A Bacteroidetes locus dedicated to fungal 1,6-β-glucan degradation: Unique substrate conformation drives specificity of the key endo-1,6-β-glucanase

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The Bacteroidetes are successful colonizers of the human gut, in large part because of their ability to rapidly adapt their metabolism to allow utilization of a wide variety of complex polysaccharides from both the diet and the host (1–4). The Bacteroidetes glycan degradation systems consist of genes arranged into co-transcribed loci called polysaccharide utilization loci (PULs).4 PULs are typically expressed at low levels in the absence of target glycan. However, when a substrate glycan is encountered, the corresponding PUL is rapidly up-regulated, often up to 1000-fold, driven by recognition of a specific oligosaccharide or monosaccharide cue (1). Surface enzyme(s) and glycan-binding proteins (SGBPs) orchestrate degradation of polysaccharides into smaller oligosaccharides that can be imported by the SusCD-like complex, a TonB-dependent membrane transporter (5). In the periplasm, additional enzymes depolymerize the imported oligosaccharides into their component monosaccharides, which are transported into the cytoplasm and then metabolized. The enzymes that degrade these glycans are mainly glycoside hydrolases (GHs); uronic acid-containing polysaccharides are depolymerized with the assistance of polysaccharide lyases. GHs are grouped into sequence-related subfamilies, which can be divided into sequence-related subfamilies, which can

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Recently, in addition to plant- and host-derived glycans, carbohydrates produced by microbes have been shown to be a source of nutrients for Bacteroides sp., and in particular Bacteroides thetaiotaomicron. B. thetaiotaomicron is able to degrade the extracellular polysaccharide of Lactobacillus spp., and the cell wall α-mannan from fungal species such as Saccharomyces cerevisiae and Candida albicans (9, 10). The ability to use microbial sources of glycans as nutrients may confer nutritional resilience upon B. thetaiotaomicron and related organisms in...
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the face of a variable supply of dietary carbohydrates. α-Mannan is an outer layer of the fungal cell wall in the species described above and covers skeletal layers of β-glucan and chitin. The heavily decorated mannoproteins of the cell wall are cross-linked through their glycosylphosphatidylinositol anchor to chains of 1,6-β-glucans that are in turn linked to both the 1,3-β-glucan and chitin chains (11).

When B. thetaiotaomicron is cultured on yeast extract, in addition to the up-regulation of loci that orchestrate α-mannan degradation, an additional PUL defined as PUL\textsubscript{1,6-β-glucan} is activated during early exponential phase (12). This locus encodes just two enzymes, which belong to GH families 3 (GH3) and 30 subfamily 3 (GH30\textsubscript{3}). Although 1,6-β-glucanase is the only activity reported for enzymes within GH30\textsubscript{3}, the majority of these GHs are fungal in origin and likely to be transglycosidases involved in cell-wall remodeling. Within the fungal mycoparasite Trichoderma harzianum, GH30\textsubscript{3} enzymes contribute to the ability of the organism to antagonize plant pathogenic fungi (13, 14). One bacterial GH30\textsubscript{3} enzyme has been characterized, from the marine bacterium Saccharophagus degradans, which is active on 1,6-β-glucan as well as the branched algal polysaccharide laminarin (1.3;1,6-β-glucan) (15). Given the role of 1,6-β-glucan in the cross-linking of mannoproteins into the fungal cell wall matrix, this glucan might comprise a target for bacteria that degrade α-mannan, because cleavage of the 1,6-β-glucosidic bonds would enable release of mannoproteins. Additionally, 1,6-β-glucan is found in edible fungi such as the basidiomycetes Agaricus bisporus (common mushroom) and Lentinula edodes (Shiitake mushroom), and thus these mushrooms comprise another source of the polysaccharide for B. thetaiotaomicron and, more widely, the human gut microbiota.

Although 1,6-β-glucans are common components of the human diet through intake of yeast cell wall and edible fungi, little is known of how these glycans are utilized by the gut microbiota. More broadly, little is known about the enzymes that degrade 1,6-β-glucans, and there is no structural data for any GH30\textsubscript{3} enzyme. Here we have tested the hypothesis that PUL\textsubscript{1,6-β-glucan} in B. thetaiotaomicron plays a role in the degradation and utilization of yeast 1,6-β-glucans and not plant 1,3;1,4-β-mixed linked glucans as previously proposed (1). Our data show that this locus orchestrates the degradation of 1,6-β-glucan, and this enables B. thetaiotaomicron to utilize this fungal polysaccharide. The surface-located endo-1,6-β-glucanase is shown to be critical for growth of the bacterium on 1,6-β-glucan. The crystal structure of the endo-1,6-β-glucanase shows that substrate recognition is mediated by shape complementarity of the substrate-binding cleft and the hooked U-shaped conformation of 1,6-β-glucan, rather than through extensive hydrogen-bonding interactions with the polysaccharide.

