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Dynamic responses of *Bacteroides thetaiotaomicron* during growth on glycan mixtures

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Summary

***Bacteroides thetaiotaomicron* (*Bt*) is a human colonic symbiont that degrades many different complex carbohydrates (glycans), the identities and amounts of which are likely to change frequently and abruptly from meal-to-meal. To understand how this organism reacts to dynamic growth conditions, we challenged it with a series of different glycan mixtures and measured responses involved in glycan catabolism. Our results demonstrate that individual *Bt* cells can simultaneously respond to multiple glycans and that responses to new glycans are extremely rapid. The presence of alternative carbohydrates does not alter response kinetics, but reduces expression of some glycan utilization genes as well as the cell's sensitivity to glycans that are present in lower concentration. Growth in a mixture containing 12 different glycans revealed that *Bt* preferentially uses some before others. This metabolic hierarchy is not changed by prior exposure to lower priority glycans because re-introducing high priority substrates late in culture re-initiates repression of genes involved in degrading those with lower priority. At least some carbohydrate prioritization effects occur at the level of monosaccharide recognition. Our results provide insight into how a bacterial glycan generalist modifies its responses in dynamic glycan environments and provide essential knowledge to interpret related metabolic behaviour *in vivo*.**

Introduction

The human distal gut is periodically inundated with a variety of complex carbohydrates (glycans) from our diet. Essentially all dietary glycans except starch transit the stomach and small intestine undigested by human enzymes. In the colon, where microbial colonization is most dense, these dietary substrates mix with endogenous glycans that are derived from secreted mucus and shed host cells to create an even more diverse glycan landscape in which gut bacteria proliferate. Microbial degradation and fermentation of these glycans produces short-chain fatty acids that can be absorbed across the gut epithelium, enabling either extraction of extra-calories from the food we eat or the recycling of energy from secreted host molecules (McNeil, 1984).

Diet is a well-established parameter that influences the taxonomic composition of the microbiota, often on the timescale of just hours (Turnbaugh *et al*., 2009; Martinez *et al*., 2010; Faith *et al*., 2011). In some cases, this effect is attributable to selective feeding of a particular nutrient that is optimally metabolized by some species (Sonnenburg *et al*., 2010). However, it is also likely that some bacterial 'generalists', which possess multiple metabolic strategies, do not undergo numerical changes in abundance, but rather shift their metabolism to accommodate changes in available nutrients. One such glycan generalist is *Bacteroides thetaiotaomicron* (*Bt*), a common human symbiont from the Bacteroidetes phylum. The sequenced type strain of this species (ATCC 29148 or VPI-5482) is capable of degrading at least a dozen different types of glycans found in plant or animal tissue by selective expression of 88 different gene clusters termed polysaccharide utilization loci (PULs) (Xu *et al*., 2003; Martens *et al*., 2008; 2011). Because *Bt* dedicates such a large portion of its genome (~ 18% of all genes) to glycan degradation, each PUL is regulated such that it is only expressed highly in the presence of a suitable substrate. Several studies in *Bt* and related gut symbionts have shown that a variety of different glycan sensor-regulators activate PUL transcription in the presence of the specific substrate that their associated PUL products target (D'Elia and Salyers, 1996; Martens *et al*., 2008; Sonnenburg *et al*., 2010). The trigger for PUL activation is most frequently an oligosaccharide fragment between two and eight sugars long that is derived from the targeted glycan (Martens *et al*., 2011).

Bacteroides thetaiotaomicron PULs typically encode between 6 and 38 proteins (Martens *et al*., 2011), most of which are predicted to traffic to the periplasm or outer membrane. The protein products of each PUL compose a

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single 'Sus-like system', named after the prototypic starch-utilization system (Sus) that was discovered in *Bt* and is required for metabolism of a variety of starch-like substrates (Reeves *et al*., 1997). Recent transcriptomic and molecular genetic studies have connected several dozen individual PULs in *Bt* with metabolism of particular glycans *in vitro*, providing a view of the molecular functions that underlie this species' vast glycan-degrading abilities (Martens *et al*., 2008; 2011; Sonnenburg *et al*., 2010). A limitation of these studies is that they were conducted with homogenous mixtures of relatively pure glycans. Generalists like *Bt* possess many different metabolic options and the availability of glycans to choose from is likely to change frequently – perhaps abruptly – as either the host changes its diet from meal-to-meal or bacteria transit along the gut and encounter new environments. Thus, it is logical to ask how *Bt* responds to a variety of different conditions that mimic the dynamic nutrient state in the gut. These questions include: (i) can multiple Sus-like systems be expressed simultaneously by single cells? (ii) do the kinetics of PUL expression change in simple versus complex glycan mixtures? (iii) is the response to a target glycan altered when it is sensed at different concentrations, either alone or in a mixture with other polysaccharides? and (iv) will *Bt* preferentially metabolize some glycans relative to others when all are present together?

In this report, we hypothesized that growth in complex nutrient environments alters the responses made by *Bt* to specific glycans relative to when they are present alone. We conducted a series of *in vitro* experiments aimed at better defining how this metabolically diverse bacterium integrates the different glycan signals that it is likely to encounter as well as which nutrients it prefers. Our results support our hypothesis and indicate that *Bt*'s responses towards individual glycans are indeed dependent on the context of other glycans that are present and that this bacterium is metabolically programmed to prioritize certain carbohydrates over others. The results of these experiments represent an essential step towards understanding how generalists like *Bt* respond in relevant timescales to the types of environmental changes that they undoubtedly encounter in the lower intestine. As such, our data provide an essential foundation for interpreting the complex physiological and adaptive responses of *Bt* and other symbiotic bacteria during growth in the mammalian intestine.

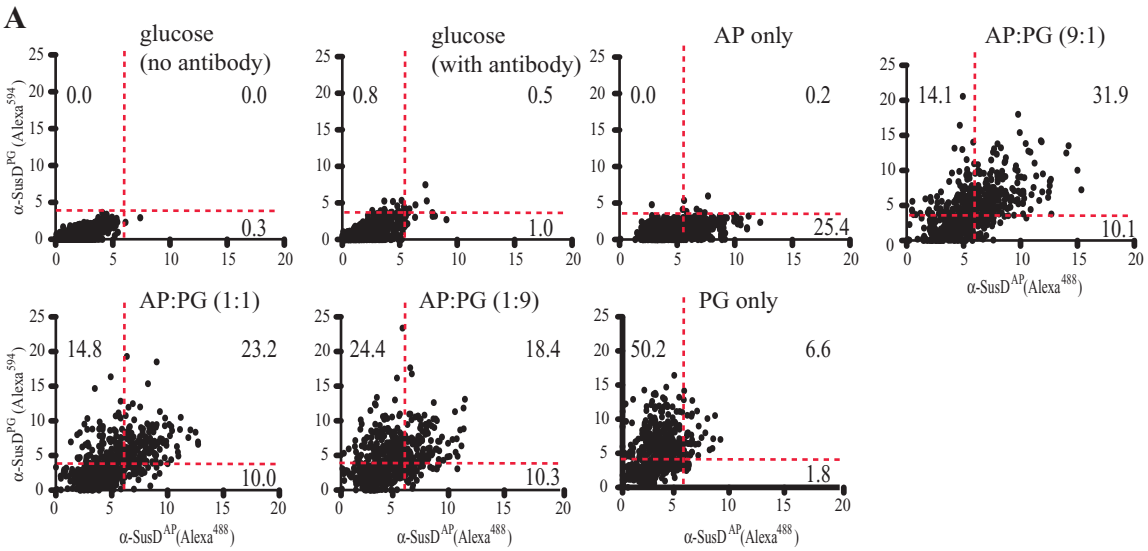
Results

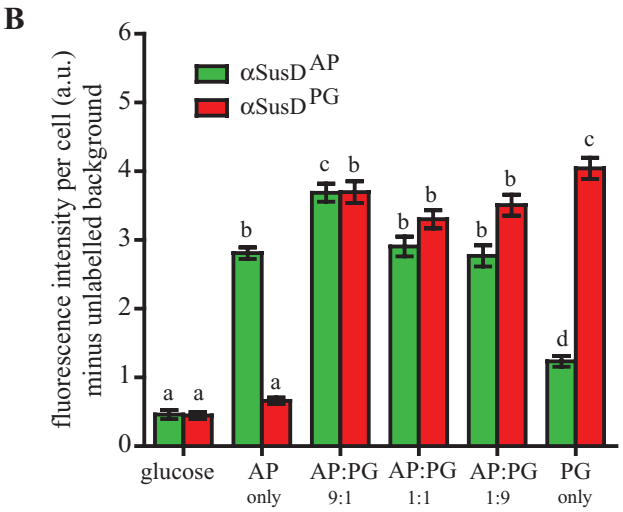
Individual Bt *cells can target multiple glycans*

