

Starch Catabolism by a Prominent Human Gut Symbiont Is Directed by the Recognition of Amylose Helices

Nicole M. Koropatkin,¹ Eric C. Martens,² Jeffrey I. Gordon,² and Thomas J. Smith^{1,*}

¹Donald Danforth Plant Science Center, St. Louis, MO 63132, USA

²Center for Genome Sciences, Washington University in St. Louis School of Medicine, St. Louis, MO 63108, USA

*Correspondence: tsmith@danforthcenter.org

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SUMMARY

The human gut microbiota performs functions that are not encoded in our *Homo sapiens* genome, including the processing of otherwise undigestible dietary polysaccharides. Defining the structures of proteins involved in the import and degradation of specific glycans by saccharolytic bacteria complements genomic analysis of the nutrient-processing capabilities of gut communities. Here, we describe the atomic structure of one such protein, SusD, required for starch binding and utilization by *Bacteroides thetaiotaomicron*, a prominent adaptive forager of glycans in the distal human gut microbiota. The binding pocket of this unique α -helical protein contains an arc of aromatic residues that complements the natural helical structure of starch and imposes this conformation on bound maltoheptaose. Furthermore, SusD binds cyclic oligosaccharides with higher affinity than linear forms. The structures of several SusD/oligosaccharide complexes reveal an inherent ligand recognition plasticity dominated by the three-dimensional conformation of the oligosaccharides rather than specific interactions with the composite sugars.

INTRODUCTION

The adult human gut is home to trillions of microbes. This community (microbiota) is dominated by members of a relatively few divisions (phyla) in the domain Bacteria (the Firmicutes and Bacteroidetes), but also contains members of Archaea and Eukarya (Eckburg et al., 2005; Ley et al., 2006a, 2006b). The distal gut microbiota is essential for digestion of a wide array of polysaccharides in our diet, because humans lack the requisite glycoside hydrolases (Sonnenburg et al., 2005; <http://cazy.org>). Short-chain fatty acids derived from polysaccharide fermentation contribute as much as 10% of daily caloric intake for persons who eat a Western-style diet (Backhed et al., 2005). Given the structural complexity and diversity of dietary glycans, a basic question is how members of the gut microbial community have evolved ways to recognize and acquire specific classes of carbohydrates among the vast and varied buffet of polysaccharides

encountered in this body habitat. The answers could provide new strategies for optimizing nutrient utilization.

Bacteroides thetaiotaomicron is a prominent member of the human gut microbiota and an adept, flexible forager of polysaccharides (Sonnenburg et al., 2005). Its capacity for glycan degradation is impressive: its proteome contains 246 known or predicted glycoside hydrolases and polysaccharide lyases (Xu et al., 2003), compared with 99 in the human proteome (<http://cazy.org>). Most *B. thetaiotaomicron* genes encoding these glycoside hydrolases occur in large clusters, termed polysaccharide utilization loci (PULs), which also contain genes for extracellular polysaccharide recognition and uptake (Bjursell et al., 2006; Xu et al., 2003). The first of these PULs to be characterized was the starch utilization system (Sus), which is required by *B. thetaiotaomicron* to grow on amylose, amylopectin, pullulan, and maltooligosaccharides (Anderson and Salyers, 1989a, 1989b). The Sus system (Figure 1) contains eight genes, *susRABCDEFG*. SusR acts as a transcriptional activator of the locus in response to maltose (D'Elia and Salyers, 1996b). SusA, SusB, and SusG have neopullulanase, α -glycosidase, and α -amylase activity, respectively (Reeves et al., 1996; Shipman et al., 2000; D'Elia and Salyers, 1996a). SusDEFG are components of an extracellular outer membrane complex that binds to and degrades large starch molecules to maltooligosaccharides, which are imported into the periplasm via SusC, a predicted TonB-dependent β -barrel porin (Cho and Salyers, 2001; Shipman et al., 2000). SusG is the only externalized glycoside hydrolase produced by the Sus system, but it has a relatively low affinity for starch ($K_m \sim 3$ mM) and therefore does not contribute to starch binding to the cell surface (Shipman et al., 1999). The roles of SusE and SusF are unclear (Cho and Salyers, 2001; Shipman et al., 2000).

The lipoproteins SusDEFG are each tethered to the outer membrane surface, and all five outer membrane proteins, SusCDEFG, co-purify on amylose resin, with SusCD being the minimum starch-binding complex (Cho and Salyers, 2001; Shipman et al., 2000). *B. thetaiotaomicron* mutants containing a polar disruption in *susD* (Δ *susDEFG*) have little to no starch-binding activity, suggesting that the putative porin SusC is not sufficient for starch or oligosaccharide binding. However, a mutant containing a polar disruption in *susE* (Δ *susEFG*) regains 70% of the wild-type starch-binding capacity (Shipman et al., 2000). Moreover, when the Δ *susEFG* cells are complemented with *susG*, *B. thetaiotaomicron* can grow on starch, whereas the Δ *susDEFG* mutants cannot (Cho and Salyers, 2001). Since SusC and SusD together promote tight binding of starch to the cell surface, it seems logical that SusD is directly involved in

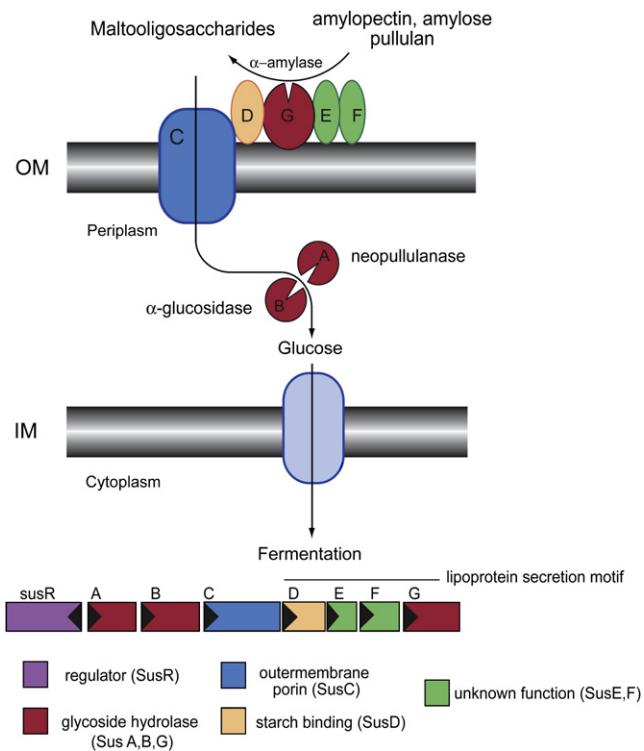


Figure 1. The Starch Utilization System (Sus) of *Bacteroides thetaiotaomicron*

Cartoon representation of the Sus operon and its protein products (Cho and Salyers, 2001; D'Elia and Salyers, 1996a; Shipman et al., 1999, 2000). The stoichiometry of the Sus complex is not known.

starch binding (Shipman et al., 1999). Interestingly, SusD does not have detectable homology to known carbohydrate-binding modules.

The *B. thetaiotaomicron* genome contains 101 pairs of genes related to *susC-susD*, with an additional 269 pairs found among four other sequenced human gut-associated *Bacteroides* species genomes sequenced to date (Xu et al., 2007). Genes encoding SusC/SusD pairs are ubiquitous components of PULs. The individual proteins may be highly divergent from one another and are often grouped with genes encoding diverse glycoside hydrolase and polysaccharide lyase activities, suggesting that each PUL has evolved to target a specific polysaccharide. Indeed, individual PULs are differentially expressed in vivo under conditions where *B. thetaiotaomicron* is forced to forage on various dietary and host glycans (Bjursell et al., 2006; Sonnenburg et al., 2005).

