
4S-Gal
D10x sulf [Dbt1636(S77A) + Dbt1622 + Dbt4683 + Dbt1624 + Dbt3109 + Dbt4631 + Dbt1628 + Dbt3177 + Dbt3057 + Dbt3796]
b
WT (Dtdk)
Dbt1636 3S-Gal
BT1636Ab
DAPI
DIC
Merged
BT1636Ab
DAPI
DIC
c
Relative abundance
Control
Dbt1636 3S-Gal
m/z 1162
m/z 1016
m/z 667
m/z 870
m/z 829
m/z 624
L-Fucose
D-Galactose
O-sulfate
N-Acetyl-D-glucosamine
N-Acetyl-D-galactosamine
Sulfated
6S-Gal
6S-GlcNAc
3S-Gal
Non-sulfated
O-sulfate linkage
d
WT (Dtdk)
D3S-Gal/GalNAc +D6S-GlcNAc
WT (Dtdk)
D3S-Gal/GalNAc
WT (Dtdk)
Dbt1636 3S-Gal
Relative abundance
Days
Small intestine
Cecum
Fecal pellet
Relative abundance
D3S-Gal/GalNAc +D6S-GlcNAc [Dbt1636(S77A) + Dbt1622 + Dbt4683 + Dbt1628 + Dbt3177 + Dbt3051]
6S-GlcNAc
D3S-Gal/GalNAc [Dbt1636(S77A) + Dbt1622 + Dbt4683]

Fig 4.