Given the diverse repertoire of glycan targeting systems in *Bt*, an important question is whether an individual cell that is exposed to multiple glycans commits to using just one carbohydrate or targets multiple substrates simultaneously. To probe expression of multiple Sus-like systems on the surface of intact *Bt* cells, we raised antisera against purified forms of two outer membrane proteins (SusD homologues) involved in degrading starch (amylopectin, AP) and pectic galactan (PG). These two systems were chosen because their expression has been simultaneously detected in *Bt* populations from the caecum of gnotobiotic mice fed a diet rich in plant-derived glycans (Sonnenburg *et al*., 2005) and the molecules that trigger their expression occur together in common food sources such as potato. The prototypic Sus is highly expressed in response to AP; whereas, PG triggers expression of a different Sus-like system that is required for its complete metabolism (Martens *et al*., 2011). In contrast to AP (α1,4-linked glucan with α1,6 branches), PG is a predominantly β1,4-linked galactan that is present in the pectic fraction of plant cell walls and is one of the glycans that fuels the most rapid growth of *Bt*.

Bacteroides thetaiotaomicron was grown on minimal medium (MM) plus glucose and then exposed for 2 h to pre-reduced MM containing different ratios of AP from maize and PG from potato (herein referred to as AP maize and PG potato respectively). In preliminary experiments, we determined that the thick *Bt* polysaccharide capsule reduces the efficiency of antibody labelling (data not shown). Thus, we performed this experiment with a mutant (*Bt* ΔCPS) in which all eight of the capsular polysaccharide synthesis gene clusters had been deleted. Because we have previously reported that expression of *Bt* surface capsules is co-ordinated with certain Sus-like systems (Martens *et al*., 2009), we conducted a control experiment to measure the ability of the *Bt* ΔCPS strain to respond to both AP maize and PG potato, which indicated that this acapsular strain exhibits no defect in its response to PG, but a small ~ 2.5-fold defect in its response to AP maize (Fig. S1). Nevertheless, this strain still activates expression of the starch system > 100-fold, indicating that it is valid for use in this experiment. Cells were fixed and incubated with primary-labelled fluorescent antibodies specific for the respective SusD proteins, $SusD^{AP}$ or $SusD^{PG}$ (superscripts indicate glycan specificity). Controls included (i) *Bt* ΔCPS grown for the same amount of time in MM-glucose, both with and without antibody staining, (ii) *Bt* ΔCPS grown in MM-AP or MM-PG alone, and (iii) deletion mutants of the respective *susD* genes grown on either substrate. As expected, exposure to either AP maize or PG potato alone specifically increased the average fluorescence intensity associated with the respective SusD antibody (Fig. 1A and B). In populations exposed to different amounts of both glycans, there was a marked increase in the intensity of staining for both SusD proteins.

Scatter plot analysis of single cells (Fig. 1A) and fluorescence microscopy images of individually labelled


A
glucose (no antibody)
glucose (with antibody)
AP only
AP:PG (9:1)
AP:PG (1:1)
AP:PG (1:9)
PG only
0.0
0.0
0.3
0.8
0.5
1.0
0.0
0.2
25.4
14.1
31.9
10.1
14.8
23.2
10.0
24.4
18.4
10.3
50.2
6.6
1.8
α-SusD^PG (Alexa^594)
α-SusD^AP (Alexa^488)


B
αSusD^AP
αSusD^PG
fluorescence intensity per cell (a.u.) minus unlabelled background
glucose
AP only
AP:PG 9:1
AP:PG 1:1
AP:PG 1:9
PG only

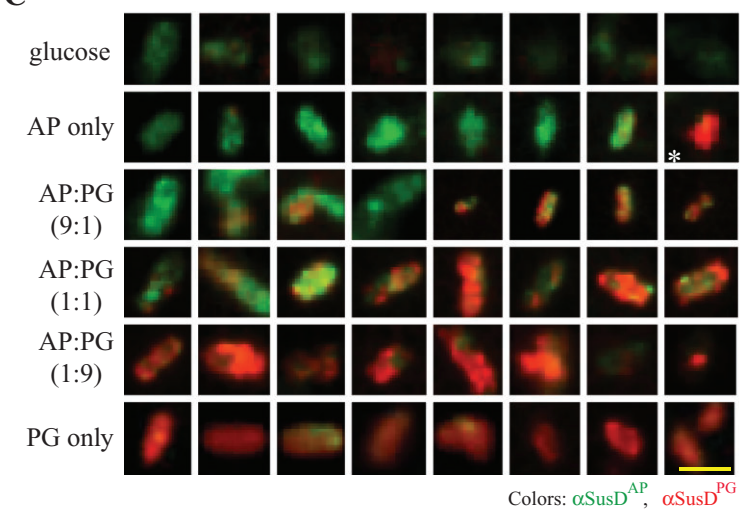

C
glucose
AP only
AP:PG (9:1)
AP:PG (1:1)
AP:PG (1:9)
PG only
Colors: αSusD^AP, αSusD^PG

Fig. 1. A. Scatter plots of fluorescent labelling intensities of cells exposed to various combinations of glucose, AP and/or PG for 2 h. A dashed red line indicates the threshold used to exclude background levels for each antibody, which was set at the high end of the MM-glucose distributions to exclude > 97.5% of the glucose-grown cells and nearly all of the cells in the no antibody control. Numbers in each quadrant represent per cent of the total population ($n = 600$ individual cells; 200 from each of three replicates). Axes are labelled with the fluorescent antibody used and units are in normalized fluorescence intensity values (arbitrary units, a.u.). See Fig. S2 for histogram representations of the same data.
B. The average fluorescence intensities of *Bt* populations grown in the various conditions from (A). Shown are mean ± standard error. Here the average background intensities for unlabelled cells were subtracted from each condition because they were disproportionately higher (2.8-fold) in the AP staining condition. Letters over each bar indicate data groupings in which the *t*-test *P*-values were < 0.001. There was slight but significant activation of the AP system in the PG only condition, suggesting that this substrate contains a small amount of starch contamination. A separate qPCR assay of $susC^{AP}$ expression in the ΔCPS strain under the same PG only growth conditions supports this notion by revealing that the starch system was activated 3.9 ± 1.2-fold ($P < 0.05$).
C. Montage of fluorescently labelled *Bt* cells taken from the treatment conditions. All images were captured at the same exposure time (2 s), processed identically to reduce background noise (*Experimental procedures*) and are at the same scale (bar in lower right = 2 μm). In very rare cases, cells with unexpectedly high labelling intensities were observed in a condition that was not predicted to promote expression of the target protein (asterisk in second row of images). The cause of these events was not investigated further. Additional negative controls in which ΔCPS strains lacking either the $susD^{AP}$ or $susD^{PG}$ genes were exposed to a 1:1 mixture of AP:PG and to the individual glycans were conducted to verify that each SusD-specific antibody was specific for the product against which it was raised. In both cases, no significant fluorescence above unlabelled background was detected at the exposure times noted above (data not shown).

bacteria (Fig. 1C) support the conclusion that single *Bt* cells can express both SusD proteins at the same time. A limitation of using antibodies that are directly coupled to fluorophores (required here to simultaneously label both systems) is that the signal intensity of labelled cells is close to the background level associated with unlabelled bacteria or those grown in non-inducing conditions. Nevertheless, by setting threshold levels that exclude > 97.5% of background events for cells grown on MM-glucose (dashed lines in Fig. 1A), we conservatively measured the number of cells that were positive for both antibodies in each condition (Fig. 1A and Fig. S2). In two of three growth conditions containing both glycans, *Bt* cells that were positive for both antibodies were most abundant. In a third condition containing a 1:9 ratio of AP:PG, cells that were detectably labelled with only the $SusD^{PG}$ antibody were most abundant, but double-positive cells were still 18.4% of the labelled cell population. Taken together, these data indicate that a substantial portion of a population exposed to these two glycans expresses both systems, regardless of alterations in glycan concentration within the range tested. Due to variation in labelling intensity, it is difficult to determine whether other cells in the population were truly expressing just one of the two systems, or if labelling for the second system fell below the threshold that we set. Moreover, the broad variation in staining intensities observed in conditions such as AP:PG 1:1 (Fig. 1C) suggest that individual cells may contain disproportionate amounts of one system versus the other. This phenomenon could influence the ability of individual bacteria to metabolize one glycan over another, but additional experiments are required to test this idea.