Despite the prominent representation of Sus-like systems in *B. thetaiotaomicron* and other sequenced human gut-associated Bacteroidetes (Xu et al., 2007), little is known about the molecular basis of carbohydrate recognition by these complexes. Therefore, we have performed structural and biochemical analyses of *B. thetaiotaomicron* SusD to elucidate its role in starch recognition and uptake. Our results indicate that SusD represents a novel class of polysaccharide-binding proteins and reveal a paradigm for how a saccharolytic bacterium recognizes and acquires a specific class of glycans.

RESULTS

SusD Is Required for Starch and Maltooligosaccharide Utilization

Previously, technical limitations for genetic manipulation of *B. thetaiotaomicron* prevented examination of the role of SusD in starch utilization in isolation from the other sus genes. We used a novel counter-selection technique to construct an in-frame deletion strain (Δ susD) that lacked codons 2–551 of susD. Quantitative real-time PCR analysis of transcripts corresponding to the remaining genes in the susBCDEFG operon demonstrated that they are still induced in the Δ susD strain in response to maltose (see Figure S1 available online), confirming that only expression of SusD is eliminated.

To determine the contribution of SusD to the growth of *B. thetaiotaomicron*, the parent (wild-type), isogenic Δ susD strain and a derivative of Δ susD containing a single, complementing copy of susD expressed from its native promoter (Δ susD:: P_{susD} -susD) were cultured in minimal medium containing a number of starch-like substrates as sole carbon sources. Consistent with previous findings that the Sus system is not required for growth on maltooligosaccharides containing ≤ 3 glucose units (Anderson and Salyers, 1989b), all three strains exhibited similar growth rates on glucose, maltose, and maltotriose (Figure 2). However, loss of SusD abolished the ability of *B. thetaiotaomicron* to grow on amylopectin and pullulan, as well as maltohexaose and maltoheptaose, suggesting an essential role in growth on starch molecules containing ≥ 6 glucose units. Additionally, the Δ susD strain exhibited a reduced growth rate on maltotetraose and malopentaose, indicating a supporting but nonessential role in utilization of shorter oligosaccharides. The loss of SusD did not affect the ability of *B. thetaiotaomicron* to utilize dextran, suggesting that SusD is preferentially recognizing the α -1,4 linked sugars.

Overall Structure of SusD

Native SusD in *B. thetaiotaomicron* includes an outer membrane signal sequence that is likely removed upon translocation, and a cysteine (C25) that is lipidated and tethers the protein to the cell membrane. Therefore, only residues 26–551 of SusD were expressed in *Escherichia coli* for these studies. Size exclusion chromatography of heterologously expressed SusD indicated that it is predominantly monomeric (~60 kDa) with a small fraction appearing as dimers (data not shown). This was confirmed by native PAGE analysis showing SusD in monomeric (~80%) and dimeric (~20%) forms (data not shown). The apo structure of SusD was determined using multiwavelength anomalous dispersion (MAD) phasing methods and seleno-methionine substituted protein crystals. The native apo-protein structure was determined to a resolution of 1.5 Å ($R_{work} = 19.3\%$; $R_{free} = 21.1\%$). The crystals belonged to the P1 space group, and each asymmetric unit contained two monomeric copies of SusD. Residues 42–59 and 70–551 were defined in the electron density of both copies of the protein. It was not surprising that the first 16 residues of the protein were disordered, because they likely form a flexible tether extending from the lipid anchor.

SusD has an α -helical fold comprising 22 α helices, three sets of 2-stranded antiparallel β sheets, and multiple reverse turns (Figure 3A). Eight α helices— α 1 (44–53), α 4 (117–139), α 5

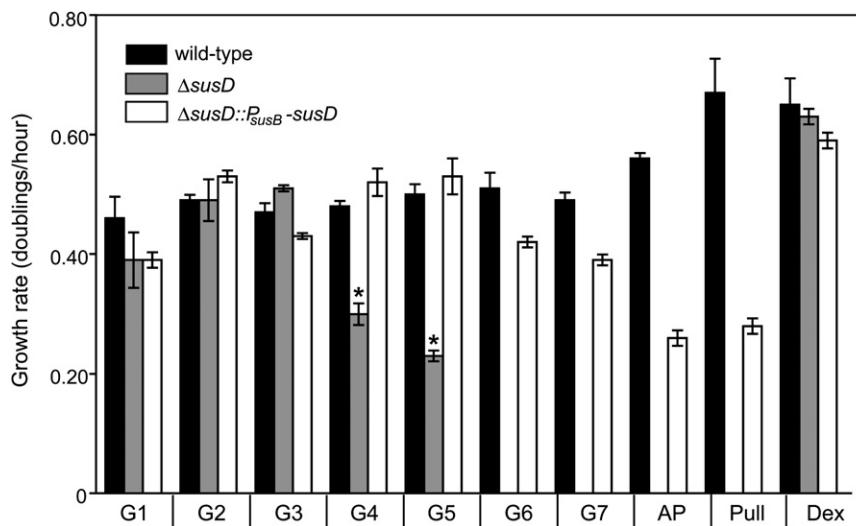


Figure 2. Growth of *Bacteroides thetaiotaomicron* and Derivative Strains on Starch-like Oligosaccharides and Polysaccharides

Shown are the log-phase growth rates of wild-type, Δ susD, and complemented Δ susD (Δ susD:: P_{susB} -susD) strains on glucose (G1), maltooligosaccharides of varying length (G2–G7), amylopectin (AP), and pullulan (Pull) and dextran (Dex). The Δ susD strain is unable to grow on substrates >5 glucose units and exhibits significantly slower rates on G4 and G5 compared to wild-type on the same substrates ($p < 0.01$, denoted by an asterisk). Complementation with a single copy of susD (expressed at ~10% of wild-type levels; see Figure S1) restores either full or partial growth characteristics depending on substrate size.

Error bars represent the standard deviation of the values (measured in triplicate).

(146–170), α 6 (189–206), α 7 (220–238), α 8 (243–255), α 17 (438–452), and α 18 (457–466)—pack together as four tetratricopeptide repeat (TPR) units that form a right-handed superhelix along one side of the structure. Two additional α helices— α 19 (478–489) and α 20 (495–502)—also adopt a helix-loop-helix motif and pack against α 17 and α 7, respectively, and connect the TPR domain to the rest of the structure. SusD was not predicted to have any TPR motifs, because it does not have the amino acid signature W₄-L₇-G₈-Y₁₁-A₂₀-F₂₄-A₂₇-P₃₂ (D'Andrea and Regan, 2003). Structural analysis using DALI (Holm and Sander, 1995) revealed the closest structural homolog of SusD is the inner membrane protein PilF from *Pseudomonas aeruginosa* (Kim et al., 2006). PilF is an entirely α -helical protein composed of 6.5 tandem TPR repeats. Despite differences in both the lengths of each helix and overall topology of the structures, the C α of residues 30–172 of PilF (PDB code: 2FI7) overlaid with an RMSD of 1.6 \AA to the four TPR units of SusD (Figure 3B).

There are a number of small molecules associated with apo SusD. A single metal ion is bound in a shallow groove on the surface of SusD adjacent to α 1 and the antiparallel β sheet formed by β 4 and β 6. The ion was modeled as Ca²⁺ because it has an octahedral coordination sphere and an average metal-ligand distance of 2.4 \AA . The Ca²⁺ is coordinated by a carboxylic acid oxygen of D432, the backbone carbonyl oxygens of D430, and Q288, an ordered water, polyethylene glycol (PEG), and ethylene glycol. From the well-ordered electron density, the PEG molecule appears to be four carbon atoms in length and hydrogen bonded to the Ca²⁺, the side chain oxygens of D273 and D432, and the guanidyl nitrogen of R287. Several other molecules of ethylene glycol were observed in this structure but are not found in identical positions in both copies of SusD.