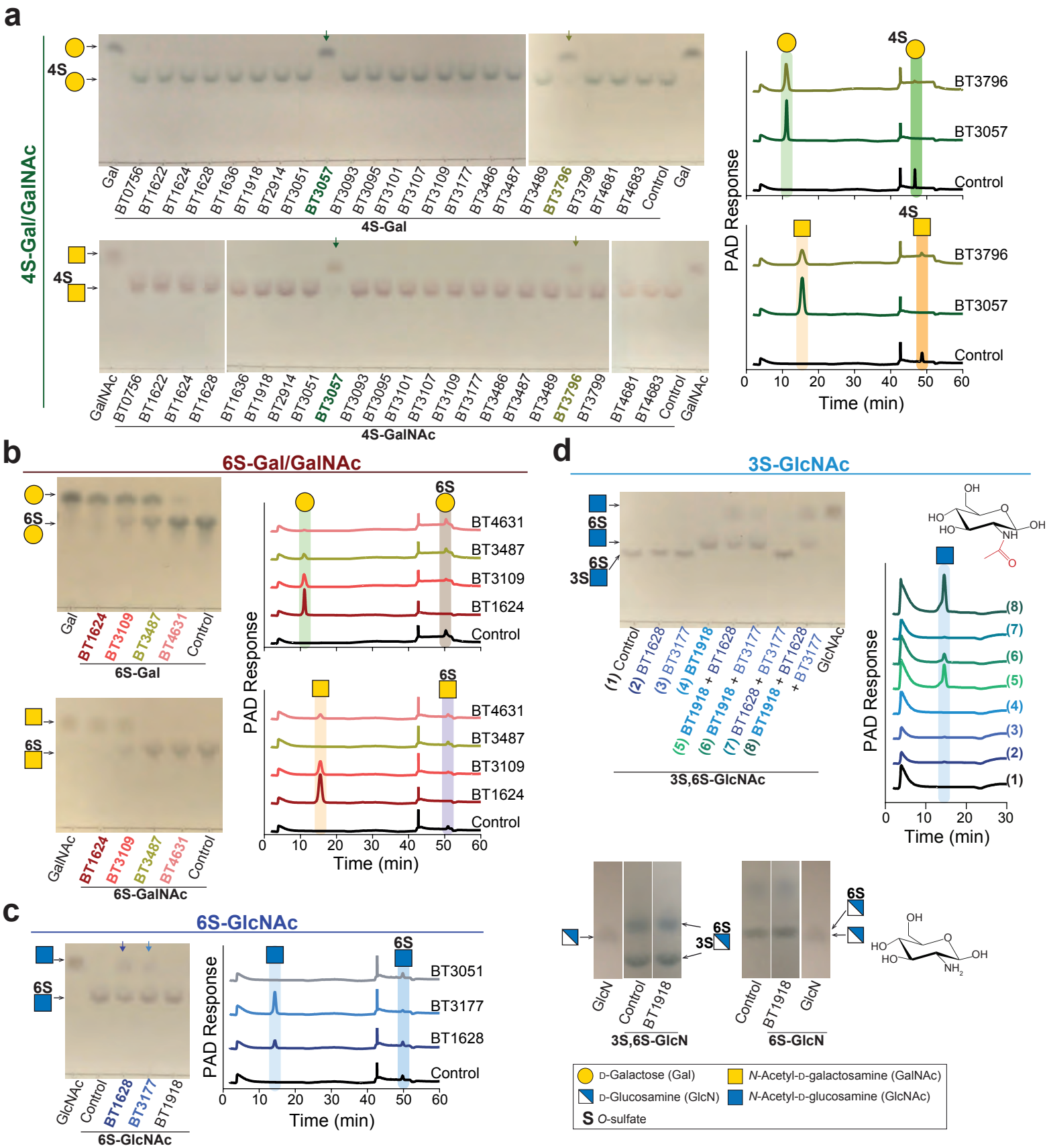



**Extended Data Figure 1**




**Extended Data Figure 2**

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