Time-dependent PUL responses after glycan exposure

In the competitive gut environment, it is important for bacteria to sense newly available nutrients and efficiently deploy the appropriate metabolic responses. We next determined how rapidly *Bt* responds to new glycans by examining the expression kinetics of two different PULs at the transcriptional and expressed surface protein levels after glycan exposure. *Bt* cells were grown to mid-exponential phase in MM-glucose and then rapidly shifted to pre-reduced MM-glucose that also contained either AP maize or PG potato (both 5.0 mg ml^{-1} final). Glucose was maintained in the growth medium because we planned to limit glycan concentration in subsequent experiments and therefore required the presence of an alternative carbohydrate to ensure that cells were actively growing. Experiments in which transcription of the *susC* genes involved in starch ($susC^{AP}$) and pectic galactan ($susC^{PG}$) metabolism were quantified during growth in the presence and absence of equal or excess glucose, suggested that neither of these glycan responses were altered by the presence of this monosaccharide; although, as discussed below, responses to some other glycans tested were variably repressed by the presence of glucose (Fig. 2).

Samples of *Bt* cells were harvested at 1 min intervals for 5 min after glycan exposure and then at 5 min intervals thereafter. Transcript levels for $susC^{AP}$ and the $susC^{PG}$ were monitored over a 30 min time-course and compared with a MM-glucose reference. Within the first 5 min after exposure to either glycan, transcript levels increased rapidly and then began to plateau (Fig. 3A and B). To determine the kinetics of PUL protein secretion to the cell surface, we measured expression of $SusD^{AP}$ and $SusD^{PG}$ (encoded by genes immediately downstream of the respective *susC*-like genes). Surface protein expression was measured at similar intervals as the *susC* transcripts in fixed intact cells using immunostaining followed by flow cytometry. Previous work has shown that these labelling conditions are specific for surface-exposed lipoproteins but not the same proteins when they are aberrantly targeted to the periplasm (Cameron *et al.*, 2012). In response

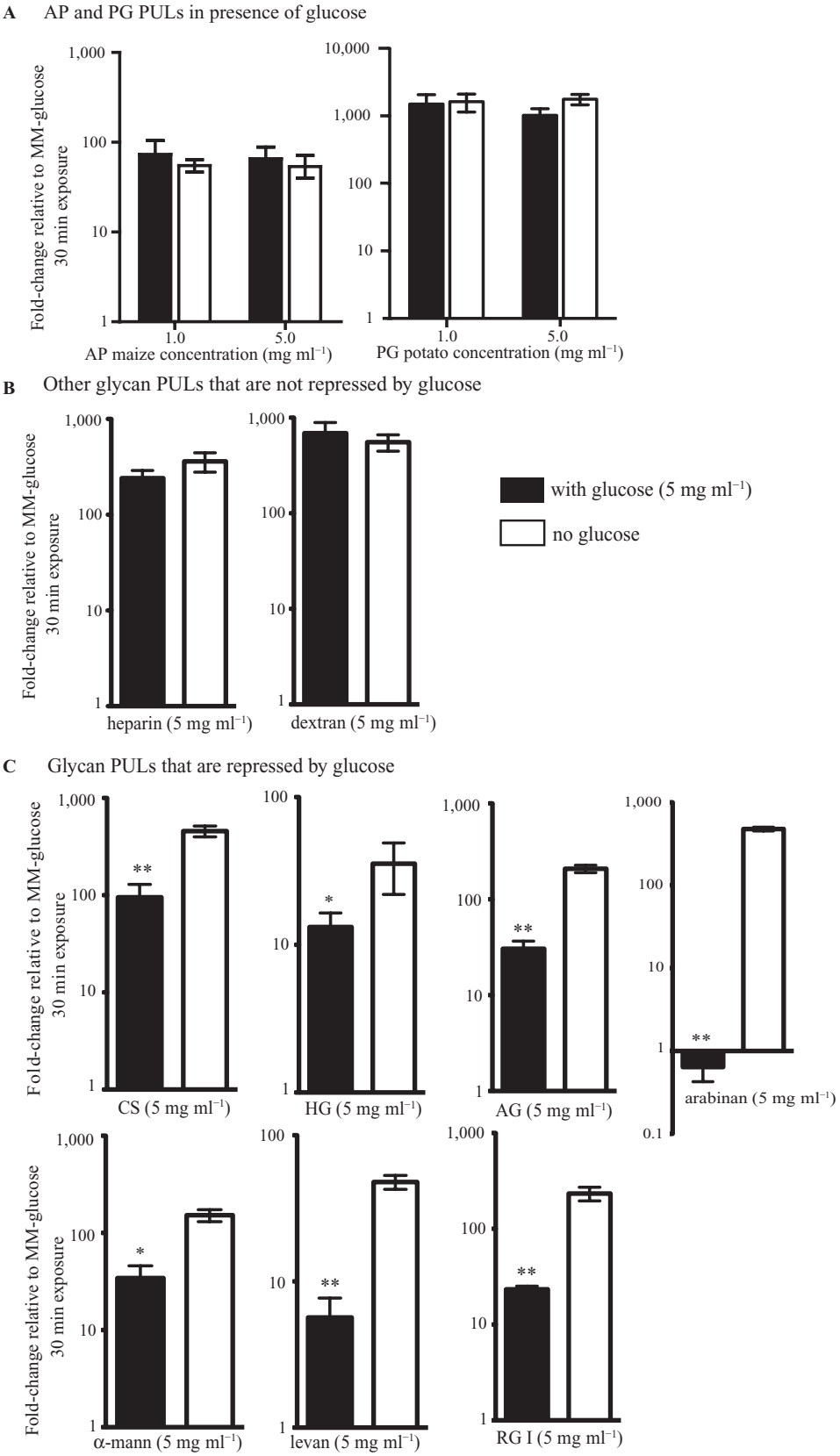


Fig. 2. A. Activation of genes required for AP and PG metabolism is not repressed by glucose. *Bt* was grown to mid-exponential phase in MM-glucose (5 mg ml^{-1}), washed in 2× MM, and resuspended in either MM-AP/PG (1 or 5 mg ml^{-1}) or MM-glucose (5 mg ml^{-1}) + AP/PG (1 or 5 mg ml^{-1}). Transcripts were measured by qPCR 30 min after exposure for *susC*^{AP} and *susC*^{PG} and are shown relative to a MM-glucose reference.
B and C. Identical experiments as described for (A), with the remaining glycans contained in PSM, except that here only a high concentration of each glycan (5 mg ml^{-1}) was tested. Graphs are separated based on whether they did not show glucose repression (B) or did show glucose repression (C). In all panels, values represent the mean ± standard deviation of three replicates (*$P \leq 0.05$; **$P \leq 0.001$, *t*-test).