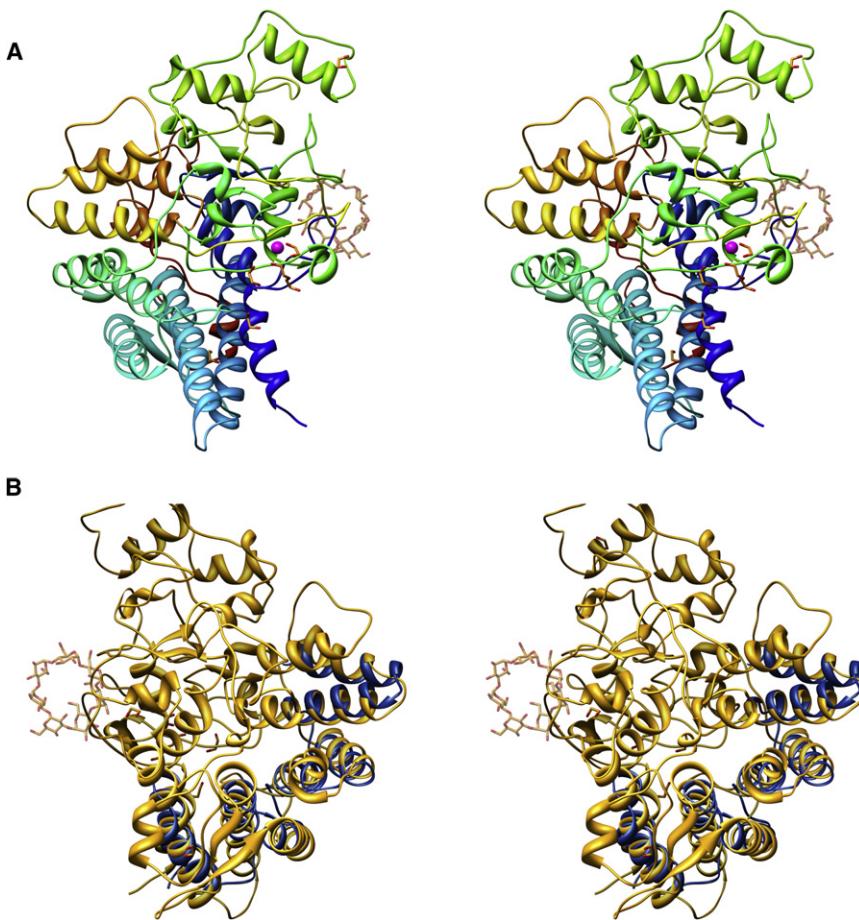
C322 was oxidized to sulfenic acid in the structure of apo SusD. Although this finding is unusual, sulfenic acid has been observed in a number of crystal structures for which it has no known function. C322 is surrounded by W96, W98, and W320, which create a hydrophobic patch on the surface of SusD and initiate starch binding (see below). Finally, a partially ordered molecule of morpholino-ethane sulfonic acid was observed to be bound within 4 \AA of W320 in chain A of the apo structure.

SusD Complexed with Maltoheptaose or β -Cyclodextrin

The structure of SusD complexed with maltoheptaose was determined to a resolution of 2.2 \AA ($R_{\text{work}} = 18.3\%$; $R_{\text{free}} = 23.1\%$). C322 was not oxidized to sulfenic acid, as in the apo structure. Maltoheptaose was observed in different relaxed helical conformations in the two copies of SusD, and this is likely due to the differences in crystal packing. Glc1, the nonreducing end of the maltoheptaose, was disordered in one copy of SusD (chain B) and therefore the interactions in chain A are described in detail.

Each glucose adopts the most favored $^4\text{C}_1$ chair conformer. In both copies of SusD, Glc5–Glc7 of maltoheptaose interact with SusD via identical ring stacking and hydrogen-bonding interactions (Figure 4A). The sugar rings of Glc5, Glc6, and Glc7 stack along the aromatic face of W98, W320, and Y296, respectively, creating an arced hydrophobic surface that complements the shape of the oligosaccharide. Maltoheptaose displays average ϕ (O5–C1–O4'–C4'), ψ (C1–O4'–C4'–C5') angles of 105.3° and –128.9°, respectively, that better reflect the natural curve and shape of double helical α -amylose ($\phi = 91.8^\circ$, $\psi = -153.2^\circ$; $\phi = 85.7^\circ$, $\psi = -145.3^\circ$; $\phi = 91.8^\circ$, $\psi = -151.3^\circ$) rather than the single helical V-amylose ($\phi = 103.6^\circ$, $\psi = 115.3^\circ$) (Gessler et al., 1999; Imberty et al., 1988). In addition to hydrophobic stacking interactions, maltoheptaose binds to SusD via both direct and solvent-mediated hydrogen-bonding networks. The 2- and 3-hydroxyl groups of Glc5 are within hydrogen-bonding distance of the carboxamide oxygen and nitrogen of N101, and the 6-hydroxyl of Glc5 hydrogen bonds with backbone carbonyl oxygen of W320. Both the 2- and 3-hydroxyl groups of Glc6 are hydrogen bonded to the guanidyl nitrogens of R81. The 2-hydroxyl oxygen of the reducing sugar Glc7 is within hydrogen-bonding distance to backbone amines of G75 and G74. At the opposite end of maltoheptaose, Glc1 is positioned for hydrogen-bonding interactions through its 2- and 3-hydroxyl groups with the carboxylic acid side chain of D73.

Although the overall structures of the apo- and maltoheptaose-bound forms of SusD are nearly identical (RMS deviation of 0.44 \AA for 495 C α atoms), two loops that form the sides of the binding cavity undergo a large conformational change upon polysaccharide binding (Figure 4B). For this comparison, chain B of both the

**Figure 3. Atomic Structure of SusD**

(A) Stereo ribbon diagram of apo-SusD, color-ramped from dark blue to red as the chain extends from the amino to the carboxyl end of the protein. An ordered Ca^{2+} ion is represented by a magenta-colored sphere, whereas polyethylene glycol and ethylene glycol are shown as ball-and-stick figures. As a reference, a molecule of maltoheptaose from the structure of the SusD-maltoheptaose complex is shown as a transparent ball-and-stick. (B) Stereo figure of SusD (yellow) highlighting residues 31–172 of PilF (blue) which contain the TPR units.

apo (no MES) and bound forms of SusD were overlaid. Residues 70–77 display the greatest amount of conformational flexibility. This finding is at least partly due to the fact that they precede residues 60–69 that are disordered in the apo structure and only ordered in chain B of the maltoheptaose bound SusD. Upon oligosaccharide binding, residues 70–77 rotate out of the binding pocket, displacing the $\text{C}\alpha$ of D70 by 18.5 Å. This rearrangement allows residues 73–76 to move proximal to the sugar, breaking the hydrogen bond between the guanidyl nitrogen of R81 and the backbone carbonyl oxygen of Q72 to form the hydrogen bond between R81 and Glc6. In turn, residues 292–297 move Y296 by 3.6 Å for ring stacking interactions with Glc7.

Maltoheptaose adopts a distinctively circular shape upon binding to SusD that is akin to the expected conformation of starch. Therefore, we hypothesized that circular polysaccharides would better represent starch binding to SusD. SusD was subsequently crystallized in the presence of β -cyclodextrin and the structure ($R_{\text{work}} = 17.05\%$; $R_{\text{free}} = 20.6\%$) was determined to a resolution of 2.1 Å. For the sake of clarity, the glucose moieties of β -cyclodextrin were labeled consistently with the bound maltoheptaose. We found that the overall structure and protein interactions with Glc4, Glc5, and Glc6 in β -cyclodextrin are essentially identical with those in maltoheptaose (Figure 5A). Because of the circular structure of β -cyclodextrin, Glc7 adopts a slightly different conformation than does maltoheptaose. To compensate for this change, the phenolic side chain of Y296 shifts slightly to

maintain hydrophobic stacking interactions with Glc7. In addition, both Glc7 and Glc1 are now beyond hydrogen-bonding distance from residues 73–75 and make only solvent-mediated interactions with the protein. A concerted movement in residues 70–77 and 292–297 is observed in the SusD/ β -cyclodextrin complex, as in the maltoheptaose complex.