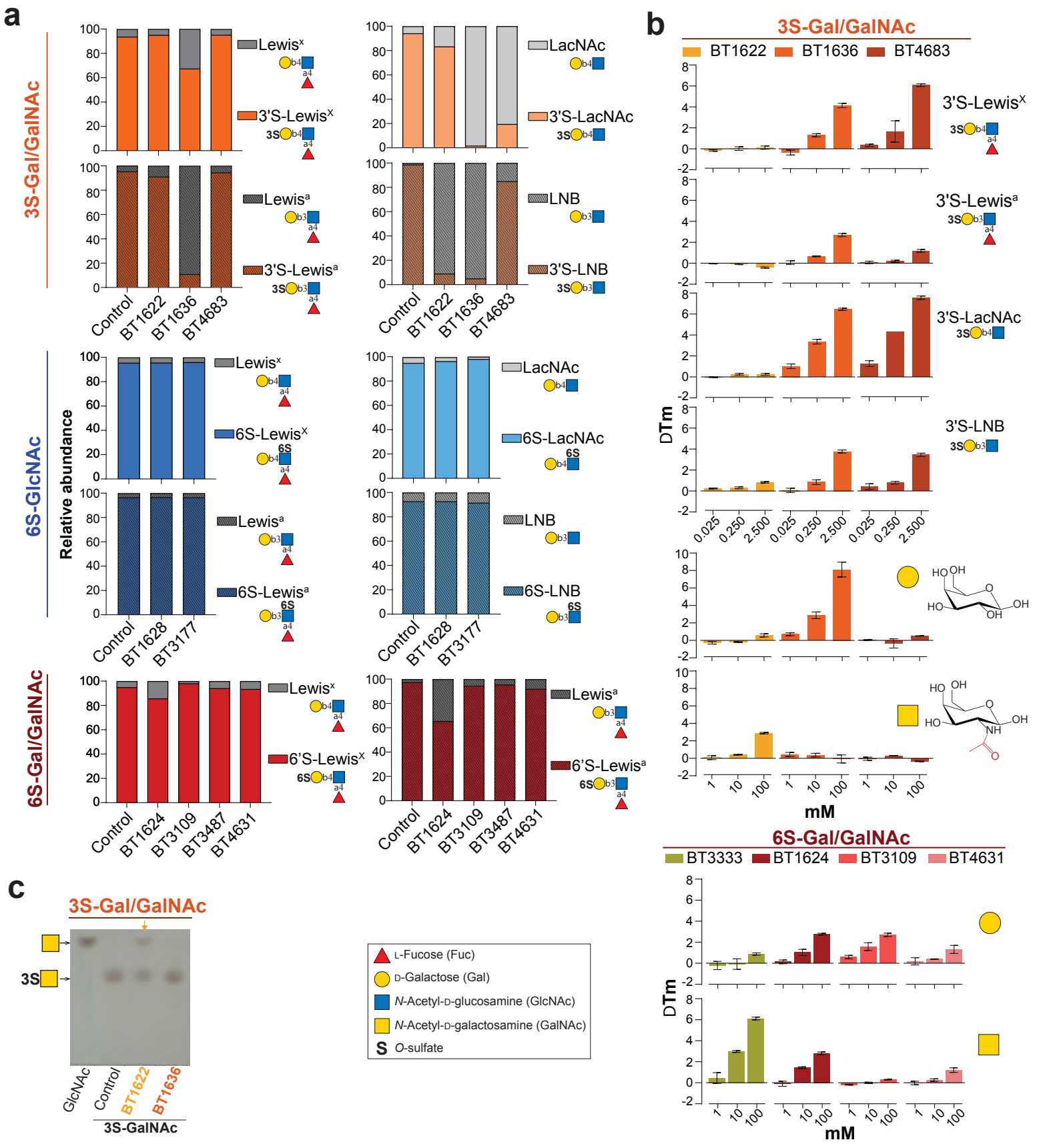
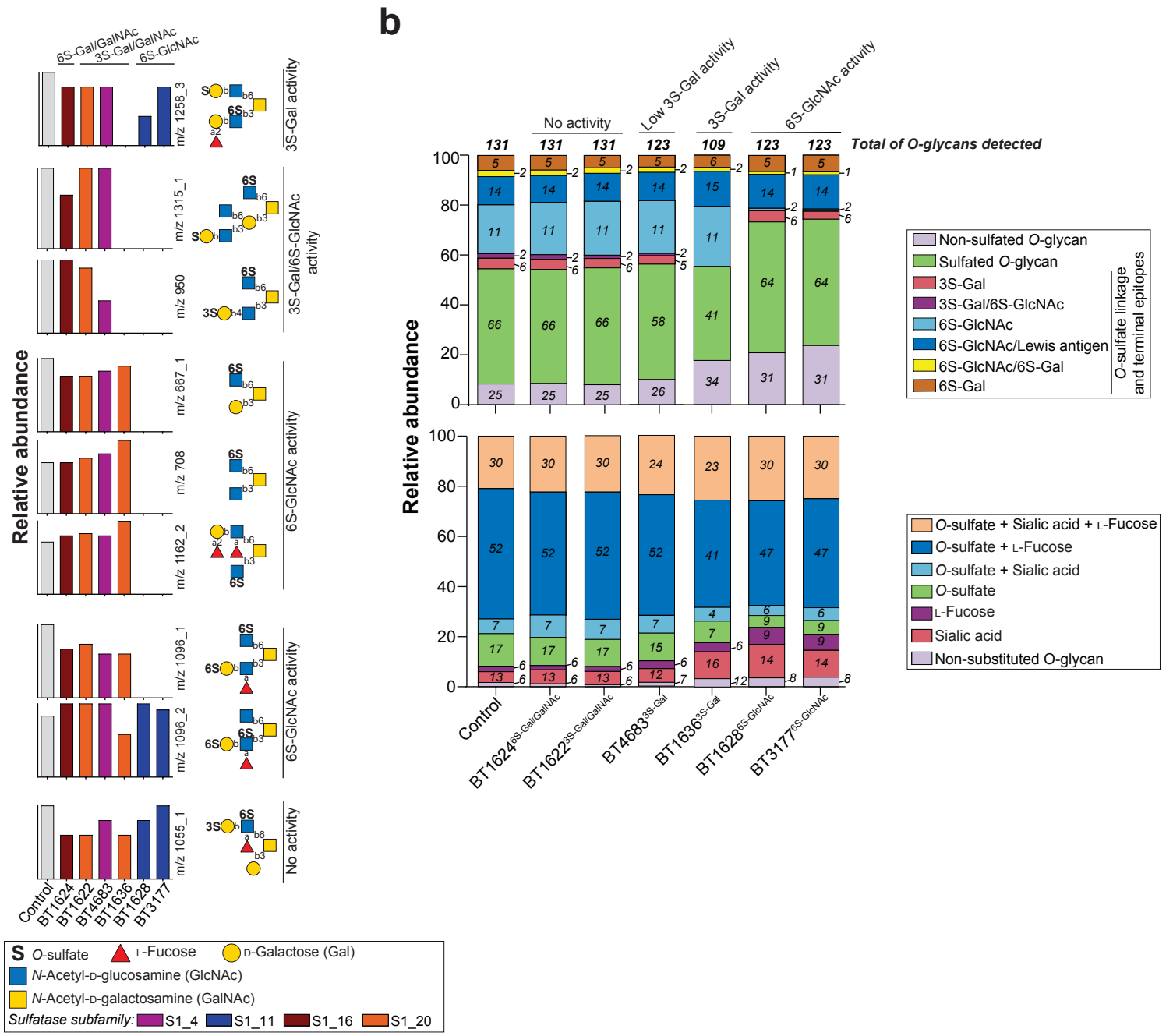
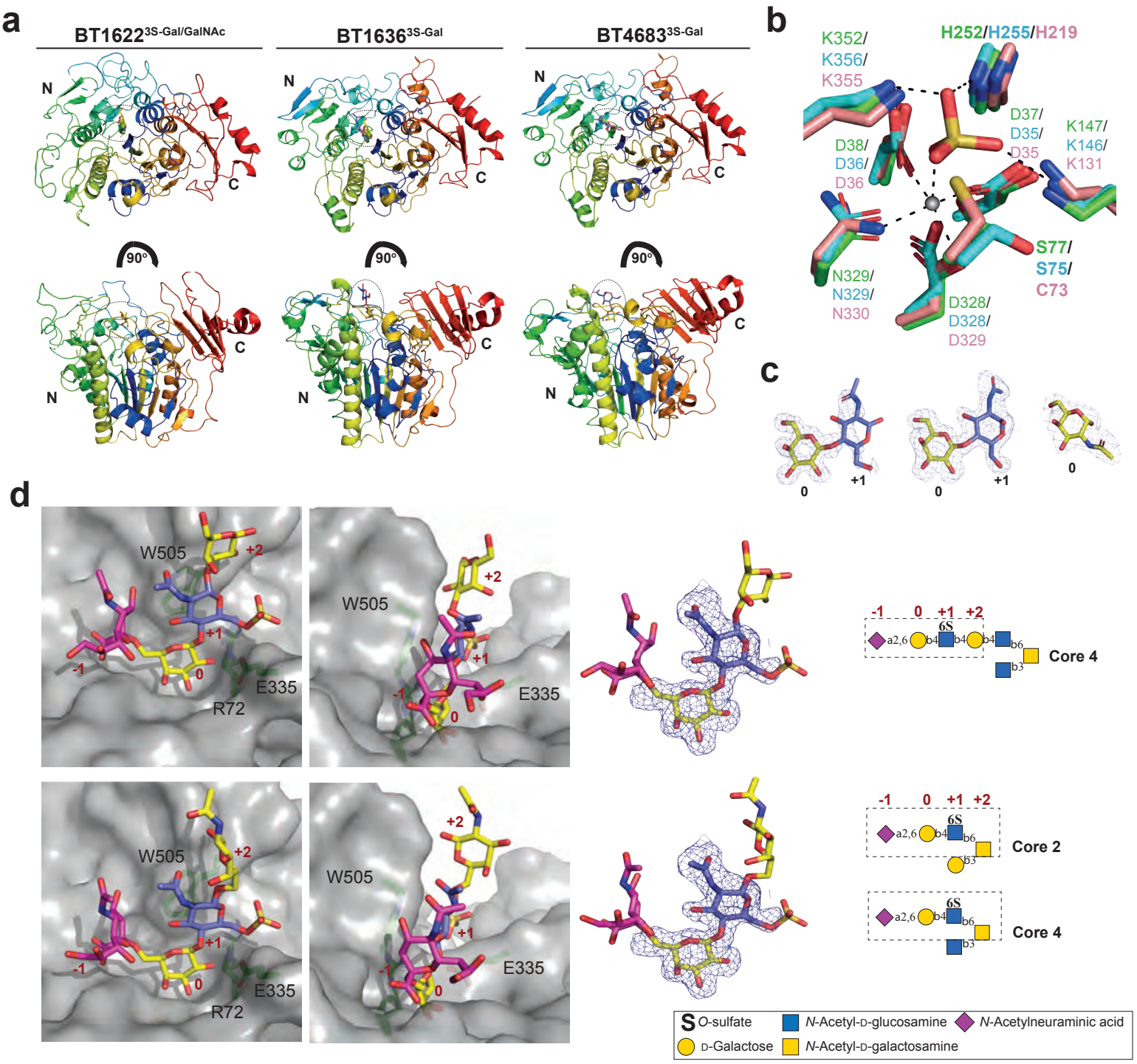