Results and discussion

**PUL\textsubscript{1,6-β-glucan} orchestrates the degradation and utilization of 1,6-β-glucan by B. thetaiotaomicron**

When B. thetaiotaomicron was cultured on the complex tryptone-yeast extract-glucose (TYG) medium, a suite of PULs were up-regulated compared with glucose minimal medium, including the locus PUL\textsubscript{1,6-β-glucan} (10, 12). PUL\textsubscript{1,6-β-glucan} was predicted to extend from bt3309 to bt3314 (Fig. 1A) and is predicted to encode five proteins: a SusR type regulator (BT3309), a SusCD-like pair (BT3310–11), and enzymes belonging to GH30\textsubscript{3} (BT3312) and GH3 (BT3314). BT3311, BT3312, and BT3313 each possess a type II signal peptide with a canonical lipoprotein box and are thus likely located on the surface of the bacterium. Because BT3313 contains multiple DUF5016 domains (also referred to as immunoglobulin domains), it is predicted to function as a SGBP (Fig. 1A) (12). The yeast-derived glycans in TYG medium include 1,6-β-glucan (also known as pustulan), 1,3-β-glucan, α-mannan, and chitin. B. thetaiotaomicron is unable to grow on laminarin, whereas the α-mannan-degrading apparatus is encoded by three PULs that are distinct from PUL\textsubscript{1,6-β-glucan} (10), suggesting that the locus may target pustulan. To test this hypothesis B. thetaiotaomicron was cultured on pustulan, and transcription of the five genes in this locus were evaluated by RT-PCR. The gene encoding the PUL regulator BT3309 was not activated by pustulan; bt3314 was up-regulated 10-fold; and transcription of the other genes in the locus increased ~100-fold. The PUL was not activated when B. thetaiotaomicron was cultured on α-mannan. These data indicate that PUL\textsubscript{1,6-β-glucan} encodes the pustulan degrading apparatus of B. thetaiotaomicron. This assumption was confirmed when the bacterium was shown to be unable to grow on pustulan when the gene encoding the predicted surface GH30\textsubscript{3} enzyme (bt3312) was deleted from the chromosome (Fig. 1E).

**Contribution of surface proteins to 1,6-β-glucan degradation**

BT3312 is a endo-1,6-β-glucanase—GH family 30 contains enzymes that are active on a variety of β-linked glycans, including xylan, glucuronoxylan, glucosyleramide, and 1,6-β-glucan. The fungal cell wall contains 1,6-β-glucan, consistent with the location of BT3312 within GH30\textsubscript{3}, a subfamily which, to date, is comprised exclusively of 1,6-β-glucanases. When cultured on 1,6-β-glucan, B. thetaiotaomicron released glucooligosaccharides into the supernatant during exponential growth, which correlates with the predicted endo-1,6-β-glucanase activity of the surface enzyme BT3312 (Fig. 1, D and E).

The catalytic activity of BT3312 was tested against a range of polysaccharides. The enzyme showed no activity on 1,3-β-glucan, 1,4-β-glucan, or 1,6-β-galactan but was active on the 1,6-β-glucan pustulan, producing a range of oligosaccharides in the initial phases of the degradative process (Fig. 2). Thus, BT3312 is a canonical and highly specific endo-1,6-β-glucanase. The limit products generated by BT3312 were primarily glucose and gentiobiose (1,6-β-glucooligosaccharide), suggesting the enzyme is active on oligosaccharides as small as glucotriose (Fig. 2). Substrate depletion assays with 1,6-β-glucooligosaccharides showed activity on oligosaccharides with a degree of polymerization ranging from 3 to 8 (Table 1). The moderate 4-fold increase in activity from glucotriose to glucococtaose suggests that the enzyme contains only three major subsites. This view is consistent with the observation that 1,6-β-glucotetraose was initially hydrolyzed to glucotriose, gentiobiose, and glucose in a molar ratio of 1:2:1 (data not shown), which showed that the substrate could bind productively in the two possible binding modes that
encapsulate -2, -1, and +1 (i.e., -3 to +1 and -2 to +2; the scissile glycosidic bond links the sugars at -1 and +1) (16). Thus, -3 and +2 are not essential for substrate binding, indicating that the only functionally significant subsites in the enzyme substrate-binding cleft extend from -2 to +1. The inability of BT3312 to hydrolyze gentiobiose, which would require binding at the -1 and +1 subsites for cleavage to occur, reveals the important contribution the -2 subsite makes to productive substrate recognition, typical of endo-acting retaining glycanases (17).

Based on the presence of a lipoprotein box within its signal peptide, BT3312 is predicted to be located on the outer membrane. Indeed, the ability of Δbt3312 to grow on a mixture of 1,6-β-glucan oligosaccharides but not pustulan strongly indicates that BT3312 is required to depolymerize the glycan at the bacterial surface, generating molecules of an appropriate size for import through the outer membrane. To provide further support for this hypothesis, whole cell polysaccharide depolymerization assays were performed under aerobic conditions, which prevents transport of oligosaccharides through the outer