SusD Complexed with α -Cyclodextrin

The structure of SusD with α -cyclodextrin (G6) was also determined because we reasoned that the tighter radius of the six-glucose sugar might be a better mimic of helical starch. SusD was crystallized in

the presence of α -cyclodextrin, and the structure was solved to 2.1 Å ($R_{\text{work}} = 19.4\%$; $R_{\text{free}} = 23.7\%$). Strikingly, α -cyclodextrin is wedged between two SusD molecules with each SusD binding three glucose residues (Figure 6). The $\text{C}\alpha$ backbones of SusD with β -cyclodextrin and with α -cyclodextrin overlay with an RMSD of 0.36 Å, with only minor shifting of the binding site residues. The two copies of SusD also interact with each other, burying $\sim 290 \text{ \AA}^2$ on each subunit upon complex formation. It is likely that these protein–protein interactions enhance the binding of the sugar to SusD, compensating for lower affinity interactions between the protein and glucose residues with higher avidity. Including contributions from bound α -cyclodextrin, a total of $\sim 1100 \text{ \AA}^2$ of protein is buried between the SusD subunits upon complex formation. Native PAGE analysis (Figure S6) and size exclusion chromatography (data not shown) both suggest that SusD forms oligomers in solution. However, it is not clear whether these interactions are relevant to what is observed in this crystal structure. The banding pattern does not appear to change when α -cyclodextrin is added to the SusD sample. However, this is not entirely unexpected with the relatively weak binding affinity of α -cyclodextrin to SusD.

Each half of α -cyclodextrin binds to SusD in an equivalent manner, with two-fold symmetry observed across the binding complex. In the trimeric complex of SusD with α -cyclodextrin, each glucose residue is involved in hydrophobic stacking interactions with W98, W320, or Y296 (Figure 6C). The loop defined

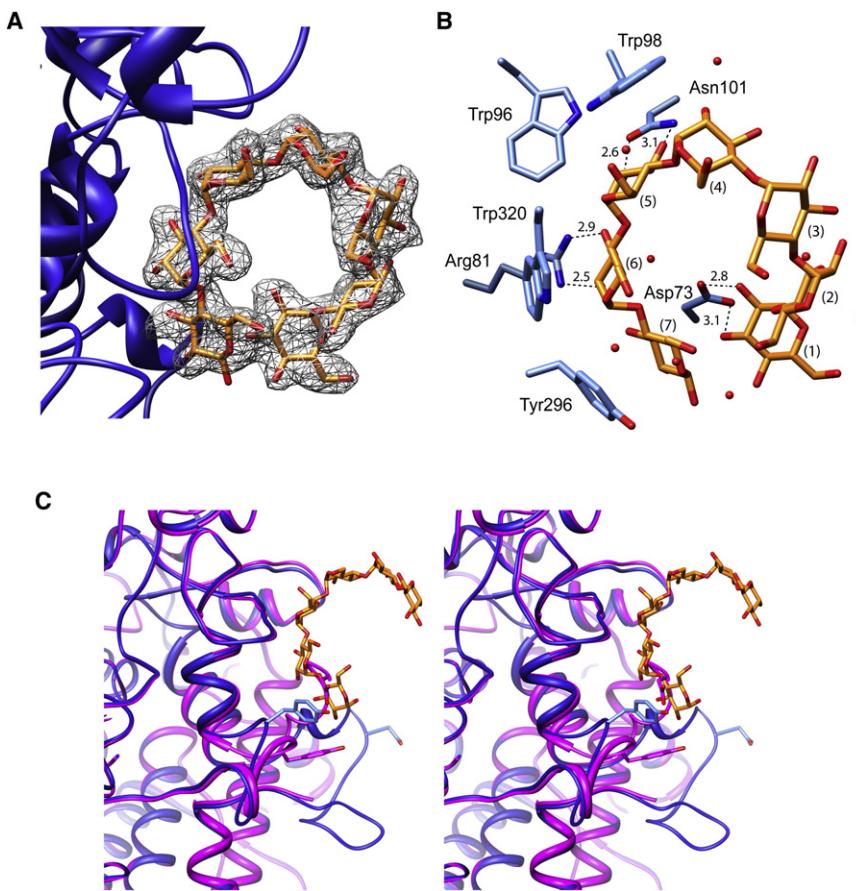


Figure 4. SusD Complexed with Maltoheptaose

(A) The electron density of bound maltoheptaose from the corresponding omit map contoured at 3σ is shown.

(B) Important hydrophobic-stacking and hydrogen-bonding interactions between the maltoheptaose and SusD are detailed.

(C) Stereo diagram of SusD in the presence (blue) and absence (mauve) of bound maltoheptaose to highlight the conformational changes that occur upon oligosaccharide binding.

hydrophobic surface created by W320 and W96 provides the initial binding surface for polysaccharide docking. As the three initial glucose residues stack along these tryptophan residues, the loops 70–77 and 290–299 open up to accommodate the larger oligosaccharides.

Isothermal Titration Calorimetry (ITC) Suggests SusD Recognizes Helical Starch

The SusD knockout mutants demonstrate that SusD plays a crucial role in growth of *B. thetaiatomicron* on starch molecules containing ≥ 6 glucose units. In addition, the crystallographic data indicate a preference for circular, or helical, oligosaccharides over linear forms. To further examine

by residues 58–72 is disordered in both subunits of this structure, as observed in the other structures except for subunit B of SusD complexed with maltoheptaose. If subunit B of SusD/maltoheptaose is superimposed on the SusD/ α -cyclodextrin complex, residues 59–72 are adjacent to the starch-binding site and near the dimeric interface of SusD. Because of their proximity to the binding pocket and the inherent flexibility of this loop, it is possible that these residues either directly assist in binding linear oligosaccharides or aid in reorganizing starch-binding residues 73–77 when longer oligosaccharides bind.

SusD Complexed with Maltotriose

To determine which contacts define the minimal polysaccharide-binding site, apo SusD was crystallized in the presence of maltotriose, and the structure was determined to a resolution of 2.3 Å ($R_{\text{work}} = 18.2\%$; $R_{\text{free}} = 23.2\%$). Maltotriose binds in an identical orientation as Glc4, Glc5, and Glc6 of both maltoheptaose and β -cyclodextrin (Figure 5B). The sugar rings of the bound polysaccharide only form stacking interactions with W320 and W98. There is no rearrangement of residues 70–77 or 292–297; therefore, Y296 does not participate in ligand binding. The only hydrogen bonds between maltotriose and SusD are between the 2- and 3-hydroxyls of Glc2 (Glc5 in β -cyclodextrin and maltoheptaose) and the side chain of N101, and the 6-hydroxyl of Glc2 with the backbone carbonyl oxygen of W320.

The complex of maltotriose and SusD likely represents the initial binding event between starch and SusD. The relatively rigid,

the substrate selectivity of SusD, ITC was performed with maltotriose, maltopentaose, maltoheptaose, α -cyclodextrin (G6), β -cyclodextrin (G7), and γ -cyclodextrin (G8). No interactions between SusD and maltotriose were detected via ITC, and the energy associated with the binding of maltopentaose was too small to accurately determine an association constant. Of the remaining oligosaccharides tested, SusD clearly prefers the cyclic derivatives of starch over the linear forms (Figure 7). The fact that SusD binds α -cyclodextrin and γ -cyclodextrin with ~ 20 -fold greater affinity than maltoheptaose suggests that recognition is dominated by the helical shape of the polysaccharide rather than the stereochemistry of the composite glucose residues.

DISCUSSION

The crystal structures of SusD complexed with maltoheptaose, β -cyclodextrin, α -cyclodextrin, and maltotriose demonstrate that SusD induces marked curvature in linear oligosaccharides that closely mimics the conformations of bound cyclic polysaccharides. Furthermore, isothermal titration calorimetry indicates that the cyclic compounds bind better than do the linear forms. Together, these results suggest that SusD has a flexible starch-binding site designed to recognize the helical nature of starch rather than the stereochemistry of its composite glucose residues. This plasticity of recognition is consistent with its relatively weak binding affinity for the oligosaccharides; however, this is likely compensated by multivalent interactions between the cell and substrate.