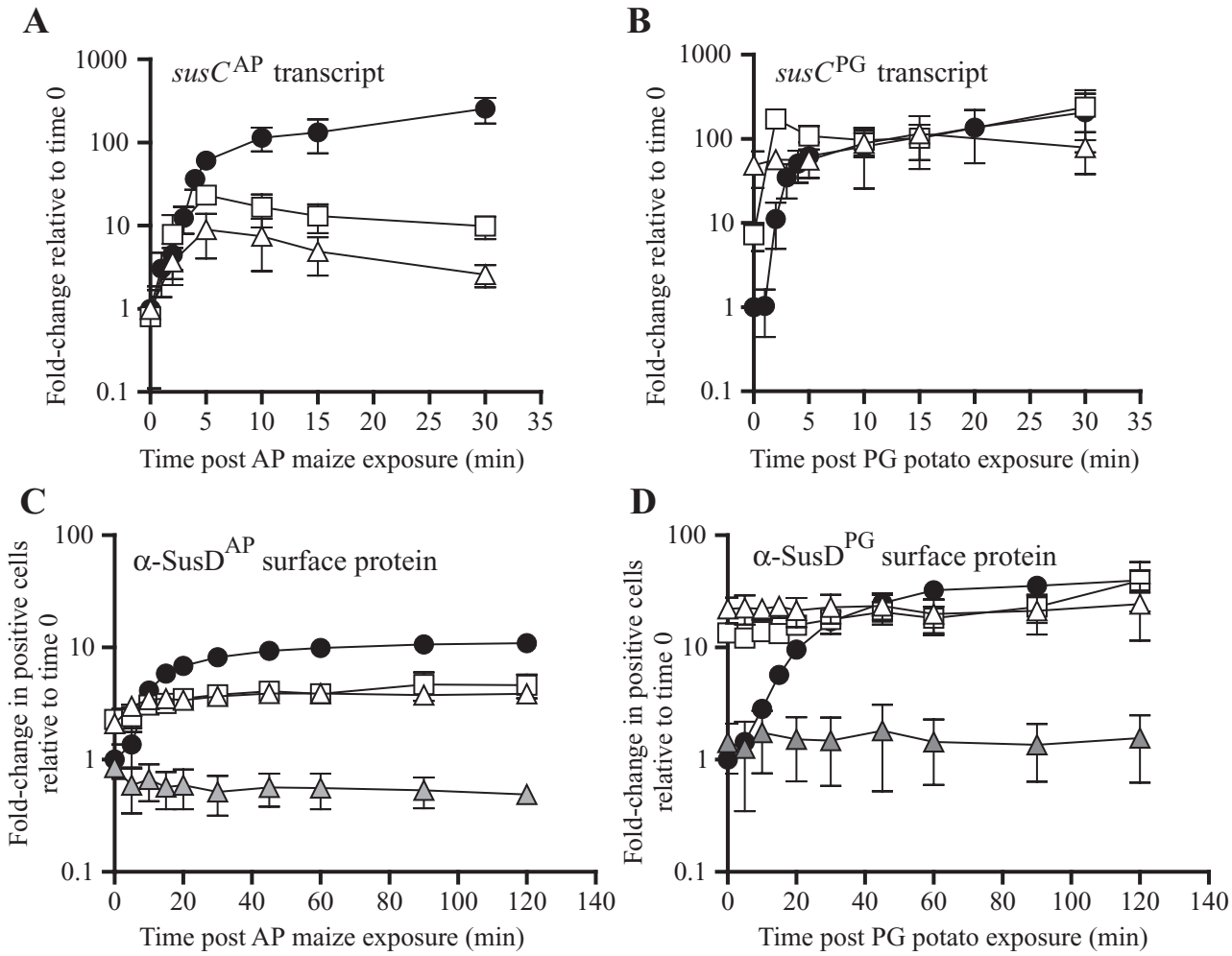


Fig. 3. Response kinetics of PUL transcripts and surface proteins when an inducing glycan is present either alone or in the context of continuous exposure to a mixture of other glycans. All induction values represent fold change relative to a MM-glucose reference taken either at t = 0 or prior to pre-growth inoculation of MM-glucose+PSM.
A. *susC*^{AP} transcript expression in response to AP.
B. *susC*^{PG} transcript expression in response to PG.
C. SusD^{AP} outer membrane protein expression in response to AP.
D. SusD^{PG} outer membrane protein expression in response to PG.
In both (C) and (D) flow-cytometry was used to measure protein expression intensity. Symbols: response to AP or PG alone (●) or in the presence of 0.5 (□) or 5 mg ml^{-1} (△) PSM. As a negative control for non-specific antibody-labelling, *Bt* Δ*susD*^{AP} (C) or Δ*susD*^{PG} (D) were grown in the presence of 5 mg ml^{-1} PSM plus 5 mg ml^{-1} AP or PG respectively (▲). The lack of detectable responses indicates that the high basal response after pre-growth on PSM is due to background contamination in the glycan mixture, rather than cross-reactivity with other SusD-like proteins that are induced by PSM. Values represent the mean ± standard deviation of three replicates.

to either glycan exposure, expression of the respective AP and PG SusD proteins increased with slower kinetics than transcript with maximum expression reached around 20 min and 40 min respectively (Fig. 3C and D).

Having established the kinetics of Sus-like system expression in naïve (glucose-grown) bacteria that are exposed to a new glycan, we next wanted to determine if expression is altered when cells are grown in the presence of multiple glycans prior to exposure to AP or PG. To test this, *Bt* was pre-grown for 2.5 h in MM-glucose containing a polysaccharide mix (PSM) with equal amounts of 10 different glycans (arabinan, arabinogalactan, chondroitin sulphate, dextran, heparin, levan, α-mannan, homogalacturonan and rhamnogalacturonan I, plus the additional AP or PG substrate for which expression was *not* being directly tested. Figure S3 contains structural schematics for each of these glycans). PSM pre-exposure was conducted at either of two different final concentrations (5.0 or 0.5 mg ml^{-1}) to determine if observed effects were dose-responsive. PSM-exposed cells were then rapidly shifted to pre-reduced MM-glucose+PSM containing either AP maize or PG potato at 5 mg ml^{-1} (the concentration of

PSM in each exposure was kept identical to that used in the pre-growth conditions). Samples were collected at identical time points as above and the transcript and protein levels were measured (Fig. 3).

Similar to the results when glucose-grown *Bt* was exposed to AP maize alone, there was a rapid increase in $susC^{AP}$ transcription that followed similar kinetics. Expression still reached maximum levels at 5 min in both PSM exposure conditions. However, maximum $susC^{AP}$ transcript levels were lower when cells were grown in MM-glucose+PSM (Fig. 3A), suggesting that continuous exposure to other glycans reduced the magnitude of the starch response. Consistent with this conclusion, the higher (5 mg ml^{-1}) PSM exposure exhibited lower maximum expression levels, indicating that repression is dose-responsive. Interestingly, *Bt* populations exposed to AP maize in the presence of PSM at either concentration exhibited consistent decreases in transcript abundance between 5 and 30 min post exposure. In contrast, cells grown in the absence of PSM showed a continued upward trend over this same period, revealing that the presence of other glycans exerts a sustained effect that may increase with prolonged exposure. $SusD^{AP}$ protein levels mirrored the effect observed with starch-inducible transcription. In this case, $SusD^{AP}$ expression levels were already elevated at t = 0 min in bacteria pre-grown on MM-glucose+PSM. We conclude that this effect is due to a small amount of contaminating starch in one or more PSM glycans (as noted above for PG, see Fig. 1 legend) and not antibody cross-reactivity as a control experiment using a strain that lacked $susD^{AP}$ showed no fluorescence increase (Fig. 3C). After full exposure to AP maize at 5 mg ml^{-1}, these levels increased only slightly and did not reach the same maximum level observed in the absence of PSM (Fig. 3C). Taken together, these data indicate that alternative glycans in the growth environment reduce both the transcription of genes involved in starch catabolism and the translation/secretion of the encoded products.

In contrast to growth on AP maize, we observed different results when cells were previously conditioned on PSM and then exposed to PSM+PG potato. In this case, maximum levels of both $susC^{PG}$ transcript and $SusD^{PG}$ protein levels were similar to the full amount observed in the absence of PSM, suggesting that the presence of alternative glycans does not exert repressive effects on PG metabolism (Fig. 3B and D). As observed with $SusD^{AP}$ expression in cells pre-grown on PSM, $SusD^{PG}$ was already high at the beginning of the time-course, indicating the possibility of PG contamination in the other PSM glycans, a notion that is supported by expression of the PG system in previous transcriptional profiling experiments on arabinan and homogalacturonan, two pectic glycans that may be covalently attached to PG in some plant cell walls (Martens *et al.*, 2011). Unlike the response to AP maize, this initially high amount of $SusD^{PG}$ expression remained constant and very similar to the maximum level achieved when naïve cells were exposed to PG potato (i.e. it was not repressed by PSM). Taken together, these results suggest that AP maize, but not PG potato, is subject to repression when alternative glycans are sensed by *Bt* and support the hypothesis that PG is among the most preferred glycans because its use was not suppressed by the presence of all others.