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**Figure 1.** The 1,6-β-glucan polysaccharide utilization loci from *B. thetaiotaomicron* and *B. ovatus*. A, schematic of the 1,6-β-glucan polysaccharide utilization loci (PUL1,6-β-glucan). The percentage identity at protein level between the PULs is indicated. B and C, RT-PCR of PUL1,6-β-glucan genes in *B. thetaiotaomicron* (B) and *B. ovatus* (C) grown on 5 mg ml⁻¹ glucose or 1,6-β-glucan, shown as fold change in expression versus glucose. D, HPAEC-PAD analysis of soluble glycans in the culture supernatant during growth of *B. thetaiotaomicron* on 1,6-β-glucan as shown in E. The A₆₀₀ at the time of sample is indicated. Oligosaccharides were eluted from the column with a 20% gradient of 100 mM NaOH. OD, optical density. E, growth of *B. thetaiotaomicron* WT or Δbt3312 deletion strain (Δbt3312) on minimal medium plus 0.5% (w/v) 1,6-β-glucan, or 1,6-β-glucan digested with 50 nM BT3312 for 5 min and then boiled to inactivate enzymes. F, HPAEC-PAD analysis of whole cell assays of WT or Δbt3312 glucose grown cells (black and red) and WT or Δbt3312 1,6-β-glucan digest grown cells (blue and orange) against 2 mg ml⁻¹ 1,6-β-glucan. Oligosaccharides were eluted from the column with a 60% gradient of 100 mM NaOH.
membrane, and thus report exclusively on the activity of surface enzymes (10, 18). When wild-type *B. thetaiotaomicron*, grown on pustulan or 1,6-β-glucooligosaccharides to activate PUL\textsubscript{1,6-β-glucan}, was subjected to aerobic whole-cell polysaccharide depolymerization assays with pustulan, the bacterium generated a range of glucooligosaccharides, consistent with the activity observed for purified BT3312 (Fig. 1F). Equivalent whole-cell assays of the Δbt3312 mutant grown on 1,6-β-glucooligosaccharides, which also activate PUL\textsubscript{1,6-β-glucan}, did not produce pustulan-derived oligosaccharides (Fig. 1F). Collectively, these data show that BT3312 is located on the surface of *B. thetaiotaomicron*.

**Crystal structure of BT3312**—No structure of a GH30_3 enzyme has been reported. Thus, to explore the mechanism of catalysis and substrate binding of an endo-1,6-β-glucanase, the crystal structure of BT3312 was determined in complex with the ligand β-glucosyl-1,6-deoxynojirimycin (GlcDNJ). The crystal structure revealed two domains. The catalytic domain adopts a β/α\textsubscript{5} barrel fold (TIM barrel), extending from residues Asp\textsuperscript{62} to Lys\textsuperscript{427}, and a β-sandwich domain comprising sequences from both the N and C termini (Fig. 3A). The TIM barrel contains a central eight-stranded β-barrel, and extending from each β-strand is an α-helix. Extended loops link the α-helices with the β-barrel, and a small β-sheet comprising three anti-parallel strands is inserted into the loop connecting β-strand-8 and α-helix-8. The β-sandwich domain contains seven antiparallel β-strands in one sheet, with two contributed by the N-terminal region of the enzyme. The other β-sheet comprises three antiparallel strands with one derived from the N terminus. Because BT3312 is classified into GH30, it is a member of clan GH-A in which the fold, catalytic apparatus, and mechanism are conserved (19). According to these criteria BT3312 is predicted to use a double-displacement mechanism in which anomic configuration is retained after bond cleavage. In this two-step mechanism, an enzymic nucleophile performs a nucleophilic attack and generates a glycosyl enzyme intermediate, which in the second step is hydrolyzed. An enzymic residue assists departure of the anomeric group in the first step, acting as a general acid, and assists the hydrolysis of the glycosyl enzyme intermediate in the second step, acting as a general base. The catalytic acid/base and nucleophile have been assigned for a GH30 family member, human β-glucocerebrosidase, and are located at the end of β-strands 4 and 7 of the barrel, respectively (20). Thus, the candidate catalytic residues are Glu\textsuperscript{238} (acid/base) and Glu\textsuperscript{339} (nucleophile). Consistent with this prediction, mutation of either of these residues to Ala resulted in the complete loss of activity (Table 1).

Based on analysis of other clan GH-A members, the substrate-binding site of BT3312 is predicted to be positioned on top of the β-barrel. Inspection of this region of the enzyme reveals a deep U-shaped cleft that houses the catalytic residues. The active site cleft of BT3312 adopts a different topology to the substrate-binding regions of other GH-A endo-acting enzymes, which typically display linear or curved clefts that are open at both ends (Figs. 3D and 4A). Interestingly, NMR analysis of the ligand complex, unambiguous electron density corresponding to GlcDNJ was evident. The crystal structure revealed that BT3312 comprises two domains. The catalytic domain adopts a β/α\textsubscript{5} barrel fold (TIM barrel), extending from residues Asp\textsuperscript{62} to Lys\textsuperscript{427}, and a β-sandwich domain comprising sequences from both the N and C termini (Fig. 3A). The TIM barrel contains a central eight-stranded β-barrel, and extending from each β-strand is an α-helix. Extended loops link the α-helices with the β-barrel, and a small β-sheet comprising three anti-parallel strands is inserted into the loop connecting β-strand-8 and α-helix-8. The β-sandwich domain contains seven antiparallel β-strands in one sheet, with two contributed by the N-terminal region of the enzyme. The other β-sheet comprises three antiparallel strands with one derived from the N terminus. Because BT3312 is classified into GH30, it is a member of clan GH-A in which the fold, catalytic apparatus, and mechanism are conserved (19). According to these criteria BT3312 is predicted to use a double-displacement mechanism in which anomic configuration is retained after bond cleavage. In this two-step mechanism, an enzymic nucleophile performs a nucleophilic attack and generates a glycosyl enzyme intermediate, which in the second step is hydrolyzed. An enzymic residue assists departure of the anomeric group in the first step, acting as a general acid, and assists the hydrolysis of the glycosyl enzyme intermediate in the second step, acting as a general base. The catalytic acid/base and nucleophile have been assigned for a GH30 family member, human β-glucocerebrosidase, and are located at the end of β-strands 4 and 7 of the barrel, respectively (20). Thus, the candidate catalytic residues are Glu\textsuperscript{238} (acid/base) and Glu\textsuperscript{339} (nucleophile). Consistent with this prediction, mutation of either of these residues to Ala resulted in the complete loss of activity (Table 1).