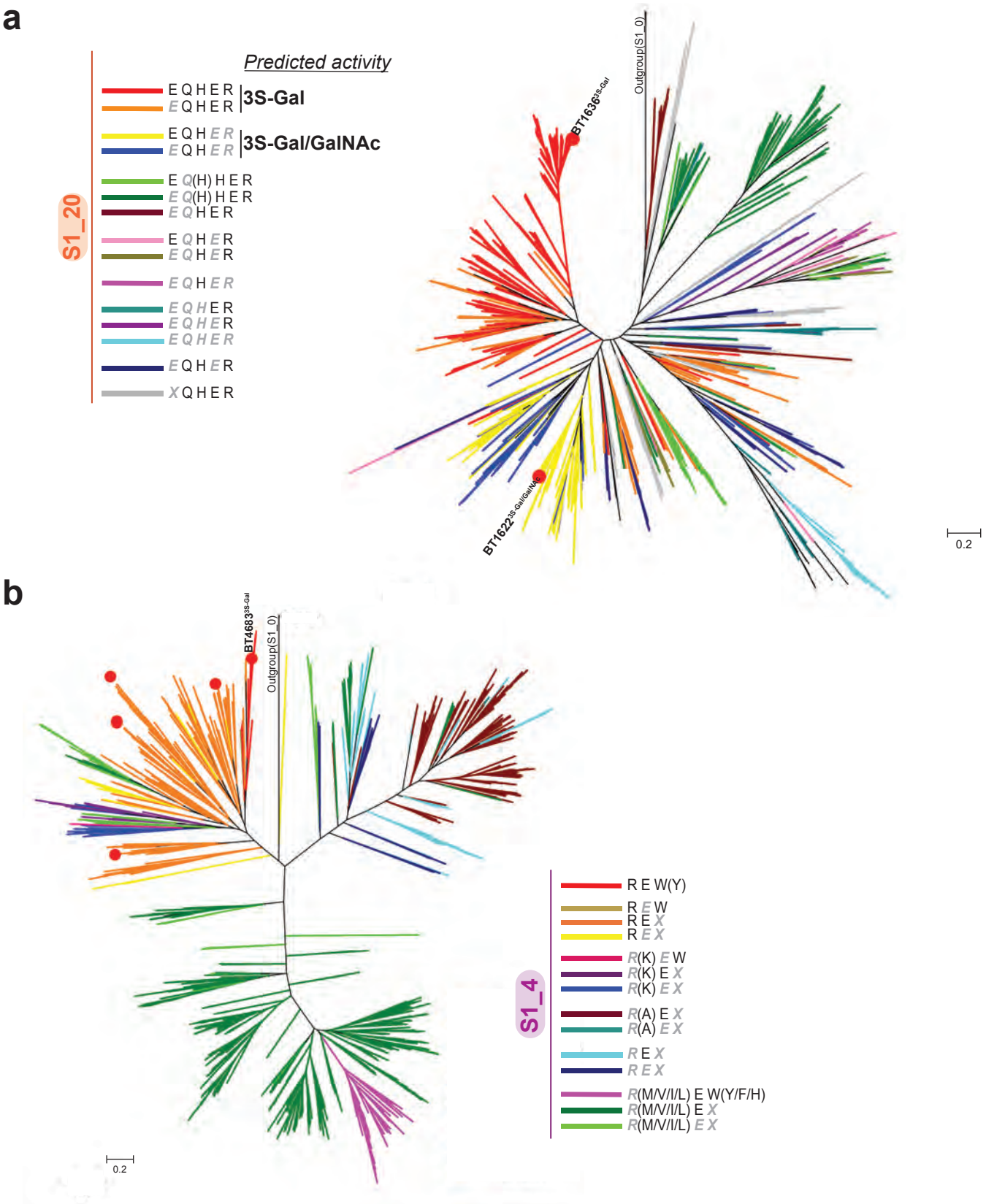

Extended Data Figure 4



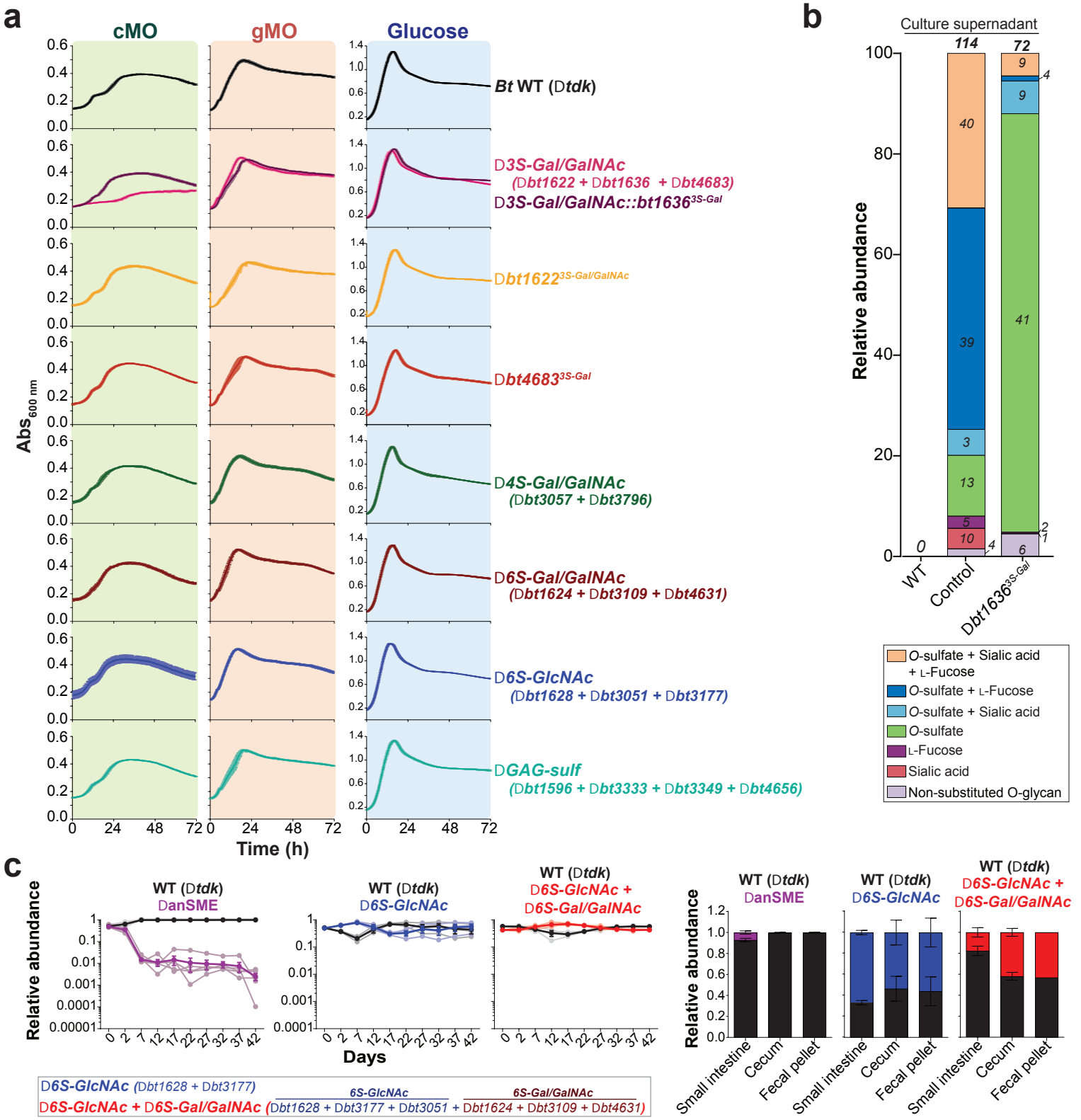

**Extended Data Figure 5**




**Extended Data Figure 6**




**Extended Data Figure 7**




# Extended Data Figure 8

## Supplemental Discussion

### 1. *Utilization of different mucin O-glycans sources by HGM*

Mucin composition varies through the gastrointestinal tract (GI), with the stomach having mainly Muc5AC and the colon mainly Muc2[1]. The glycosylation of these respective mucins also varies along the GI tract with higher levels of sulfation and sialylated structures observed in the distal colon compared to the upper GI tract[2]. From the 20 bacterial strains tested for growth in this study, 12 failed to grow on gastric mucin *O*-glycans (gMO) or colonic mucin *O*-glycans (cMOs) (**Fig. 1**). Only 6 bacteria were able to utilize both *O*-glycans substrates but growth was variable. In both *O*-glycans substrates, *Bacteroides thetaiotaomicron* (*Bt*), *B. caccae*, *B. fragilis* and *B. fluxus* grew better than *B. dorei* and B*. vulgatus* (**Fig. 1** and **Extended Data Fig. 1**). The differences observed in the growth profiles were reproducible in two different batches of purified cMOs (**Fig. 1**) Indeed, it is likely that different HGM members evolve to target different (or only a subset) of the available *O*-glycans and that this fine-tuning of host glycan utilization may have important implications in gut colonization and symbiosis. Additionally, *B. massilensiens* and *Akkermansia mucinipila* (*Amuc*) grew on gMO but failed to utilize cMO (**Fig. 1** and **Extended Data Fig. 1**). Both strains were able to grow on *N*-acetylglucosamine (GlcNAc) and *Amuc* grew on GlcNAc in the presence of cMO suggesting that these *O*-glycans do not inhibit the growth of this bacterium. Previous studies have determined *B. massiliensis* and *Amuc* to be mucin-degraders by demonstrating growth on gastric mucins[3,4]. However, the lack of growth in colonic *O*-glycans suggests that these bacteria are not able to initiate the degradation of more complex and sulfated colonic glycans. This finding highlights the importance of taking in account *O*-glycosylation differences along the GI tract and the need to utilize colonic mucins to draw conclusions regarding the colonic HGM.

### 2. **Sulfatase activity in cMO**

Despite all 12 sulfatases being active on defined oligosaccharides, of those tested on cMO, BT1622$^{3S\text{-}Gal/GalNAc}$ and BT1624$^{6S\text{-}Gal/GalNAc}$ did not show any activity on this complex substrate (**Fig. 2, Extended Data Fig. 5** and **Supplementary Table 5)**.