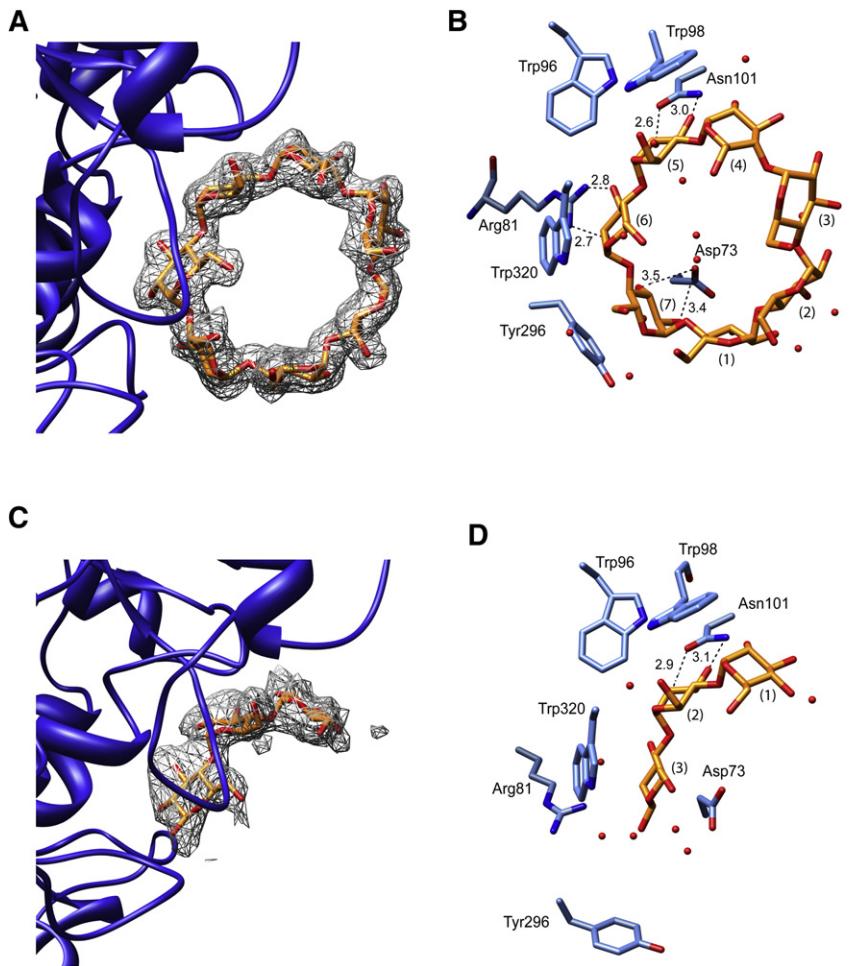


Figure 5. SusD Complexed with β -Cyclodextrin and Maltotriose

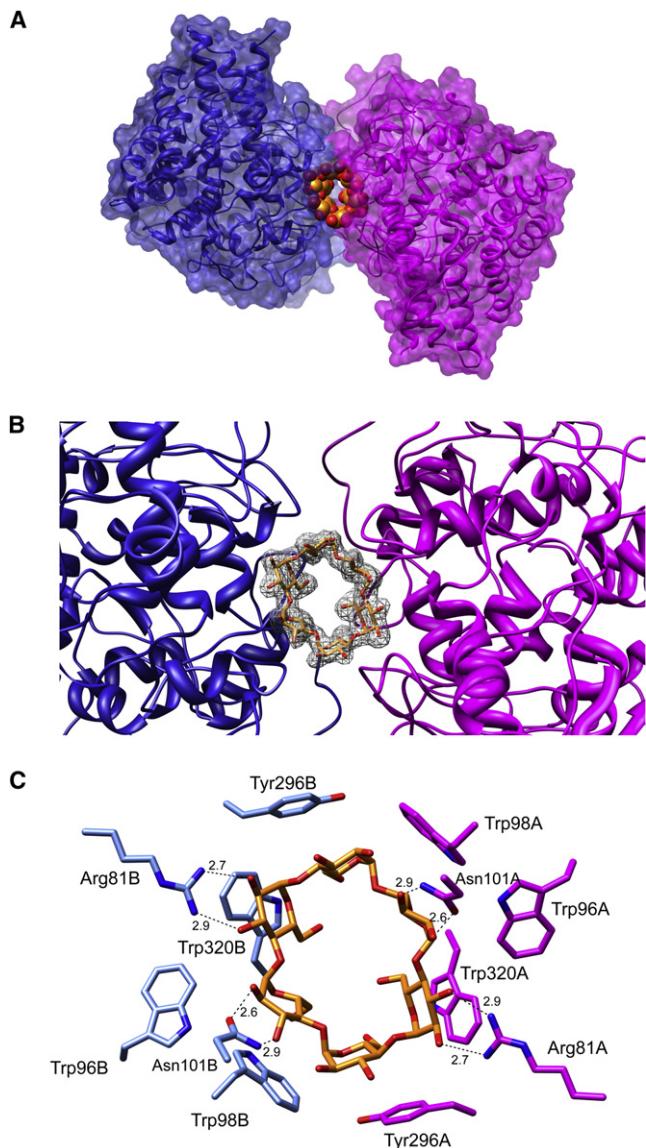
(A and C) The electron densities of β -cyclodextrin and maltotriose, respectively, from omit maps contoured at 3σ .

(B and D) Important hydrophobic-stacking and hydrogen-bonding interactions for bound β -cyclodextrin and maltotriose, respectively, are highlighted.

Our studies of wild-type *B. thetaiotaomicron*, an isogenic in-frame deletion strain (Δ susD), and a Δ susD strain containing a single complementing copy of susD expressed from its native promoter demonstrate that SusD is required for the utilization of maltooligosaccharides comprising ≥ 6 glucose units, including pullulan and amylopectin, which contain α 1,6 branch points. SusD plays a nonessential role in growth on maltotetraose or maltopentaose, since Δ susD is able to grow on these substrates, albeit at about half the growth rate of wild-type. It is possible that these intermediate length oligosaccharides represent a transition point in the glycan-utilizing capabilities of *B. thetaiotaomicron* and that maltotriose and smaller sugars enter the cell via a different (non-Sus) pathway with maltotetraose and maltopentaose lying between the size limitations for these two systems. The decreased growth rate of the complemented Δ susD::*P*_{susB}-susD strain on pullulan and amylopectin, compared with its wild-type parent, is likely due to decreased SusD expression. Quantitative RT-PCR techniques demonstrated that this strain produces ten-fold less of the susD mRNA transcript (Figure S1) upon induction. Because the stoichiometry of the Sus complex is unknown, it is difficult to know whether the defect arises from less SusD incorporated per Sus complex or whether fewer Sus complexes are present on the cell surface.

SusD has a novel α -helical fold unlike that observed for any other carbohydrate-binding modules (CBMs), which are mainly composed of β -sheets arranged in a β -barrel (Machovic and Janecek, 2006). On the basis of the arrangement of tetratricopeptide repeat units, the closest structural homolog to SusD is PilF, an inner membrane protein produced by *P. aeruginosa* that is involved in type IV pilus biogenesis (Kim et al., 2006). Although TPR proteins have a wide variety of functions, they tend to be involved in protein-protein interactions and assembling multiprotein complexes (D'Andrea and Regan, 2003). Previous studies suggested that PilF interacts with other members of the pilus assembly complex via the concave inner surface formed from the right-handed superhelical twist of the assembled TPR units (Kim et al., 2006). However, the concave face created by the TPR units of SusD, which comprise roughly half of the full-length PilF structure, cradles the remainder of the α -helical SusD structure in a way that the TPR domain of protein farnesyl transferase supports its catalytic domain (Kim et al., 2006). It is tempting to speculate that the TPR units of SusD form an assembly scaffold for the SusCDEFG complex since the entire Sus complex can be purified using an amylose column and SusC and SusD copurify in the absence of SusE, SusF and SusG (Shipman et al., 2000).