Responses of Bt *to varying glycan concentrations*

Because *Bt* had different prioritization responses to the two glycans tested above, we next chose to determine its sensitivity to varying concentrations of AP maize and PG potato and see if these responses are altered by the presence of other glycans. Because of the minor glycan contaminant issues noted above with PSM, we chose to instead use an equal amount of a single alternative glycan to test the effect of its presence on sensitivity to various AP and PG concentrations. In the absence of another glycan, we found that expression of the PULs involved in AP and PG utilization is remarkably sensitive (note that glucose was included in all assays to ensure that there was a consistent backup carbon source). AP maize induced maximum $susC^{AP}$ expression (370-fold) at only 8.0 μg ml^{-1} and expression decreased slightly with higher concentrations (Fig. 4A). The response to PG potato was even more sensitive, with a measurable response below 10^{-4} mg ml^{-1} (Fig. 4B). The dose-responsiveness of this PUL was much broader compared with AP maize and near full expression (250-fold) was not achieved until 40 μg ml^{-1}. Overall, the dose-responsiveness of the PG PUL was nearly linear over three orders of magnitude of glycan concentrations. In contrast, the AP maize response was quite narrow.

To determine the concentration-dependent response to AP maize and PG potato in the presence of another glycan, transcript levels were determined in cells exposed to the same conditions used above (MM-glucose with varying amounts of AP or PG); however, this time the other glycan was also included in the medium at the time of exposure at a constant high concentration of 5 mg ml^{-1}. Thus, the AP maize dose-response was measured in the presence of abundant PG potato and vice versa.

In both cases, the effect of an abundant alternative substrate shifted the glycan dose-response to higher concentrations, rendering *Bt* less sensitive to the limiting glycan. The most obvious effect of abundant PG potato on the starch response was reduced transcription at lower AP maize concentrations (Fig. 4A). The repression of glycan sensitivity was more striking for the PG PUL, where the amount of PG potato required to stimulate similar fold changes with and without AP maize present

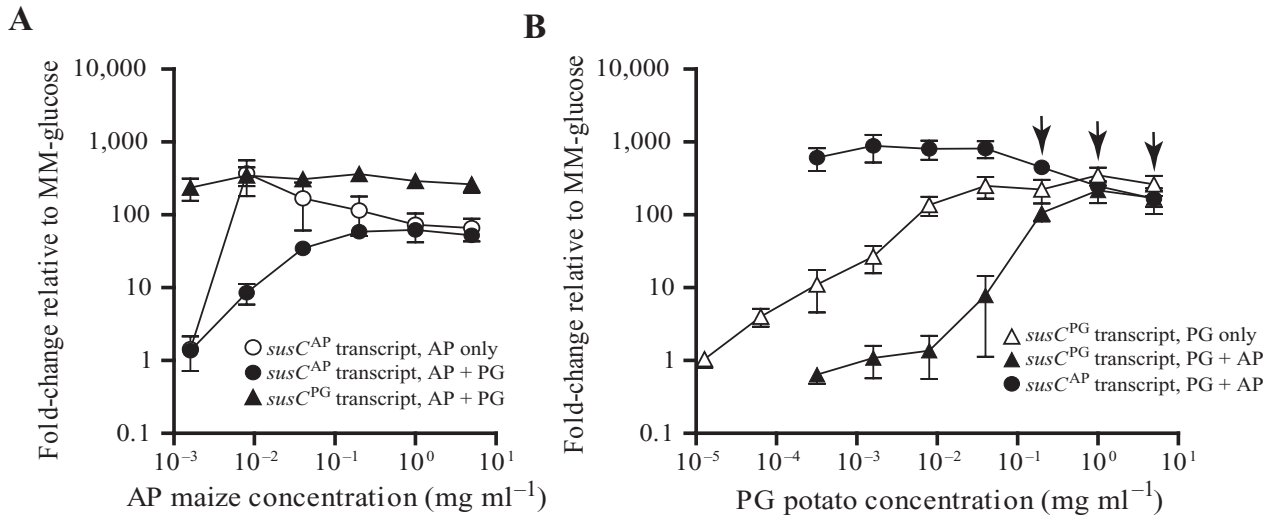


Fig. 4. *Bt* PUL expression is dependent on the concentration of the inducing glycan and presence of additional glycans.
A. *susC*^{AP} transcript levels in *Bt* cells exposed for 30 min to various AP maize concentrations either alone (○) or with 5 mg ml^{-1} PG (●), relative to MM-glucose. Transcript levels for *susC*^{PG} were also monitored when PG was present at constant 5 mg ml^{-1} (▲).
B. *susC*^{PG} transcript levels in *Bt* cells exposed for 30 min to various PG potato concentrations either alone (△) or with 5 mg ml^{-1} AP (▲). Transcript levels for *susC*^{AP} in the AP PUL were also monitored when AP was present at 5 mg ml^{-1} (●).
Values represent the mean ± standard deviation of three replicates. Filled arrows indicate points at which *susC*^{AP} expression is reduced by increasing PG concentration.

varied by up to three orders of magnitude until the PG concentration exceeded 10^{-1} mg ml^{-1} (Fig. 4B).

In both experiments, we monitored expression of the *susC*-like transcript corresponding to the glycan that was provided at constant high concentration. These transcripts were highly expressed at all times. However, *susC*^{PG} and *susC*^{AP} exhibited different behaviour in response to the variable glycan: *susC*^{PG} remained at nearly constant 200- to 300-fold induction in all AP maize concentrations (Fig. 4A); in contrast, *susC*^{AP} exhibited decreasing expression at the three highest PG potato concentrations (filled arrows in Fig. 4B). This observation adds to those made above in Fig. 3 and further suggests that PG itself causes some repression of starch-utilization genes and this effect is concentration dependent.

Prioritization of glycan utilization in a complex mixture

The data so far indicate that *Bt* populations alter their responses as the glycan cues become more complex or an alternative glycan becomes more abundant. To extend these findings, we examined the expression of PULs that are known to target the other glycans present in PSM (Martens *et al*., 2011). Because the presence of PSM exerted a repressive effect on the response of *Bt* cells to AP but not PG, we hypothesized that some of these other PULs would exhibit reduced expression even though their cognate glycans were present in the growth medium. Because 30 min post AP maize exposure exhibited the largest difference in *susC*^{AP} expression in the presence of PSM (Fig. 3A), we selected this time point to measure the expression of *susC*-like genes that are responsive to other glycans in cells exposed to starch both with and without PSM and glucose (Fig. 5).

Besides induction of *susC*^{AP}, AP maize alone induced modest expression of just two other PULs that are involved in degrading dextran (primarily α1,6-linked glucan) and levan (β2,6-linked fructan). The response to dextran might be explained by the presence of α1,6 linkages in AP maize. The *susC*-like gene for levan is induced by the monosaccharide fructose (Sonnenburg *et al*., 2010), which may be a minor contaminant in the AP maize preparation used. In response to PSM with glucose present, seven out of 10 additional PULs showed activation, while three did not (Fig. 5). The fold changes for most of the activated loci were high (between 50 and 100). However, two PULs that target α-mannan and levan exhibited modest fold changes that were below 10 and were considerably lower than previous reports from cells grown in those glycans alone (Sonnenburg *et al*., 2010; Martens *et al*., 2011), suggesting that their full expression is inhibited by the presence of other glycans in PSM. Most strikingly, three of the 10 PULs probed (arabinan, arabinogalactan and rhamnogalacturonan I) remained inactive under these conditions, despite being highly expressed in response to their cognate glycans in previous studies (Martens *et al*., 2011).