These findings are consistent the results observed in defined commercial oligosaccharides where BT1622$^{3S\text{-}Gal/GalNAc}$ showed a preference to for sulfated GalNAc over Gal glycans (**Extended Data Fig. 4**) and BT1624$^{6S\text{-}Gal/GalNAc}$ activity is blocked by the presence of additional substitutions (such as Lewis antigens) (**Extended Data Fig. 4a**). Additionally, 2 of 6 detected 6S-Gal structures contained a capping sialic acid and a terminal blood group H type 2 [Fuc-α1,2-(6S)Gal-] (**Fig. 2**). The lack of activity of BT1624$^{6S\text{-}Gal/GalNAc}$ towards such structures confirms the exo-mode of action that we described for this sulfatase using commercial substrates.

Overall, when compared to the non-enzyme treated control, we detected an increase of non-sulfated structures and decrease of sulfated oligosaccharides in all samples with the active enzymes (BT4683$^{3S\text{-}Gal}$, BT1636$^{3S\text{-}Gal}$, BT1628$^{6S\text{-}GlcNAc}$ and BT3177$^{6S\text{-}GlcNAc}$) (**Extended Data Fig. 5b**). BT1636$^{3S\text{-}Gal}$ was active towards all detected 3S-Gal structures with the exception of glycan m/z 1055_1 that is a doubly sulfated 3S-Gal/6S-GlcNAc fucosylated structure (**Extended Data Fig. 5a**). As we observed using commercial substrates , the presence of Lewis a/x epitopes leads to a decrease in the activity of this sulfatase (**Extended Data Fig. 4a** and **Supplementary Table 5**) and the presence of a second sulfate group might exacerbate this negative effect leading to the lack of activity towards this complex sulfated *O*-glycan. The incubation of the 6S-GlcNAc sulfatases BT1628$^{6S\text{-}GlcNAc}$ and BT3177$^{6S\text{-}GlcNAc}$ with cMO suggests that these enzymes are redundant, but because they are encoded in different PULs they could be expressed in response to different activating cues (**Fig. 2** and **Supplementary Table 5)**. Compared to the non-enzyme treated control, 16 glycans were not detected after incubation with these sulfatases, 14 of these structures have a terminal 6S-GlcNAc (**Fig. 2** and **Supplementary Table 5)**. BT1628$^{6S\text{-}GlcNAc}$ and BT3177$^{6S\text{-}GlcNAc}$ were active in 6S-GlcNAc core 3 (GlcNAc-β1,3-GalNAc) and core 4 (GlcNAc-β1,6-GalNAc) structures (**Extended Data Fig. 5a**), suggesting that these sulfatases are well suited to accommodate the variations in linkages/sugars found in mucin *O*-glycans. Additionally, we also detect 7 new glycans that are likely to be reaction products of BT1628$^{6S\text{-}GlcNAc}$ and BT3177$^{6S\text{-}GlcNAc}$ (**Fig. 2** and **Supplementary Table 5)**.