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**Table 1**

| Kinetic parameters for wild type and variants of BT3312 |
|--------------------------------------------------------|
| **Substrate**                                           | **$k_{cat}/K_m$** | **Relative activity** |
| Wild type 1,6-β-Glucan (pustulan)                       | 1776 ± 20.6       | 1                      |
| Wild type 1,3-β-Glucan                                   | 0                | 0                      |
| Wild type 1,4-β-Glucan                                   | 0                | 0                      |
| Wild type 1,6-β-Galactan                                 | 0                | 0                      |
| E339A 1,6-β-Glucan (pustulan)                            | 0                | 0                      |
| N281A 1,6-β-Glucan (pustulan)                            | 10 ± 1           | 0.006                  |
| D286A 1,6-β-Glucan (pustulan)                            | 17 ± 1           | 0.009                  |
| E339A 1,6-β-Glucan (pustulan)                            | 475 ± 30         | 0.27                   |
| E339Q 1,6-β-Glucan (pustulan)                            | 0                | 0                      |
| W345A 1,6-β-Glucan (pustulan)                            | 0                | 0                      |
| C393S 1,6-β-Glucan (pustulan)                            | 7 ± 1            | 0.004                  |
| C396S 1,6-β-Glucan (pustulan)                            | 9 ± 1            | 0.005                  |
| Oligosaccharides                                        |                  |                        |
| Wild type 1,6-β-Glactotriose                             | $1.7 \times 10^6 \pm 1.1 \times 10^4$ |
| Wild type 1,6-β-Glactotetraose                           | $2.6 \times 10^6 \pm 3.4 \times 10^4$ |
| Wild type 1,6-β-Glactohexaose                            | $6.0 \times 10^6 \pm 6.0 \times 10^2$ |
| Wild type 1,6-β-Glactoheptaose                           | $6.4 \times 10^6 \pm 1.4 \times 10^2$ |

*The kinetic parameter is mg ml\textsuperscript{−1} min\textsuperscript{−1} for pustulan and s\textsuperscript{−1} min\textsuperscript{−1} for glucooligosaccharides.*

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**Figure 2.** HPAEC-PAD analysis of BT3312. BT3312 (50 nM) was incubated with 2 mg ml\textsuperscript{−1} 1,6-β-glucan at 37 °C as described under “Materials and methods.” Samples were taken at the indicated time points and analyzed by HPAEC-PAD.
Candida glabrata 1,6-β-glucan revealed a hooked, U-shaped conformation (21), which is complementary to the topology of the substrate-binding region of BT3312 (see below).

The structure of BT3312 bound to GlcDNJ reveals details of the active site and the distal substrate-binding subsites (Fig. 3, B and C). The Glc at the −2 subsite adopts a relaxed chair (4C1) conformation, whereas DNJ bound in the −1 subsite, the active site, is distorted and adopts a 4E envelope conformation. Substrate distortion at the active site is a characteristic feature of GHs, because distorted conformations require less nuclear movements to achieve the transition state structure and allow the geometric and stereoelectronic requirements of the reaction to be achieved. In particular, GHs undergo reactions involving “exploded” transition states with significant oxocarbonium-ion like character, in which the endocyclic oxygen and anomeric carbon are required to be in the same plane to share the developing positive charge (22). Because most β-gluco-active enzymes proceed through a transition state in a 4C1 (half-chair) conformation and because the 4E and 4H1 conformations are adjacent to each other on a “Stoddard” conformational plot of pyranose ring conformations, this result is suggestive that this enzyme utilizes a 1S3−4H1−4C1 conformational itinerary along the reaction coordinate (23, 24). Despite the resemblance of GlcDNJ to the proposed transition state, the inhibitor bound