Because glucose was maintained in the three experimental conditions tested above and contributes to catabolite repression in other bacteria, we performed an

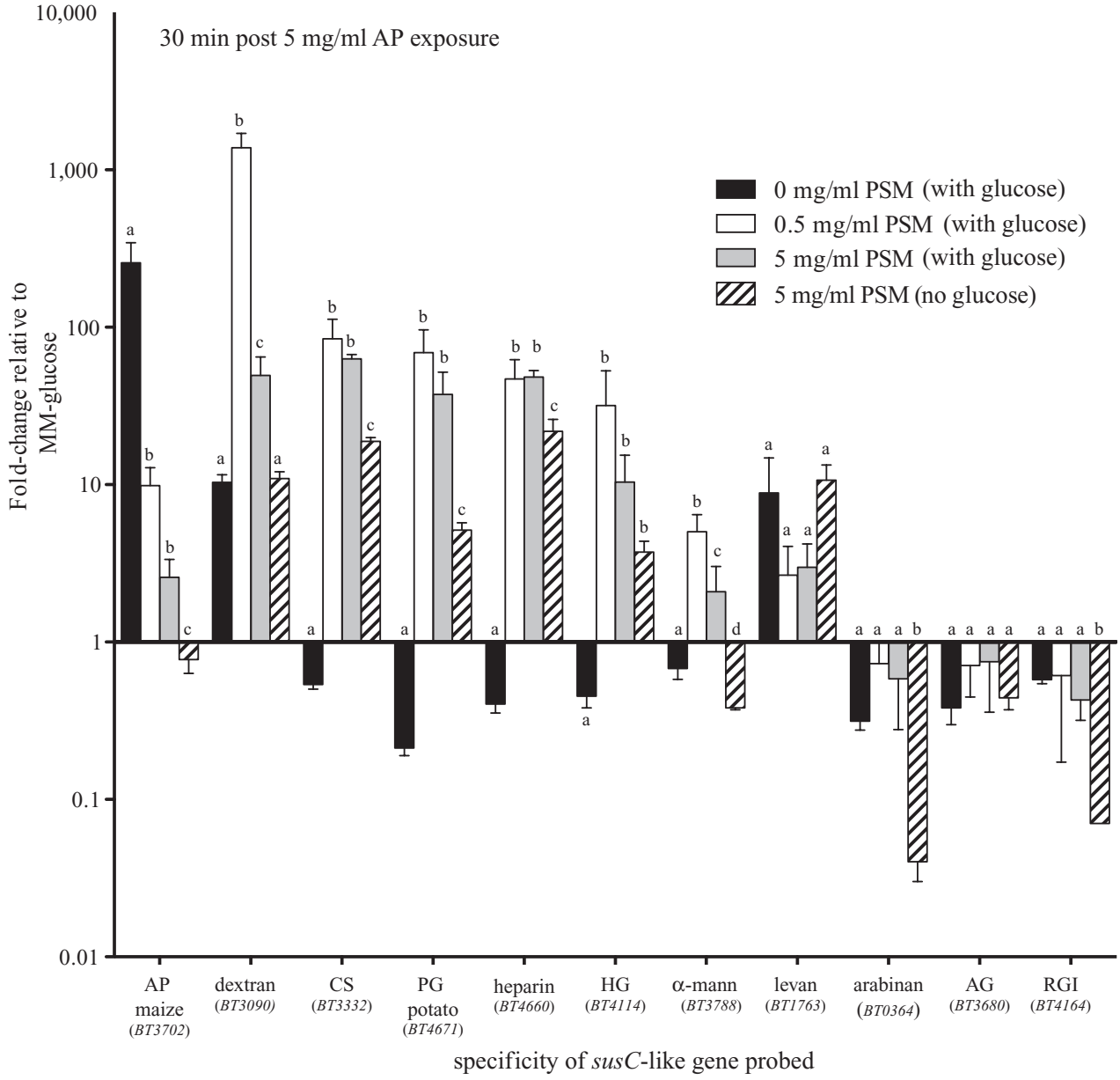


Fig. 5. Expression of *Bt* PULs in response to media containing AP maize with glucose, either alone or in combination with two different concentrations of PSM glycans. An additional comparison shows responses to AP maize and the higher concentration of PSM, but without glucose. Transcript levels for sentinel *susC*-like genes contained in PULs that target AP, PG and nine other glycans (locus tag numbers indicated) were monitored in *Bt* cells that were exposed to 5 mg ml^{-1} AP for 30 min following previous incubation in either MM-glucose or MM-glucose containing 0.5 or 5 mg ml^{-1} of a polysaccharide mix (PSM) lacking AP. The polysaccharide mix contained equal concentrations of dextran, chondroitin sulphate (CS), PG, heparin, homogalacturonan (HG), α-mannan (α-Mann), levan, arabinan, arabinogalactan (AG) and rhamnogalacturonan I (RGI). Values represent the mean ± standard deviation of three replicates. Letters above histogram bars are used to label measurements within each substrate category that were significantly different from one another ($P < 0.05$, *t*-test).

additional experiment in which *Bt* was pre-grown in PSM (5 mg ml^{-1}) lacking glucose and then exposed for the same amount of time to medium containing fresh PSM plus AP maize (both at 5 mg ml^{-1}). This experiment, which was designed to eliminate the role of glucose as a direct mediator of repression, revealed a similar overall trend in PUL expression, with 5 PULs exhibiting repressed levels compared with a glucose-grown reference. There were two surprising features of these data: the first is that, in the absence of glucose, the AP system was not detectably activated despite AP being abundant; the second was that expression levels were reduced overall, suggesting that removing glucose may have reduced the propensity of the cells to activate the sentinel PULs probed in this experi-

ment or altered the timing of the response. Taken together, the PUL expression profile in the absence of glucose supports the conclusion that some other carbohydrates (glycans and monosaccharides), and not just glucose, influence metabolic prioritization effects in *Bt*.

To further probe the effects of glucose on the prioritization of other glycans beyond just AP and PG, we performed additional pair-wise comparisons of the effect of glucose on each individual glycan that is contained in PSM (Fig. 2B and C). Our results revealed that the presence of glucose in equal amount as the single other glycan (5 mg ml^{-1} each) has the ability to repress some, but not all glycans. Interestingly, the same glycans that were most repressed in the presence of PSM with/without glucose were also strongly repressed in the presence of just glucose: arabinan, arabinogalactan and rhamnogalacturonan I. Like starch, the response to a different α-glucan, dextran, was also not suppressed by glucose, suggesting that this monosaccharide does not repress use of polymers in which it is contained. The response to heparin also showed no repression. Utilization of other glycans showed intermediate responses: levan, chondroitin sulphate, homogalacturonan and α-mannan all exhibited some degree of repression by glucose. Taken together, these results suggest that related carbohydrate signals – at least glucose and perhaps the polymers in which it occurs – have the capacity to repress utilization of some lower priority substrates, but not others with higher priority.

The PUL expression data for a single time point 30 min post exposure support our hypothesis that *Bt* prioritizes some glycans in a mixture while repressing use of others. These data provide a 'snapshot' of *Bt* responses after exposure to a diverse glycan mixture, but they do not address the question of how the bacteria proceed to then metabolize this mixture over time. To determine this, we performed time-course experiments on *Bt* grown in a combination of 12 different glycans that it is capable of degrading: the PSM mixture used above, plus AP maize, PG potato and purified mucin *O*-linked glycans (PSM-12). The latter substrate represents the endogenous glycans that are abundant in host mucus and *Bt* has previously been shown to repress utilization of this family of host-derived glycans when alternative carbohydrates are present (Martens *et al.*, 2008; Lynch and Sonnenburg, 2012). To ensure that *Bt* would process all of the glycans present, we titrated the glycan concentration to ensure that it was growth limiting in the conditions used (Fig. S4). We sampled cultures growing in the PSM-12 mixture every 30 min for a total duration of 12 h and measured temporal expression of PULs involved in degrading each glycan (solid lines in Fig. 6).