The identification and characterization of the first sulfatases active on mucin *O*-glycans creates the opportunity to improve our understanding of *O*-glycan structures by using these enzymes as analytical tools. After the treatment with BT1636$^{3S\text{-}Gal}$

several oligosaccharides predicted to contain a terminal sulfate linked to Gal were not detected. Although we could not determine the specific sulfate linkage by mass spectrometry, the activity of the 3S-sulfatase suggests that these oligos contain a terminal 3S-Gal (**Extended Data Fig. 5a** and **Supplementary Table 5**). The specificity of the 6S-GlcNAc sulfatases for non-fucosylated *O*-glycans also illuminates their potential use as tools to characterize the structure of these complex structures since it allows the differentiation of different isomers. For example, we detect two oligosaccharides with m/z 1096, however, after incubation with BT1628$^{6S\text{-}GlcNAc}$ or BT3177$^{6S\text{-}GlcNAc}$, only the isomer 1096_2 was detected, indicating that the isomer 1096_1 contain a terminal 6S-GlcNAc (**Extended Data Fig. 5a** and **Supplementary Table 5**).

3. ***Conserved structural features of the S1 formylglycine family***

*Protein fold and subsites nomenclature*

S1 sulfatases comprise the most common and largest family of sulfatases, currently encompass 36,816 members in sulfAtlas and are found in all domains of life[5]. S1 sulfatases are part of the alkaline phosphatase superfamily and adopt an alkaline phosphatase-like fold. This is an N-terminal $\alpha/\beta/\alpha$ domain with S1 sulfatases also possessing a smaller C-terminal 'sub domain'. The active site is located in the N-terminal domain that has a large mixed $\beta$-sheet composed of ~10 $\beta$ strands, sandwiched between $\alpha$ helices above and below. The C-terminal 'sub-domain' is composed of a 4 stranded antiparallel $\beta$-sheet and a single amphipathic terminal helix. This C-terminal domain abuts the N-terminal domain through the antiparallel $\beta$-sheet with loops from the $\beta$ strands sometimes contributing to the active site architecture (**Extended data Fig. 6a**). The subsite nomenclature for carbohydrate sulfatases is such that the invariant sulfate binding site is denoted the S site. The S site sulfate is appended to is the 0 subsite sugar. Subsites then increase in number (i.e. +1, +2, +3) as the sugar moves toward the reducing end (free O1) and decreases in number as the sugar chain moves towards the non-reducing end (i.e. -1, -2, -3)[6].

*S1 formylglycine active site conservation*

The sulfate binding site (S site) is invariant across the S1 family and comprises the catalytic residues (nucleophile and catalytic acid) and a calcium binding site (**Extended data Fig. 7b**). An invariant histidine is likely the potential catalytic acid but a lysine has also been suggested to possibly fulfill this role[7]. The pKa of His is ~6.0, whilst Lys has a pKa of >10, making it more chemically feasible that His performs the role of the catalytic acid. Homologues of these residues (H252 and K352 in BT1636$^{3S\text{-}Gal}$) hydrogen bond to the scissile sulfoester linkage (**Extended data Fig. 6b**). Previously published work with BT1596 and BT4656, a 2S-Uronic acid and 6S-GlcNAc sulfatase, respectively, showed that the mutation of either residue to alanine causes inactivation[7]. Consistent with this work, a BT4683$^{3S\text{-}Gal}$ H219A mutant was inactive. However, in BT1622$^{3S\text{-}Gal/GalNAc,}$ the mutation of H255 to Ala caused only a ~30 fold decrease in activity (**Supplementary Table 3**). Thus, it is possible that in BT1622$^{3S\text{-}Gal/GalNAc}$ the loss of H255 is compensated by the invariant residue K356 and interestingly BT1622$^{3S\text{-}Gal/GalNAc}$ has a pH optimum ~2 units higher than most sulfatases assayed (**Supplementary Fig. 4**).

The calcium binding site is located at the base of the S site interacting with the sulfate group. This calcium ion is an essential component of the catalytic mechanism helping to stabilise negative charges that occur during the catalysis. All three of the solved structures had occupation for calcium. In BT1636$^{3S\text{-}Gal}$ D328 and the sulfate group of the substrate coordinate above and below the calcium with D37, D38, N329 and the formylglycine binding in a plane completing an octahedral coordination (**Extended Data Fig. 6b**). These three Asp and the Asn coordinated with calcium are structurally conserved in all 3S-Gal/GalNAc sulfatases structures (Extended data Fig. 6b).

The solved structures of BT1636$^{3S\text{-}Gal}$ and BT1622$^{3S\text{-}Gal/GalNAc}$ were native *Bt* proteins having a Ser at the formylglycine position. However, structure of BT4683$^{3S\text{-}Gal}$ was obtained with the active protein where S73 was mutated to Cys (as *E. coli* can only convert Cys, not Ser, to formylglycine). The analysis of BT4683$^{3S\text{-}Gal}$ reveals that the crystallized protein still has the Cys and not formyglycine indicating poor installation of the formylglycine. This observation means the kinetic data, (although the rates are significant and readily measurable) may be an underestimation of true catalytic performance. This will affect the $k_{cat}$ component of the $k_{cat}/K_M$ measurement and thus the $k_{cat}/K_M$ reported in **Supplementary Table 3** is an underestimate of the true activity.