As noted above, our results indicate that SusD recognizes the three-dimensional fold of the starch molecule rather than the stereochemistry of its composite glucose residues. Although the crystal structures of SusD with maltoheptaose or β -cyclodextrin have nearly identical ligand-protein interactions, ITC demonstrates that cyclodextrins bind with ~ 20 -fold higher affinity than the linear maltoheptaose. If the binding site of SusD is designed to recognize the helical structure of α -amylose, then linear oligosaccharides would be expected to bind more weakly because of the imposition of the curved conformation. In contrast, the cyclic oligosaccharides are expected to bind better since they are already constrained to a curved conformation. The ends of bound maltoheptaose do not interact with SusD, consistent with the idea that SusD recognizes the internal helical structure of starch ("endo" versus "exo" recognition) rather than a discrete oligosaccharide length.



The starch-binding site of SusD is dominated by tryptophan and tyrosine residues that create an arched hydrophobic surface, complementing the shape of helical oligosaccharides. In particular, W98 and W320 are juxtaposed at an angle of $\sim 130^\circ$ to each other, and their conformations are relatively unaffected by oligosaccharide binding. The sugar rings of maltotriose were found to stack along these tryptophans nearly the same as that observed with the bound maltoheptaose and β -cyclodextrin. Thus, the geometry of these two tryptophans likely aids in the initial docking of sugars based on their overall shape. This dual tryptophan motif has also been observed in several glycoside hydrolases capable of degrading raw starch, such as glucoamylase, cyclodextrin glu-

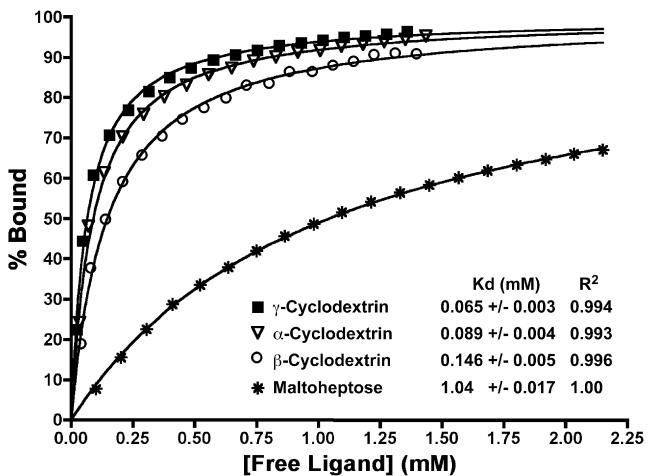


Figure 7. Isothermal Titration Calorimetry of the Binding of Various Oligosaccharides to SusD

As described in the [Experimental Procedures](#), the heat of binding was converted to a percentage of the maximum binding of the ligands to SusD and fitted to a single class of binding sites equation.

cosyltransferase, and barley α -amylase (Kadziola et al., 1998; Penninga et al., 1996; Robert et al., 2005; Sogaard et al., 1993; Sorimachi et al., 1997). The starch-binding site of SusD shares the most similarity with the surface starch granule-binding site of the barley α -amylase AMY1 (Robert et al., 2005). This starch granule-binding site has a K_d value of ~ 2 mM for maltoheptaose, a value very similar to SusD and maltoheptaose. Like SusD, the starch granule site of AMY1 is dominated by two tryptophans, W278 and W279, that are situated at an angle of 159° to each other and provide a ring-stacking environment that holds adjacent glucose moieties in a helical conformation. Thus, as with SusD, the AMY1 starch-granule binding site acts as a “geometric filter,” capable of binding to helical starch polymers and thus selecting substrate by overall shape.

SusD displays some striking differences from the starch granules-binding sites of such glycoside hydrolyases as AMY1. Although W98 and W320 of SusD likely initiate oligosaccharide recognition, the lack of measurable binding of maltotriose by ITC demonstrates that this interaction alone is not sufficient for high affinity binding. A reorganization of the loops defined by residues 70–77, and 292–297 occurs when a longer oligosaccharide binds and brings R81 and Y296 into docking position. In addition, residues 73–75 are brought into the binding pocket so that the side chain of D73 is able to interact with Glc7 of maltoheptaose. The inherent plasticity in the SusD starch-binding pocket may allow SusD to initiate starch binding, facilitating recognition of both amylopectin and pullulan. The intermittent α 1,6 linkages in these polysaccharides would likely distort the helical shape of an α 1,4 glucose polymer (amylose), and therefore any protein capable of recognizing all three polysaccharides would require a plastic recognition site. As reflected in the *B. thetaiaotaomicron* growth curves, SusD is essential for both amylopectin and pullulan utilization.

The structure of SusD with α -cyclodextrin revealed two SusD subunits binding to a single ring of sugar. Although the *in vivo* relevance of this complex is unknown, both size exclusion

Table 1. MAD Data Collection Statistics

	Peak	Inflection	Remote
Wavelength (Å)	0.97934	0.97951	0.97167
Resolution (Å)	50–1.76 (1.82–1.76)	50–1.76 (1.82–1.76)	50–1.75 (1.81–1.75)
Independent reflections	112,867 (10,400)	112,750 (10,235)	115,258 (10,735)
Completeness	96.7 (89.0)	96.3 (87.7)	96.9 (90.8)
Redundancy	3.8 (3.6)	3.8 (3.6)	3.9 (3.6)
Avg I/Avg σ(I)	46.2 (15.9)	46.7 (16.6)	44.5 (12.4)
R _{sym} (%)	8.5 (14.9)	6.6 (12.7)	6.3 (12.8)

chromatography and native PAGE analysis (Figure S6) suggest that ~20% of heterologously expressed SusD exists as oligomers. The ability of SusD to bind oligosaccharides as either a monomer or dimer further highlights the inherent flexibility of SusD's starch-binding capacity; it is capable of monovalent interactions with flexible linear oligosaccharides as well as multivalent contacts with the more structurally static cyclic sugars.

Exactly how SusD confers the ability of the Sus complex to utilize starch is not fully understood. In previously published studies, both SusC and SusD were required, but not by themselves sufficient, for starch binding to *B. thetaiotaomicron* (Shipman et al., 2000). Interestingly, SusC and SusD expressed alone are more protease sensitive than when coexpressed, suggesting SusC/SusD interactions (Shipman et al., 2000). SusC might directly participate in binding starch in conjunction with SusD or indirectly facilitate starch binding by organizing multiple copies of SusD.

Perhaps the more important question is whether SusD is involved solely in initial starch binding to the cell surface, or whether

it is also involved in the transit of smaller oligosaccharides to SusC for import. From the data presented here, it seems most likely that the main role of SusD is to bind starch to the surface of *B. thetaiotaomicron*. The plasticity of the SusD-binding site increases the spectrum of recognized polysaccharides as made evident by the structural results and growth on α 1,6-branched pullulan and amylopectin. This plasticity in the recognition site is the likely cause of the relatively low affinity of interaction between SusD and oligosaccharides. As exemplified in the SusD/ α -cyclodextrin structure, it also seems likely that relatively weak intrinsic affinity is compensated by multivalent binding. Because of their long tethers (~16 amino acids) to the bacterial surface, multiple SusD molecules could bind to starch either through multiple copies of SusD in a given outer membrane Sus complex or through the interactions of multiple Sus complexes with the large starch polymer. Multivalent binding can improve apparent affinity (avidity), as exemplified by studies with antibodies showing that the difference between the apparent affinity of Fab (monovalent)