Over the time-course, we observed three general PUL expression trends. The first trend is typified by the top five profiles in the leftmost column of Fig. 6 and is characterized by rapid, intense expression of the involved PUL, followed by a decline in expression that is suggestive of substrate depletion. The second trend is exemplified by the remaining PULs except mucin *O*-linked glycans and is characterized by an initial burst of PUL expression – sometimes of diminished magnitude compared with the single glycan exposures in Fig. 2 – followed first by a decrease in expression and then by an increase at later times. The magnitude of this transient decrease was variable as portrayed by the remaining profiles beginning with AP maize in Fig. 6. Utilization of mucin *O*-linked glycans represented a third trend, in which an early response could not be detected but instead the sentinel PUL was immediately repressed. In cases where repression was most severe (bottom four profiles in right column of Fig. 6), expression levels decreased in the middle of the profile to either reach or go below onefold relative to the glycan-free reference (i.e. lack of activating substrate). In each of these cases, as well as those with less severe repression at intermediate times, PUL expression later increased, suggesting that these PULs resume expression late in the mixed growth, perhaps when more preferred substrates are gone. While the exact timing and level of expression for these PULs were not identical between three separate replicates of this experiment, similar patterns of higher and lower priority PUL expression are observed (Figs S5 and S6).

Because our results suggest that *Bt* preferentially metabolizes some of the glycans in our mixture, we were curious to see if the PUL expression pattern would be altered if fresh PSM-12 were introduced to bacteria that were in the process of metabolizing glycans of lower priority. This question is of particular biological importance because it tests the hypothesis that *Bt* changes its glycan preference if it has been pre-conditioned on lower priority nutrients, which could subsequently be given higher priority because the cell already possess the metabolic machinery for their metabolism. To test this, we removed a portion of the PSM-12 culture described above at 6 h into the time-course, washed it with pre-reduced MM lacking glycans, and re-introduced the bacteria (all under fully anaerobic conditions) into fresh MM-PSM-12. The response of each PUL in this refreshed culture (dashed lines in Fig. 6) was measured for the remaining 6 h and compared with expression in the parallel non-refreshed culture. Consistent with the conclusion that *Bt*'s glycan preferences are 'hard-wired' and do not change upon pre-conditioning, the top four profiles in the leftmost column of Fig. 6 showed either rapid increases in expression (heparin and chondroitin sulphate) or extensions of a trend that was waning prior to introduction of fresh PSM-12 (homogalacturonan and PG potato). In contrast, each of the remaining profiles exhibited the opposite response. In these cases, introduction of fresh PSM-12

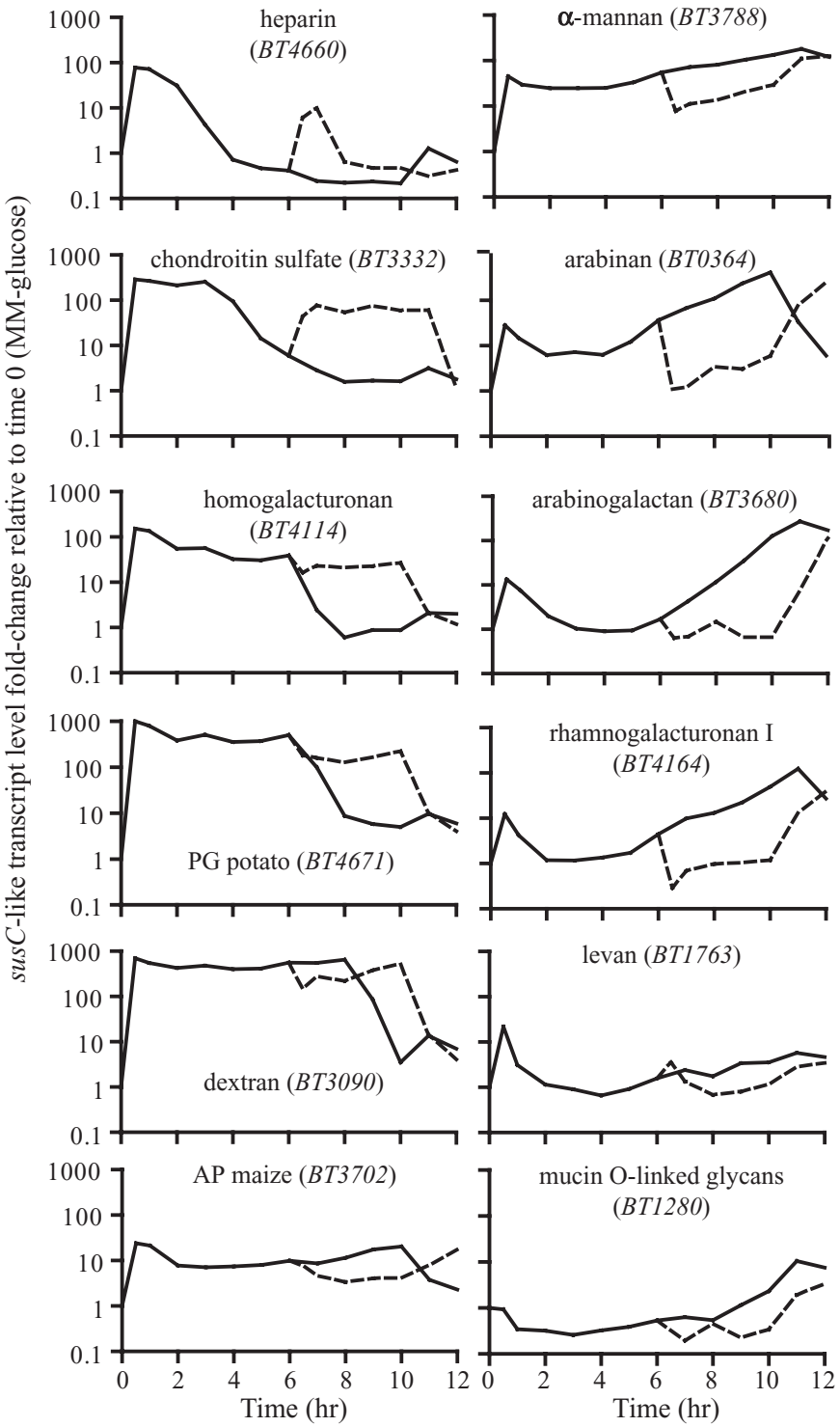


Fig. 6. Temporal expression of *Bt* PULs during unperturbed growth in a mixture of 12 polysaccharides (solid lines). The PSM-12 mixture contained equal amounts (0.1 mg ml^{-1} each) of the glycans listed in Fig. 5, plus *O*-linked glycans harvested from porcine gastric mucin, at a final combined concentration of 1.2 mg ml^{-1} total polysaccharides. At 6 h post PSM-12 exposure, the culture was split, and one half was anaerobically washed in 2× MM then resuspended in fresh MM+PSM-12 (dashed line). Samples were removed at 30 min time points for RNA preparation. Transcript levels were determined using qPCR assays against sentinel *susC*-like genes representing PULs that are induced by each of the 12 polysaccharides (locus tag numbers for each gene are indicated). All transcript level changes are relative to time 0, prior to PSM-12 exposure. Two additional replicates of this time-course are shown in Figs S5 and S6, two additional PSM spike-in experiments are shown in Figs S7 and S8. The 12 plots are organized in approximate order from high to low priority based on depletion of the target glycan with respect to time and the response to fresh PSM exposure. Only one of several PULs implicated in *O*-linked glycan metabolism is shown here (*BT1280*), but similar responses for three additional PULs implicated in degradation of these glycans is shown in Fig. S9.

caused some amount of repression of the current trend, suggesting that new exposure to a prioritized glycan represses active metabolism of other lower priority substrates. Notably, the PULs that showed the greatest repression upon introduction of fresh PSM-12 (arabinan, arabinogalactan and rhamnogalacturonan I) were the same three that showed repressed responses at the 30 min time point in response to glucose or PSM (Figs 2 and 5). Similar effects were observed in replicate experiments (Figs S7 and S8); however, given the differences in

timing in each experiment, and the fact that some trends were waning in the middle of the time-course, the directionality of the shift depended on whether a particular response was high or low at the 6 h time point when fresh medium was added. Taken together, these data support the idea that *Bt* prioritizes utilization of some glycans regardless of its prior glycan exposure. When these high-priority substrates become available, the cell represses other utilization pathways – even if they are active – until the preferred substrates are depleted.

Discussion

Bacteria that successfully colonize the human gut must be capable of adapting to rapidly changing nutrient environments because the intestine is directly influenced by changes in host diet. Some bacteria like *Bt* have evolved as glycan generalists and are capable of altering their metabolic approach as different nutrients become available. A consequence of this metabolic breadth is that these species may need to make decisions when multiple glycans are present.