*Additional 3S-Gal/GalNAc specificity determinants based on structures*

BT1636$^{3S\text{-}Gal}$ was solved in complex with the product LacNAc; the 0subsite galactose is well ordered and makes extensive interactions, whilst the +1 GlcNAc is highly disordered and appears to make no interactions with the protein (**Fig. 3** and **Extended Data Fig. 6c**). O2 of the 0 Gal hydrogen bonds with Oε1 of E334 and NH2 of R353. Mutation of these residues to Ala causes ~300 and ~60-fold reductions in $k_{cat}/K_{M,}$ respectively. The O6 group of Gal potentially coordinates with Oε2 of E100 and Nε2 of Q173 and mutations of these residues to Ala cause ~80 and ~50-fold decreases in $k_{cat}/K_{M,}$ respectively (**Fig. 3** and **Supplementary Table 3**). Comparison of the BT1622$^{3S\text{-}Gal/GaNAc}$ structure with BT1636$^{3S\text{-}Gal}$ shows that E98 and Q172 (which correspond to E100 and Q173 in BT1636$^{3S\text{-}Gal}$) are conserved **(Fig. 3)** and mutating E98 to Ala caused only a 15-fold decrease in $k_{cat}/K_{M}$ (**Supplementary Table 3**). Additionally, in BT1622$^{3S\text{-}Gal/GaNAc}$ the hydrophobic interactions with the *N*-acetyl group, and the more open pocket, may offset the effects H176A (300-fold loss in activity) when compared to H177A (complete loss in activity) in BT1636$^{3S\text{-}Gal}$ (**Fig. 3** and **Supplementary Table 3**).

BT4683$^{Gal\text{-}3s}$ also displayed the same 3S Gal activity as the S1_20 enzymes but showed a preference for 3S-LacNAc, reciprocal to BT1622$^{3S\text{-}Gal}$. BT4683$^{3S\text{-}Gal}$ bound the O2 Gal of LacNAc via Oε2 of E335 (equivalent to E334 in BT1636$^{3S\text{-}Gal}$) and through either Nε or NH1 of R72 **(Fig. 3)**. Although R72 is sequentially distal to R353 in BT1636$^{3S\text{-}Gal}$ it is spatially similar and likely contributes in a similar capacity **(Fig. 3)**. Despite the mutations R72A and E335A resulting in loss of activity, the Glu and Arg are only conserved in 62 % and 19 % of S1_4 sequences, respectively, suggesting there is a significant but not absolute selection for an equatorial O2 in this subfamily (**Extended Data Fig. 7** and **Supplementary Fig. 2**). Uniquely among the 3S-Gal sulfatases identified, BT4683$^{3S\text{-}Gal}$ utilises a hydrophobic stacking interaction through W505 to provide a platform for the +1 GlcNAc and partially the 0 Gal. Mutation of W505 to Ala almost completely abolishes activity on 3S-LacNAc (**Supplementary Table 3**) but surprisingly this residue is not conserved in our phylogenetic analyses of S1_4 being present in only 8 other sequences (**Extended Data 7** and **Supplementary Fig. 3**). It is important to note here, that overall W505 is not well conserved; potential equivalent aromatic residues can be found in some additional clades, coloured light brown (or bronze), pink or dark red, but it is not evident from the alignment that these

are true equivalents. Only 3D structural work will be able to show if these aromatic residues take equivalent positions in those sulfatases. Additionally, the BT4683$^{3S\text{-}Gal}$ activity against defined sulfated saccharides was suggestive of an exo-acting enzyme that cleaves terminal 3S-Gal (**Extended Data Fig. 4**). However, a close analysis of this sulfatase structure shows that the active site is located in an open cleft characteristic of an endo-active enzyme[6]. This more open cleft of BT4683$^{3S\text{-}Gal}$ may allow additional sugars/sulfates to be accommodated on the O6 of both the 0 Gal and +1 GlcNAc. Indeed, the activity determined in cMO shows that this sulfatase can act on sialylated *O*-glycan (**Fig. 2**). Further modelling of different *O*-glycan structures (using the crystallographically solved LacNAc as an 'anchor') indicate that this enzyme can accommodate complex *O*-glycans with internal sulfation **(Fig. 3 and Extended Data 7)**. Together, these results suggest that BT4683$^{Gal\text{-}3S}$, and its close homologues, could be endo 3S sulfatases where the 0 subsite specificity for Gal is driven by glycan context and/or distal subsites such as -1 and +2, rather than an axial O4 as in S1_20.

Additionally, it is unclear why BT1636$^{Gal\text{-}3S}$ acts better on LacNAc substrates than BT1622$^{3S\text{-}Gal/GalNAc}$. It is interesting to note, however, that both BT1636$^{3S\text{-}Gal}$ and BT4683$^{3S\text{-}Gal}$ perform well on LacNAc configured substrates and utilise an Arg and Glu to coordinate O2 whilst BT1622$^{3S\text{-}Gal}$ lacks these residues **(Fig. 3)**. These residues may lead to the enhanced activity on LacNAc (β1,4 glycan) vs. LNB (β1,3 substrate). Another thing to note is that a β1,4 vs β1,3 linkage will rotate the GlcNAc ~60° but switch the position of the Fuc residue from being on the '*N*-acetyl side' of the glycosidic bond to the 'O6 side' of the glycosidic bond, and this may also be the cause of the differential activities on β1,4 vs β1,3 linked substrates.

*Phylogenetic analyses of S1_20 specificity determinants*

The essential His that acts as a key specificity determinant of galacto- over gluco-substrates (H177 and H176 in BT1636$^{3S\text{-}Gal}$ and BT1622$^{3S\text{-}Gal/GalNAc}$, respectively) is highly conserved (92% of S1_20 sequences) (**Extended data Fig. 7**). The Gln (Q173 and Q172 in BT1636$^{3S\text{-}Gal}$ and BT1622$^{3S\text{-}Gal/GalNAc}$, respectively) is only conserved in 66% of sequences and in 25% of the cases is substituted with a histidine, a residue that can also fulfil the same role of Gln interacting with Gal O6. Indeed, these conserved residues are located in a highly conserved domain with the consensus sequence [CDNS]-[QH]-[RVF]-[QHLD]-[AG]-H-[NRST]-[YHF]-[YF]-P (Prosite syntax).