Table 2. Data Collection and Refinement Statistics

Structure	Apo	Maltotriose	Maltoheptaose	β -cyclodextrin	α -cyclodextrin
PDB accession	3CKC	3CKB	3CK9	3CK8	3CK7
Resolution (Å)	50–1.47 (1.52–1.47)	59.5–2.3 (2.44–2.3)	60.2–2.2 (2.35–2.2)	60.2–2.1 (2.21–2.1)	67.5–2.1 (2.21–2.1)
Unique reflections	190,539 (17,881)	47,571 (5979)	56,299 (7038)	66,380 (8307)	127,524 (16,027)
% Completeness	95.7 (90.0)	91.9 (73.2)	94.6 (72.0)	94.2 (82.7)	93.4 (81.2)
Redundancy	4.2 (3.7)	3.5 (2.2)	4.6 (2.3)	4.3 (1.7)	4.5 (1.5)
I/sig(I)	54.0 (19.7)	6.1 (2.5)	7.3 (2.0)	16.0 (6.6)	6.9 (1.9)
R _{sym} (%)	4.5 (8.4)	7.85 (17.7)	8.65 (19.8)	3.3 (7.3)	9.6 (21.3)
No. proteins atoms	8048	8075	8196	8049	16,002
No. hetero atoms	1299	705	925	1186	1468
R _{work} (%)	19.2 (22.8)	18.0 (22.5)	18.5 (21.4)	16.9 (17.7)	19.4 (25.5)
no. of reflections	163,414 (3298)	42,749 (517)	50,168 (629)	58,823 (996)	114,786 (1432)
R _{free} (%)	21.0 (24.5)	21.6 (26.2)	22.2 (26.6)	20.0 (22.0)	23.7 (26.8)
no. of reflections	18,043 (369)	4791 (60)	5665 (70)	6638 (117)	12,682 (168)
Average B values (Å ²)					
Protein atoms	16.09	7.5	16.2	15.1	12.0
Ligand (sugar)	n/a	18.3	28.2	29.3	10.5
Heteroatoms	25.73	11.5	28.9	24.1	15.8
RMS deviations					
Bond length (Å)	0.0047	0.0059	0.0064	0.0052	0.0057
Bond angles (°)	1.21	1.16	1.22	1.18	1.19

Numbers in the parentheses represent the statistics of the highest resolution shell.

and intact IgG (bivalent) proteins for their ligand can be three to four orders of magnitude (Hornick and Karush, 1972). However, these interactions could be an artifact of crystal packing or might be indicative of interactions between SusD and the other proteins in this complex.

The binding of starch to the Sus complex could greatly enhance the catalytic efficiency of the Sus-associated α -amylase SusG by keeping the starch molecule and its products from diffusing from the complex until it is cleaved into oligosaccharides small enough for transport. The molecular weight cutoff observed in SusD binding, defined from ITC and the growth studies of the isogenic wild-type and Δ susD strains, suggests that SusD could direct these sugars into or near the SusC porin once they are smaller than six sugar units in length. Additional studies are necessary to better understand the interactions between SusD and the other proteins in this complex.

The Sus system is just one of 101 SusC/SusD paralog pairs found in the *B. thetaiotaomicron* genome. Whole genome transcriptional profiling of *B. thetaiotaomicron* in vitro and in the intestines of gnotobiotic mice (E.C.M. and J.I.G., unpublished data) suggests that each SusC/SusD paralog is specific for a particular glycan, with the SusD homologs binding the carbohydrate to the cell surface for initial degradation by glycoside hydrolases and subsequent import into the periplasmic space (Bjursell et al., 2006; Sonnenburg et al., 2005). SusC/SusD paralogs are also present in soil and marine *Bacteroidetes*, such as *Cytophaga hutchinsonii*, *Croceibacter atlanticus*, *Gramella forsetii*, and *Leeuwenhoekella blandensis* (Bauer et al., 2006; Cho and Giovannoni, 2003; Pinhassi et al., 2006; Xie et al., 2007). These bacteria are capable of degrading a variety of polysaccharides, including starch, cellulose, chitin, and algal material. Thus, the Sus system is likely a prototype for a nutrient acquisition complex that is ubiquitous among the bacteria in the division *Bacteroidetes*. Understanding the binding specificities, capacities, and transport mechanisms of Sus and Sus-like systems may not only aid the development of novel strategies for manipulating the nutrient utilization functions of the human gut microbiota but also may elucidate new ways of degrading polysaccharides for biofuels.

EXPERIMENTAL PROCEDURES

Heterologous Protein Expression

The *susD* gene (residues 26–551) was amplified by PCR from genomic DNA prepared from *Bacteroides thetaiotaomicron* ATCC 29148. The amplicon was cloned into pET28rTEV, where the thrombin cleavage site of pET-28a (Novagen) has been modified to a tobacco etch virus (TEV) protease cleavage site. pET28rTEV-*susD* was transformed into Rosetta (DE3) pLysS (Novagen) for protein expression. Cells were grown in TB medium at 37°C with shaking (225 rpm) until they reached an OD of ~0.4, at which time the temperature was adjusted to 22°C. Once the cultures reached an OD of ~0.8, cells were treated with 0.2 mM IPTG to induce SusD expression and were allowed to grow 16 hr at 22°C. Cells were subsequently harvested by centrifugation, frozen in liquid nitrogen, and stored at –80°C. Selenomethionine-substituted protein was produced via the methionine inhibitory pathway (Van Duyne et al., 1993), as described elsewhere (Koropatkin et al., 2007).

Purification of Native and Selenomethionine-Substituted SusD

Both native and selenomethionine-substituted SusD were purified using a 5 ml Hi-Trap metal affinity cartridge, according to the manufacturer's recommendations (GE Healthcare). The cell lysate was loaded onto the column in His

Buffer (25 mM NaH₂PO₄, 300 mM NaCl, and 10 mM imidazole [pH 8.0]), and SusD was eluted using an imidazole (10–300 mM) gradient. The His-tag was removed by incubation with rTEV (1:100 molar ratio relative to SusD) at room temperature for 16 hr. The cleaved protein was then dialyzed against His Buffer and passed over the affinity column to remove the His-tagged rTEV and undigested SusD. Purified SusD was dialyzed against 20 mM HEPES/100 mM NaCl (pH 7.0) and concentrated to an OD₂₈₀ of ~22 for crystallization.

Crystallization and Data Collection

Initial crystallization conditions for apo-SusD were determined via hanging drop using the Hampton Screen (Hampton Research). Large single crystals for both SeMet SusD and the native SusD were grown at 4°C in batch plates by seeding small crystals into mother liquor that contained 4.5 mg/ml SusD, 14% poly(ethylene) glycol 8000, 50 mM NaCl, and 50 mM morpholino-ethane sulfonic acid (MES). Crystals grew to dimensions of ~0.3 × 0.3 × 0.5 mm in 1–3 weeks. All apo crystals were triclinic with unit cell dimensions of $a = 62.19 \text{ \AA}$, $b = 68.06 \text{ \AA}$, $c = 83.05 \text{ \AA}$, $\alpha = 111.1^\circ$, $\beta = 93.2^\circ$, and $\gamma = 109.2^\circ$. The solvent content of the crystals was approximately 51%, with two molecules in the asymmetric unit (Tables 1 and 2).

Crystals of SusD complexed with maltoheptaose, β -cyclodextrin, or maltriose were grown at room temperature by streak seeding the native apo crystals into batch plates containing 7.0 mg/ml SusD, 50 mM sodium cacodylate (pH 6–6.5), 50–75 mM calcium acetate, 13%–15% PEG 8000, and 70 mM maltriose, 50 mM maltoheptaose, or 10 mM β -cyclodextrin. Crystals of SusD with α -cyclodextrin were grown at room temperature by streak seeding into batch plates containing 20 mg/ml SusD, 50 mM Tris-HCl (pH 8.5), 100 mM sodium acetate, 14%–15% PEG 4000, and 2.5 mM α -cyclodextrin.