Table 2

Data collection and refinement statistics

|                     | BT3312 no ligand | BT3312 ligand |
|---------------------|------------------|--------------|
| **Data collection** |                  |              |
| Date                | 26/01/14         | 04/07/15     |
| Source              | I02              | I03          |
| Wavelength (Å)      | 0.9794           | 0.7749       |
| Space group         | P2₁              | P2₁          |
| Cell dimensions     |                  |              |
| a, b, c (Å)         | 62.4, 156.0, 78.0| 62.4, 78.8, 145.5|
| α, β, γ (°)         | 90.0, 95.0, 90.0 | 90.0, 100.4, 90.0 |
| No. of measured reflections | 429,705 (10,374) | 445,926 (22,895) |
| No. of independent reflections | 114,209 (4871) | 116,665 (5762) |
| Resolution (Å)      | 48.24–1.90 (1.93–1.90) | 48.79–1.85 (1.88–1.85) |
| CC1/2               | 0.996 (0.466)    | 0.996 (0.644) |
| Rmerge               | 9.0 (1.8)        | 7.8 (1.6)    |
| Completeness (%)    | 97.4 (97.6)      | 99.2 (96.2)  |
| Redundancy           | 3.8 (4.0)        | 3.8 (2.1)    |
| **Refinement**      |                  |              |
| Rwork/Rfree         | 18.19/22.12      | 18.84/22.80  |
| No. atoms           |                  |              |
| Protein             | 10,813           | 10,738       |
| Ligand/ions         | 0                | 68           |
| Water               | 607              | 606          |
| B-factors           |                  |              |
| Protein             | 34.3             | 30.6         |
| Ligand/ions         | N.A.             | 29.5         |
| Water               | 37.5             | 32.5         |
| Root mean square deviations | 0.013 | 0.013 |
| Bond lengths (Å)    | 1.53             | 1.52         |
| PDB code            | 5NGK             | 5NGL         |
extremely weakly to the enzyme, with an estimated $K_i$ of $\sim 1$ mM, suggesting that additional sugar residues may be required to fully capitalize on interactions along the active site cleft. Indeed, mutagenesis data described below suggest the enzyme has at least four functional subsites for the polysaccharide pustulan. The polar interactions between the active site of BT3312 and GlcDNJ are displayed in Fig. 3 (B and C). Within the $-1$ subsite, the carboxylate of Glu$^{339}$ and N$^2$ of Asn$^{237}$ make polar contacts with O$_2$. O$_3$ interacts with N$^2$ of Trp$^{179}$, N$^2$ of Asn$^{244}$, and the carboxylate of Asp$^{131}$. The O$^2$ of Asp$^{131}$ and N$^1$ of Trp$^{377}$ make hydrogen bonds with O$_4$. Finally, the endocyclic nitrogen donates a hydrogen bond to O$_2$ of Glu$^{339}$. Binding of GlcDNJ in the active site is also mediated through apolar contacts between the sugar ring and Trp$^{377}$, which provides the hydrophobic platform in the $-1$ subsite. The catalytic nucleophile, Glu$^{339}$, is 3.2 Å from the anomeric carbon of DNJ and is thus in an appropriate position to mount a nucleophilic attack on the anomeric carbon. Glu$^{238}$ is in an optimal position (3.4 Å) to donate a proton to the glycosidic oxygen appended to C$_1$ and thus is able to function as the acid-base catalyst.

The $-2$ subsite makes only apolar interactions with the Glc in GlcDNJ. Trp$^{345}$ stacks against the pyranose ring, whereas the disulfide formed by Cys$^{393}$ and Cys$^{396}$ also makes apolar contacts with the $-2$ Glc. Given that O$_6$ of the non-reducing glucose points into solvent, it is unlikely that there are additional negative subsites in BT3312. It is difficult to establish the number of positive subsites without bound ligand in this region of the enzyme. However, based on the height of the wall of the substrate-binding cleft, there are likely to be one or possibly two positive sites in which His$^{281}$, Asn$^{282}$, and Asp$^{286}$ contribute to substrate binding. To examine the contribution of these residues to catalysis by BT3312, alanine-scanning mutagenesis was performed (Table 1). As stated above, the catalytic residues including Glu$^{238}$ are essential for activity. Mutation of the other residues in the active site caused a significant reduction in the activity observed, except Asp$^{286}$, which only resulted in a 3-fold loss of catalytic efficiency. The relatively few polar interactions, which are confined to the active site, suggest that specificity is driven by the complementary conformation of the substrate and the topology of the enzyme. The biochemical data presented above point to only one positive subsite, which is surprising because the crystal structure of the enzyme suggests four observable subsites extending from $-2$ to $+2$. It is possible that the U-shaped structure of the substrate, which is required to fully occupy the four subsites, can only be achieved through extensive intrachain hydrogen bonds within the polysaccharide. Thus, short oligosaccharides may not be able to adopt the U-shaped conformation required to occupy the $+2$ subsite.

Comparison of the structure of BT3312 with GH30 enzymes with different specificities sheds light on the structural basis for substrate recognition in this family. These structural comparisons show that specificity is dominated by the topology of the substrate-binding regions of the respective proteins (Fig. 4, A and B). Thus, when BT3312 is compared with the glucuronoxylanase from Dickeya chrysanthemi D1 (PDB code 2Y24), the B. thetaiotaomicron enzyme contains a long loop extending from residues 381–398 that sterically occludes the $-2$ and $-3$ subsites in the glucuronoxylanase (25). This loop is stabilized by a disulfide bond between Cys$^{393}$ and Cys$^{396}$, and three short $\beta$ strands behind the loop. BT3312 contains a second extended loop comprising Glu$^{236}$ to Trp$^{252}$ that prevents access to the positive subsites in the glucuronoxylanase. The lack of residues that target O$_6$ of the substrate bound at the $-1$ or $-2$ subsites reinforces the view that it is the topology of the proximal sub-
The Homo sapiens over 1,4- by 1,6- distinct from the highly curved, U-shaped structure exhibited. The active site region of BT3312 that confers specificity for 1,6-β-glucan over 1,4-β-xylan. 1,4-β-Xylan, which lacks O6 substitution, adopts a linear 3-fold screw axis conformation (26, 27) that is distinct from the highly curved, U-shaped structure exhibited by 1,6-β-glucan (21).