With H177 targeting the axial O4 of Gal directly, a Q173 may function indirectly to select for an axial O4 and thus these residues may operate as a selectivity 'dyad' for Gal with S1_20. Additionally, the residues implicated in recognition of Gal over GalNAc, E335 and R353 in BT1636$^{3S\text{-}Gal}$ are conserved in 64 and 74% of S1_20 sequences, whilst the residue that allows the accommodation of O2 N-acetyl and activity in GalNAc (N334 in BT1622$^{3S\text{-}Gal/GalNAc}$) is only found in 8% of members of this family (**Extended Data Fig. 7** and **Supplementary Fig. 3**). This observation suggests that the majority of the S1_20 sulfatases evolved to target sulfated Gal and only a subset of this subfamily's members can actually also be active on GalNAc. Interestingly, all of the close homologs of BT1636$^{3S\text{-}Gal}$ and BT1622$^{3S\text{-}Gal/GalNAc}$ that share the critical specificity determinants of these proteins (**Supplementary Tables 6 and 7**) were isolated from mammals at body regions rich in mucins, highlighting the role of these sulfatases in accessing sulfated host glycans.

4. **Growth of sulfatase mutants on *O*-glycans**

The deletion strain lacking 4S-Gal/GalNAc sulfatases (*Δbt3057* + *Δbt3796*) did not show any phenotype in cMO (**Extended Data Fig. 9a**), a result that is consistent with the lack of these sulfated linkages in colonic mucins (**Supplementary Table 5**). Unexpectedly, the deletion strains lacking the identified 6S-Gal/GalNAc sulfatases (*Δbt1624* + *Δbt3109* + *Δbt4631*) and 6S-GlcNAc sulfatases (*Δbt1628* + *Δbt3051* + *Δbt3177*) also did not show any phenotype on cMOs (**Extended Data Fig. 8a**). Analysis of cMO by MS showed that this substrate contains a low abundance of sulfate linked to O6 of Gal but a relatively high abundance of sulfate linked to *O*6 of GlcNAc, especially in shorter structures (**Supplementary Table 5**). Although the low abundance of O6-sulfated Gal could explain the lack of phenotype of the 6S-Gal/GalNAc sulfatase deficient strain, the lack of a phenotype of the *Δ6S-GlcNAc* mutant in cMO was unexpected (**Extended Data Fig. 8a**). Due to the limitations of the MS technique it is not possible to analyse sulfation in longer oligos, making it unclear what the real complexity of structures found in colonic mucins are. Indeed, the lack of phenotype of *Δ6S-GlcNAc* mutant in cMO suggests that 6S-GlcNAc might not be a major terminal epitope in colonic mucins. It is also important to note that the mutant *Δ6S-GlcNAc* is the deletion of two characterized 6S-GlcNAc sulfatases active on cMO (BT1628$^{6S\text{-}GlcNAc}$ and BT3177$^{6S\text{-}GlcNAc}$) and a third closely related S1_11 sulfatase

(BT3051$^{putative_6S\text{-}GlcNAc}$) for which no activity was also deleted to avoid possible compensation of function after loss of BT1628$^{6S\text{-}GlcNAc}$ and BT3177$^{6S\text{-}GlcNAc}$ activities.

The deletion of previously characterized GAG-specific sulfatases[8] (*Δbt1596* + *Δbt3333* + *Δbt3349* + *Δbt4656*) did not result in any observable phenotype in cMO (**Extended Data Fig. 8a**), indicating that this substrate is not contaminated with additional endogenous host glycans. Additionally, despite some mutants exhibiting growth defects on sulfated cMO, all of the mutants grew well on gMO and glucose (**Fig. 4** and **Extended Data Fig. 9a**), suggesting that the phenotypes observed are dependent on the mucin source (colon) and cannot be observed utilizing mucins from other regions of the gastrointestinal tract. Together these results highlight the contribution of sulfatases in utilization of colonic mucins by the HGM.

5. **Analysis of Δ*bt1636*$^{Gal3Sulf}$ culture supernatant by MS**

The analysis of the oligosaccharides present in *Δbt1636*$^{3S\text{-}Gal}$ culture supernantant after 96h incubation revealed that this sample is different from the cMO profile in the starting material (**Fig. 4c**, **Extended Data Fig. 8b** and **Supplementary Table 8**). We detected 114 glycans in the cMO sample, of which 39 were substituted with *O*-sulfate and L-fucose (44% total) (**Extended Data Fig. 8b**) and, the three most common structures (12% total) were 6S-GlcNAc oligosaccharides (**Fig. 4c** and **Supplementary Table 8**). In the control sample, the levels of sulfation, sialylation and fucosylation were 92%, 40% and 77% (**Supplementary Table 8**). In the *Δbt1636*$^{Gal\text{-}3Sulf}$ culture supernatant, we detected 72 glycans, of which 41 were substituted only with *O*-sulfate (84% total) (**Extended Data Fig. 8b**). In the mutant supernatant the levels of sulfation (95%) were similar to cMO, however the levels of sialylation (11%) and fucosylation (5%) decreased substantially (**Supplementary Table 8**), suggesting that the mutant is not able to utilize sulfated structures and these accumulate in culture media.

Additionally, a total of 98 of the 114 structures present in cMO were not detected in *Δbt1636*$^{3S\text{-}Gal}$ culture supernatant whereas in mutant supernatant, we detected 49 glycans that were not detected in the initial substrate (**Supplementary Table 8**). This unexpected result suggests that some of the oligosaccharides present in cMO can support the limited growth of *Δbt1636*$^{3S\text{-}Gal}$ and, although this mutant is not able to utilize many sulfated cMO structures it can still modify the glycans to create new structures. It remains unclear which enzymes are encoded by the mutant to modify

the *O*-glycans but the presence of a cell surface sialidase[9] can explain the decrease of sialylation levels in structures found in *Δbt1636*$^{3S\text{-}Gal}$ supernatant. Additionally, the presence of surface endo acting glycoside hydrolases able to cleave *O*-glycans into shorter oligosaccharides[10] while preserving structures like sulfate at the non-reducing end can also contribute to new glycan structures in the mutant culture supernatant. Together these results show that *Δbt1636*$^{3S\text{-}Gal}$ is not able to utilize most sulfated *O*-glycans explaining the limited growth of this mutant in cMO.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