In preparation for freezing, apo native and selenomethionine-substituted protein crystals were serially transferred to a final cryoprotectant solution containing 21% PEG 8000, 100 mM NaCl, 50 mM MES, and 20% ethylene glycol. The crystals were flash-frozen with liquid nitrogen, and diffraction maxima were collected on a 3 × 3 tiled "SBC3" CCD detector at the Structural Biology Center 19-BM beamline (Advanced Photon Source, Argonne National Laboratory, Argonne, IL). X-ray data were processed with HKL3000 and scaled with SCALEPACK (Otwinowski and Minor, 1997). Crystals of SusD complexed with maltriose, maltoheptaose, or β -cyclodextrin were serially transferred to final cryoprotectant solutions containing 17%–18% PEG 8000, 60–75 mM sodium cacodylate (pH 6.5), 30–75 mM calcium acetate, 20% ethylene glycol, and the concentration of oligosaccharide noted above. Crystals of SusD with α -cyclodextrin were serially transferred to a final cryoprotectant solution consisting of 75 mM Tris-HCl (pH 8.5), 100 mM sodium acetate, 20% PEG 4000, 17% ethylene glycol, and 5 mM α -cyclodextrin. Diffraction maxima were collected using an Oxford cryosystem and a Proteum R Smart 6000 CCD detector connected to a Bruker-Nonius FR591 rotating anode generator. Data were processed using the program SAINT from the Proteum2 software (Bruker AXS) and merged using XPREP (SHELXTL 6.10, Bruker AXS) or ProScale (Bruker AXS). Data collection statistics are displayed in Table S1.

X-Ray Structure Determination

The structure of SusD was solved via MAD phasing from the X-ray data collected from the selenomethionine-substituted crystals. The program SOLVE (Terwilliger and Berendzen, 1999) was used to determine and refine the initial positions of the selenomethionines, and RESOLVE (Terwilliger, 2000) was then applied for solvent flattening and initial model building. Alternate cycles of manual model building in O (Jones et al., 1991) with maximum-likelihood refinement with CNS (Brünger et al., 1998) was used to build and refine the 2.0 \AA selenomethionine-substituted SusD ($R_{\text{work}} = 20.4\%$; $R_{\text{free}} = 24.1\%$). This structure was then refined against with the native SusD X-ray data collected to 1.5 \AA . In all cases, the reflections used for the calculation of R_{free} were selected, at random, immediately after scaling and were never used for refinement. The structures of SusD complexed with the various oligosaccharides were determined via molecular replacement using the program AMoRe (Navaza, 1994) from the CCP4 suite of programs (CCP4, 1994) with the apo SusD structure as a search model. Alternate cycles of manual model building in O and refinement using CNS were combined to complete the models. Initial coordinates and geometric constraints for the four oligosaccharides were downloaded from the HIC-Up server (<http://xray.bmc.uu.se/hicup/>). Relevant refinement statistics are presented in Table S2.

Isothermal Titration Calorimetry

ITC measurements were performed using a MicroCal VP-ITC titration calorimeter (MicroCal). SusD was dialyzed overnight against a solution containing 20 mM HEPES (pH 7.0), 100 mM NaCl, and 0.5 mM CaCl₂ prior to the experiment. Oligosaccharides solutions were prepared using dialysis buffer. Protein samples (0.34–0.48 mM) were placed in the reaction cell, and the reference cell was filled with deionized water. After the temperature was equilibrated to 25°C, 19 successive 15 μ l injections of oligosaccharide solution (10–20 mM) were made while stirring at 460 rpm, and the resulting heat of reaction was measured. Baseline measurements were made using an identical injection regime in the absence of protein. Although these measurements yielded classic saturation curves for binding, the relatively low binding constants made analysis with the MicroCal Origin software package problematic. Therefore, the raw data were analyzed by other means with emphasis on limiting the number of variables for the nonlinear curve-fitting analysis in the program Prism. Using a linear extrapolation from the first few injection data points, the Δ heat value at zero ligand concentration was estimated and this value was used to adjust the baseline for the data set. Using the data at the higher ligand concentrations, the Δ heat at equilibrium was estimated by fitting the data to an exponential equation, $Y = Y_{\max}(1 - e^{-kX})$, where Y is the Δ heat, X is the ligand concentration, and k is the exponential rate of association. Using this Y_{\max} value and the known protein concentration, the ratio of Δ heat per mole of bound ligand was determined and used to calculate the concentration of bound ligand. The concentration of free ligand was then determined by subtracting the bound ligand from total ligand concentration at each point of the curve. The resulting curves were then analyzed using the standard disassociation constant formula, $Y = (B_{\max} - X)/(K_d + X)$, where Y was the concentration of bound ligand, X the concentration of free ligand, B_{\max} the maximum concentration of bound ligand, and K_d is the disassociation constant. As expected, B_{\max} always refined to the concentration of protein used for the reaction. The original ITC data are shown in Figures S2–S5.

Construction of *B. thetaiotaomicron* Gene Deletions

All bacterial strains, plasmids, and primers are referenced in Tables S3 and S4. Deletion of *susD* by allelic exchange was done using a novel counter-selectable system involving a modified *B. thetaiotaomicron* strain with a deletion of *tdk* (BT2275), a gene encoding thymidine kinase. A *tdk* deletion strain, resistant to the toxic nucleotide analog 5-fluoro-2'-deoxyuridine (FUDR), was constructed by ligating 3.5 kb fragments flanking the *tdk* gene into the suicide vector pKNOCK-*bla*-*erm*Gb. The resulting construct was conjugated into *B. thetaiotaomicron*, a single-recombinant merodiploid selected on erythromycin, and plated on BHI-blood agar containing FUDR (200 μ g ml⁻¹) to select for recombinants. Subsequent deletion of *susD*, using genomic fragments flanking the gene, was performed similarly as described for *tdk*, except that the Δ *tdk* strain was used as a parent in conjunction with a different suicide vector (pExchange-*tdk*) that contains a cloned copy of *tdk* to facilitate counter-selection. Candidate *susD* deletions were screened by PCR and by DNA sequencing to identify isolates that had lost the gene.

Complementation of Δ *susD* was accomplished by joining the *susB* promoter (197bp *susA*-*susB* intergenic region) to a promoterless copy of *susD* in the *Bacteroides* species integrative vector pNBU2-*bla*-*erm*Gb (Supplemental Data) and inserting this construct into one of two tRNA^{ser} attachment sites targeted by NBU2 (Wang et al., 2000).

B. thetaiotaomicron Growth on Oligosaccharides

The minimal medium used for analysis of growth rates contained 100 mM KH₂PO₄ (pH 7.2), 15 mM NaCl, 8.5 mM (NH₄)₂SO₄, 4 mM L-cysteine, 1.9 μ M hematin, 200 μ M L-histidine, 100 nM MgCl₂, 1.4 nM FeSO₄ • 7 H₂O, 50 μ M CaCl₂, 1 μ g/ml vitamin K₃, 5 ng/ml vitamin B₁₂, plus individual carbon sources (0.5% wt/vol, Sigma). Five-milliliter cultures were inoculated with a 1:50 dilution of bacterial cells that were freshly grown in TYG medium and washed once in minimal medium lacking any added carbon source. Cultures were grown at 37°C using the NaHCO₃/pyrogallol anaerobiosis method (Holdeman et al., 1977) and were monitored by OD₆₀₀ at 30 min intervals. Rates were calculated from \geq 4 data points from each growth curve (OD₆₀₀ range, 0.2–0.5) that had been fit to an exponential function.

Note that suppressor mutants (5/20 cultures) were consistently observed when the Δ *susD* strain was grown in medium containing maltohexaose and

maltoheptaose. Analysis of four of these suppressor strains revealed that, despite lacking *susD*, they had regained the ability to grow on maltoheptaose and pullulan, albeit more slowly than wild-type (data not shown).

ACCESSION NUMBERS

X-ray coordinates have been deposited in the Protein Data Bank under accession numbers 3CKC, 3CKB, 3CK9, 3CK8, and 3CK7.

SUPPLEMENTAL DATA

Supplemental Data include six figures, two tables, Supplemental References, and Supplemental Experimental Procedures and can be found with this article online at <http://www.structure.org/cgi/content/full/16/7/1105/DC1>.

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