The active site (−1) subsite of BT3312 was compared with the Homo sapiens β-glucosylceramidase (PDB code 2V3D) and the D. chrysanthemi glucuronoxylanase (PDB code 2Y24), representatives of subfamilies GH30_1 and GH30_8, respectively. In addition to the catalytic nucleophile and acid-base residues, many of the other substrate-binding amino acids are conserved in the three enzymes. Indeed the only two residues in the active site of BT3312 that are not conserved in other GH30 enzymes are Asn224 and Asp131. The asparagine in BT3312 is replaced in the other two enzymes by an aromatic residue that makes apolar contacts with the substrate. Though Asp131 is conserved in the β-glucosylceramidase, the loop containing this residue adopts a different position in the glucuronoxylanase (to accommodate the linear extended substrate), and thus there is no residue equivalent to the aspartate in this enzyme. The additional distal subsites in the glucuronoxylanase likely compensate for this loss of binding energy in the active site that would have been provided by this aspartate.

Surface glycan-binding proteins—Similar to other characterized PULs, PUL1,6-β-glucan encodes two putative surface lipoproteins predicted to contribute to polysaccharide binding. The genes encoding both BT3311, the SusD-like protein, and BT3313, a predicted lipoprotein, were expressed in E. coli, and their ability to bind glucans was evaluated. Affinity gel electrophoresis showed that both BT3311 and BT3313 bound specifically to 1,6-β-glucan and showed no affinity for laminarin (1,3-β-glucan) or hydroxy-ethyl cellulose (1,4-β-glucan).

**Table 3**

| Protein | Ligand                | $K_a$ (μM) | $\Delta H$ (kcal/mol) | $N$ |
|---------|-----------------------|------------|-----------------------|-----|
| BT3311  | 1,6-β-Glucan (fit as 1 mM) | 6.5 × 10^4 ± 0.2 | -19 × 10^4 ± 0 | 0.8 ± 0.02 |
| BT3311  | 1,6-β-Glucoceratoase | 2.1 × 10^4 ± 0.1 | -14 × 10^4 ± 0 | 1.2 ± 0.02 |
| BT3311  | 1,6-β-Glucohexosease | 1.0 × 10^4 ± 0.1 | -14 × 10^4 ± 1 | 1.0 ± 0.04 |
| BT3311  | 1,6-β-Glucopentaose | 8.3 × 10^3 ± 0.3 | -16 × 10^4 ± 3 | 1.1 ± 0.1 |
| BT3311  | 1,6-β-Glucotetraose | Low        | Low                  | Low |
| BT3311  | 1,6-β-Glucosido     | NB         | Low                  | Low |
| BT3313  | 1,6-β-Glucan (fit as 1 mM) | 5.3 × 10^2 ± 0.1 | -36 × 10^4 ± 1 | 1.02 ± 0.04 |
| BT3313  | 1,6-β-Glucoceratoase | 1.5 × 10^2 ± 0.2 | -24 × 10^4 ± 3 | 1.25 ± 0.1 |
| BT3313  | 1,6-β-Glucohexosease | 8.0 × 10^2 ± 0.2 | -15 × 10^4 ± 2 | 1.38 ± 0.2 |
| BT3313  | 1,6-β-Glucopentaose | 3.6 × 10^2 ± 0.2 | -15 × 10^4 ± 3 | 1.28 ± 0.2 |
| BT3313  | 1,6-β-Glucotetraose | Low        | Low                  | Low |
| BT3313  | 1,6-β-Glucosido     | NB         | NB                   | NB |

a Low, binding too low to quantify.

b NB, no binding was evident.

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**Figure 5. Isothermal titration calorimetry of glycan-binding proteins.** Shown are representative traces of the binding of 50 μM SusD BT3311 (left panel) or SGBP BT3313 (right panel) to 5 mg ml⁻¹ 1,6-β-glucan in 50 mM HEPES, pH 7.5 (estimated at 1 mM to fit the data).
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Table 4

| Enzyme       | Substrate       | $K_m$ (μM) | $k_{cat}$ (sec$^{-1}$) | $K_m$ (μM) | $k_{cat}/K_m$ (sec$^{-1}$ μM$^{-1}$) |
|--------------|-----------------|------------|------------------------|------------|--------------------------------------|
| BT3314       | pNP-β-D-Glc$^a$ | 240 ± 379  | 40.8 ± 2.9             | 0.02       |                                      |
| BT3314       | 1,3-β-Glucozyme | ND         | 1.8 ± 0.2              | 0.6 ± 0.0  |                                      |
| BT3314       | 1,4-β-Glucoside | ND         | 0.05 ± 0.01            |            |                                      |
| BT3314       | 1,6-β-Glucoside | ND         | 5.6 ± 0.2              |            |                                      |
| BT3314       | 1,6-β-Glucozyme | ND         | 6.7 ± 0.6              |            |                                      |
| BACOVA_00946 | pNP-β-D-Glc     | 1052 ± 87  | 195.8 ± 5.5            | 0.19       |                                      |
| BACOVA_00946 | 1,3-β-Glucoside | ND         | 3.7 ± 0.1              |            |                                      |
| BACOVA_00946 | 1,4-β-Glucoside | ND         | 0.6 ± 0.02             |            |                                      |
| BACOVA_00946 | 1,6-β-Glucoside | ND         | 38.8 ± 0.8             |            |                                      |

$^a$ pNP-β-D-Glc, 4-nitrophenyl β-D-glucopyranoside.

$^b$ ND, not determined.

Bacteroidetes

The distribution of the PUL$^{1,6-β}$-glucan within other Bacteroidetes genomes was examined using the Gene Ortholog Neighborhood viewer on the Integrated Microbial Genome and Microbiome sample database at img.jgi.doe.gov, and the Polysaccharide Utilization Loci database (PULDB; www.cazy.org/PULDB) (29). Manual comparison of the strains containing a PUL with synergy to PUL$^{1,6-β}$-glucan to the ~350 strains screened for growth on α-mannan in Ref. 10 revealed that the PUL is more widely distributed than the ability to degrade α-mannan, which is mostly limited to strains of B. thetaiotaomicron, Bacteroides ovatus, and Bacteroides xylanisolvens (Fig. 6). Although several of the mannann-degrading strains possess a putative PUL$^{1,6-β}$-glucan, the Bacteroidetes Capnocytophaga canimorsus (a commensal of the canine mouth and occasional human pathogen) and Prevotella copri, which are not predicted to grow on yeast α-mannan because of a lack of α-mannanases and mannosidases encoded in the genome, also contain a PUL that displays substantial synergy with PUL$^{1,6-β}$-glucan. The functional significance of syntenic PULs to PUL$^{1,6-β}$-glucan is illustrated by the observation that (i) B. ovatus can grow on pustulan, (ii) pustulan up-regulates the locus, and (iii) bacova_00946 encodes a β-glucosidase that displays a strong preference for 1,6-β-glucooligosaccharides (Fig. 1, A and C, and Table 4).

Conclusions

Our data show that PUL$^{1,6-β}$-glucan (bt3309–bt3314) does not depolymerize mixed linkage 1,3;1,6-β-glucans from cereals, as proposed previously for B. ovatus (1); rather, it is highly specific for fungal 1,6-β-glucans. A model for the degradative pathway of the glucan is shown in Fig. 7. The critical enzyme encoded by the PUL is BT3312, an endo-1,6-β-glucanase that is displayed on the bacterial surface, allowing direct access to the intact glucan. The SusD-like protein and SGBP show a preference for longer substrates, suggesting that the secondary structure of the polysaccharide is required for recognition by these proteins. The “hook-like” secondary structure of 1,6-β-glucan is distinct from other β-glucans, and the U-shaped topology of the substrate-binding cleft of the endo-1,6-β-glucanase, which makes limited polar interactions with the ligand GlcDNJ, matches the conformation of its target glucan, suggesting that it is shape complementarity between substrate and enzyme that is the driving force for substrate specificity. It has been shown that a GH30_3 enzyme from Aspergillus fumigatus promotes the release of cell wall proteins from C. albicans cell walls, which suggests that another role for PUL$^{1,6-β}$-glucan could be to increase access of yeast cell wall components for other B. thetaiotaomicron enzyme systems, such as the α-mannann-degrading PUL (30). 1,6-β-glucan is also a common component of fungal cell walls in mushrooms, as well as gut commensal fungi such as S. cerevisiae, C. glabrata, and C. albicans (the latter pair are also important human pathogens). The observation that this PUL is more widely distributed within the Bacteroidetes than the α-mannan PULs could reflect the multiple sources of this glucan from both mannann-free and mannann-rich fungi and yeasts. The presence of the locus in bacteria that do not use the more abundant mannann glucans in S. cerevisiae sug-
Bacterial metabolism of 1,6-β-glucan

Organism utilises α-mannan (experimental)

Organism does not utilise α-mannan (experimental or *predicted from Cazyome analysis)

Figure 6. Distribution of PUL1,6-β-glucan in Bacteroidetes. PULs with synteny to PUL1,6-β-glucan were identified using the Integrated Microbial Genome and Microbiome sample database at www.img.jgi.doe.gov using the Gene Ortholog Neighborhood viewer and PULDB at www.cazy.org/PULDB.3 Example PULs from strains that can and cannot grow on α-mannan are indicated by asterisks. The functions or enzyme families of the gene products are indicated. The basis for assigning genes encoding SGBPs was based on significant sequence identity (>35%) with BT3313 (SGBP of B. thetaiotaomicron) and the presence of a lipoprotein signal peptide. The gray genes encode proteins of unknown function.

Figure 7. Cartoon representation of 1,6-β-glucan degradation by B. thetaiotaomicron. A schematic of 1,6-β-glucan degradation by B. thetaiotaomicron is shown. Gene products are colored as in Fig. 1A. 1,6-β-Glucan is represented by blue circles. The inner membrane transporter to transport monosaccharides into the cytoplasm for fermentation is assumed to exist but is not within PUL1,6-β-glucan.

Gene expression studies

Comparison of the levels of transcript expression from the PUL was performed by RT–PCR. B. thetaiotaomicron or B. ovatus were cultured in 5 ml of minimal medium containing 0.5% (w/v) carbon source, as described above. Triplicate bacterial cultures were harvested at mid-log phase (A600 ~0.8), placed in RNAprotect (Qiagen), and then stored at −80 °C overnight, before purification with RNaseasy kit (Qiagen). RNA purity was assessed spectrophotometrically, and 1 μg of RNA was used immediately for reverse transcription (QuantiTect reverse transcription kit; Qiagen). RT–PCR was performed in a 96-well plate on a LightCycler 96 System (Roche) with FastStart Essential DNA Green Master (Roche) using the primers shown in supplemental Table S1. The reactions were carried out in 10 μl, consisting of 5 μl of SYBR Green mix, 20 ng of cDNA, and 1 μM (PUL genes) or 0.125 μM (16 S rRNA) primer mix. The reaction conditions were 95 °C 600 s, followed by 45 cycles of 95 °C for 10 s, 55 °C for 10 s, and 72 °C for 10 s. Cq values were calculated using LightCycler 96 SW 1.1. The data were normalized to 16 S rRNA transcript levels, and changes in expression level were calculated as fold change compared with cultures of minimal medium plus glucose.

Oligosaccharide production

One gram of 1,6-β-glucan (Eliciyl Oligotech) was digested with 50 nM BT3312 in 20 mM Tris, pH 8.0, for 30 min to produce small oligosaccharides or by acid hydrolysis with 20 mM HCl for 2 h to generate longer oligosaccharides. The mixture was subjected to size-exclusion chromatography on two Biogel P2 columns (120 × 2.5 cm) in series in 50 mM acetic acid. Fractions were screened by thin layer chromatography, and those containing pure oligosaccharide were pooled and freeze-dried.

Materials and methods

Bacterial strains

B. thetaiotaomicron VPI-5482 and B. ovatus ATCC 8483 were cultured anaerobically in TYG medium, brain-heart infusion (Sigma) plus 2% (w/v) agar or minimal medium containing 0.5% (w/v) 1,6-β-glucan (Eliciyl Oligotech) or glucose as sole carbon source, as described previously (2).
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Recombinant protein production

Genes encoding proteins of interest from PUL1,6-β-glucan were amplified by PCR from B. thetaiotaomicron VPI-5482 genomic DNA and cloned into a pET28 derivative, including an N-terminal hexahistidine tag, with any native signal peptide removed. Proteins were expressed in E. coli Tuner cells by culturing cells in Luria broth with 10 mg ml⁻¹ kanamycin to A₆₀₀ of ~1.0 at 37 °C before cooling to 16 °C and inducing with 1 mM isopropyl β-D-thiogalactopyranoside overnight. The cells were harvested by centrifugation at 5000 × g for 5 min and resuspended in 20 mM Tris–HCl buffer, pH 8.0, containing 300 mM NaCl (buffer A). The cells were lysed by sonication, and the cell-free extract was recovered by centrifugation at 15,000 × g for 30 min. The proteins were purified from the cell-free extract using immobilized metal affinity chromatography using Talon™, a cobalt-based matrix. Proteins were eluted from the column in buffer A containing 100 mM imidazole. For crystallization trials, immobilized metal affinity chromatography-purified protein was concentrated and further purified by gel filtration chromatography using a Superdex S200 16/600 column equilibrated in buffer A. Protein concentration was measured at 280 nm using a NanoDrop spectrophotometer.

Enzyme assays

Reducing sugar assays on polysaccharide were carried out in buffer A, including 0.1 mg ml⁻¹ BSA, following the method of Miller (31). Briefly, 2 mg ml⁻¹ 1,6-β-glucan (Pustulan, Elicityl Oligotech; containing ~2% O-acetylation) was incubated with concentrations of enzyme from 50 nM to 1 mM at 37 °C. A reaction against oligosaccharides were performed in 50 mM sodium acetate. The peak areas of oligosaccharides were monitored at 280 nm using a NanoDrop spectrophotometer.

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CH₂Ph), 5.02 (1 H, d, J = 12.6 Hz, CH₂Ph), 5.24 (1 H, d, J₁,₂ = 2.1 Hz, H2), 5.47 (1 H, m, H4), 5.50 (1 H, t, J₁,₂ = 3.0 Hz, H3), 7.14–8.09 (20 H, m, Ar); 1³C NMR (101 MHz, d₆-DMSO, 25 °C): δ = 2.4–2.3 (2 × 1 C, Me), 17.7 (3 C, CMe₂), 25.5 (1 C, CMe), 38.8 (1 C, C1), 55.6 (1 C, C5), 59.2 (1 C, C6), 66.5 (1 C, C4), 66.6 (1 C, CH₂Ph), 67.2 (1 C, C3), 67.7 (1 C, C2), 126.0, 127.1, 127.7, 128.5, 128.6, 128.7, 128.8, 128.9, 129.1, 129.2, 129.30, 129.8, 133.6, 134.0, 136.4 (24 C, Ar), 155.6, 163.9, 164.5, 164.8 (4 × 2 C, = O); HRMS (ESI⁺) m/z 724.2941 (C₄₉H₄₆NO₁₅Si (M + H⁺) requires 724.2936).

The silylated ester (68 mg, 0.094 mmol) was dissolved in AcOH:H₂O:THF (3:1:1, 9.0 ml) and the mixture was stirred at room temperature overnight at 40 °C. The solution was concentrated under reduced pressure, and the residue was purified by flash chromatography (AcOEt/hexane, 19:1 to 6:4) to afford the alcohol (4 mg, 0.0006 mmol) was then added, the mixture was purified by ion-exchange chromatography (Dowex 1X-8, form, eluted with H₂O; Amberlite CG50 Type I, H⁺ form, eluted with H₂O, and then 6 m aqueous NH₃ followed by C₈ reversed phase chromatography (CH₃CN:CH₃OH:H₂O:CH₃CO₂H = 95:5:5:0.1) to afford the azasugar 5 (2.9 mg, 75%) with NMR data matching the previously reported (41). [α]D = +7.7 (c 0.1, H₂O); HRMS (ESI⁺) m/z 326.1460 (C₁₄H₂₄NO₃ (M + H⁺) requires 326.1464) (41).

### Genetic manipulation of B. thetaiotaomicron

The gene bt3312 gene was removed from the genome of B. thetaiotaomicron by in-frame deletion using the vector pExchange, as described in Ref. 42.

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