Using antibodies to components of two different Sus-like systems, we show that single *Bt* cells are capable of expressing systems to target at least two different glycans (AP and PG). Despite being able to express both systems concurrently, the results of several experiments suggest that one of these substrates (PG) is preferred. First, when PG or AP were presented to bacteria that were pre-conditioned on PSM, expression of the starch utilization system was repressed relative to the levels observed when AP was present alone. In contrast, the presence of PSM did not alter the maximum level of gene expression triggered by PG relative to when it was the only substrate. Additional data supporting a preference for PG comes from the time-course experiment in PSM-12 in which PG was one of a few glycans for which PUL expression was strongly activated early and declined before 6 h, presumably when PG was depleted to a level that it was no longer sensed. Finally, PSM-12 refreshment had opposite effects on the PG and AP PULs; the former was re-activated by the introduction of new substrate, while the latter was repressed.

Bacteroides thetaiotaomicron was able to respond to both AP maize and PG potato extremely quickly, reaching full mRNA expression within ~ 5 min of exposure. Although other bacterial transcriptional responses proceed with similar kinetics, it is worth noting the metabolic steps that must occur in order to relay these glycan signals to the corresponding transcriptional machinery. For example, the average AP maize molecule contains up to ~ 10^6 glucose units, which upon being introduced need to permeate the cell's external polysaccharide capsule, be bound by outer membrane-binding proteins, cleaved into oligosaccharides, transported into the periplasm and further digested into the maltose signal that stimulates *sus* gene expression. All of these steps are precedent to transcription being activated, underscoring how rapidly these events must occur to observe the rates shown in Fig. 3. It is possible that *Bt* has evolved to optimize the rate of some of these steps, which would increase its fitness in the competitive gut ecosystem, for example by deploying proteins with multiple starch-binding domains (SusE, SusF and SusG) on its surface (Cameron *et al*., 2012). Another example is the use of inner membrane-spanning sensor regulators like SusR, which bypass additional membrane transport steps (note that PG degradation is also controlled by a membrane-spanning hybrid two-component system).

Inverse to the rapid expression of PUL transcripts involved in glycan utilization, *Bt* is also capable of quickly decreasing these responses. Evidence for this comes from PUL transcript dynamics measured over time in PSM-12 when fresh glycan mixture was added to cultures at 6 h (Fig. 6). In cases such as growth on arabinan, PUL transcription decreased 36.8-fold within 30 min after PSM-12 exposure even though the bacterial population was doubling at a rate far slower than this interval (Fig. S4). One can envision *Bt* cells experiencing an influx of new nutrients from the upper intestine – an event that would trigger expression of PULs that target prioritized or more abundant glycans in the new meal while reducing transcripts for PULs that are no longer optimal. The mechanism and specificity of this transcript turnover remain to be explored.

Equally impressive as *Bt*'s ability to respond temporally to changing nutrient conditions is its ability to sense these nutrients at low concentrations. Of the two glycans tested (AP and PG), this species was more sensitive to PG. Remarkably, *Bt* achieved ~ 10-fold induction of the PG PUL after exposure to only 320 ng ml^{-1} PG potato (Fig. 4B). To put this amount into perspective, fresh wet potato has been reported to contain ~ 0.2% galactan (Oxenboll Sorensen *et al*., 2000), suggesting that *Bt* could sense the PG contained in the digestive residue from as little as 160 mg of potato per ml of intestinal contents. Full expression of the PG system would require the residue from several orders of magnitude more material (~ 20 g); although, much of this original mass is water that would be removed by absorption in the colon.

Another important finding is that the sensitivity of *Bt* towards a particular glycan can be modified by the presence of other substrates. In both cases documented here, the sensitivity towards an individual glycan decreases with additional nutrient complexity. Several mechanisms could account for this behaviour. Perhaps the simplest is for *Bt* to reduce the number of 'surveillance' Sus-like systems (i.e. those targeting glycans other than one(s) being actively metabolized) on its surface. We have previously shown

that *Bt* expresses low amounts of some PUL proteins in the absence of substrate, possibly to start the process of glycan degradation upon encounter and generate the oligosaccharide cues required to activate strong expression (Martens *et al*., 2008; Martens *et al*., 2009). If activation of a single PUL during growth on a particular glycan reduced basal expression of other PULs, or inhibited secretion of their products to the cell surface, this could reduce sensitivity to a new glycan because there would be fewer systems to initiate catalysis. Since the *Bt* cell surface has finite area in which various Sus-like systems can be deployed to interact with their substrates, it is also possible that binding of one abundant glycan to the cell surface reduces access of other glycans to their cognate Sus-like systems or that multiple Sus-like systems compete for common nodes in their metabolic functions, such as the TonB/ExbBD proteins that are predicted to energize transport in these systems. Finally, it is possible that active regulation akin to carbon catabolite repression is at work to prioritize *Bt* glycan metabolism. Obvious candidates to mediate this event, such as phosphoenol pyruvate-dependent phosphotransferase systems, are lacking in *Bacteroides* genomes (Brigham and Malamy, 2005), as are detectable levels of 3′-5′-cyclic adenosine monophosphate (cAMP) in *Bt* (Cotta *et al*., 1994). However, the data presented in Fig. 6, in which introduction of fresh high-priority glycans induced repression of active PULs that target lower priority substrates, suggest that *Bt* is programmed to respond this way regardless of its prior exposure. Additional studies will be required to elucidate the molecular mechanism(s) mediating these phenomena.

In addition to PG, at least three other glycans (heparin, chondroitin sulphate and homogalacturonan) were preferentially metabolized relative to others substrates. Interestingly, these glycans share very little in common: PG is neutral, while the remaining three are negatively charged; two are derived from plants, while two occur in animal connective tissue (present in carnivorous meals); only heparin and chondroitin sulphate share overlapping sugar content. Thus, there is no clear logic regarding why *Bt* prioritizes these glycans. One possibility is that these glycans contain high value sugars; although, this idea is discounted by the different responses to PG and arabinogalactan, which are both predominantly composed of D-galactose. Another possibility is that prioritized glycans fuel the most rapid *Bt* growth, presumably due to a combination of sensitivity and enzymatic ability to degrade these substrates. Evidence against this hypothesis comes from growth rate analysis on each of these glycans in pure form, which shows that at least heparin is among the substrates that fuels the slowest growth; although, PG and chondroitin sulphate support two of the most rapid growth rates (Martens *et al*., 2011) (N. Pudlo and E. Martens, unpubl. data). A final possibility is that these glycans are targeted because they are more soluble, and therefore accessible, in the context of their natural sources. However, it is worth noting that in the *in vitro* experiments performed here, all of the glycans were in purified soluble form excluding the possibility that solubility plays a direct role. Thus, examination of this hypothesis will require *in vivo* experiments using gnotobiotic mice in which natural sources of these glycans are introduced in the context of either *Bt* alone or other microbiota species that could facilitate nutrient digestion. It will also be quite interesting to measure the dynamic responses of other species such as *Bacteroides ovatus*, for which we have recently reported a detailed analysis of glycan utilization connected with PUL expression (Martens *et al*., 2011), to determine if the same or different glycans are prioritized by other species.

In summary, our results underscore the idea that *Bt* is a metabolically complex organism that responds dynamically to changing glycan environments, which are likely to be the norm in its gut habitat. The results of this study provide a platform on which to build additional experiments to probe the molecular mechanisms underlying these glycan prioritization phenomena, as well as investigate the behaviour of *Bt in vivo* in gnotobiotic mice fed similar combinations of glycans as those used here either in purified forms or from natural sources. Such experiments will test if the glycan prioritization patterns observed *in vitro* extend to the gut environment where additional complexity is created by the unidirectional flow along the intestine and the mucus layer that covers the epithelium. By increasing microbial competition in these experiments, it will be possible to determine if the already complex metabolic choices made by a symbiont like *Bt* are altered further when it is forced into competition with additional species, some of which may be better suited to compete for its preferred nutrients. Together, the results of this and future studies will unveil general paradigms about the glycan physiology of the microbiota, reveal its metabolic plasticity in the face of changing diet and elucidate practical knowledge required to intervene in the function of particular bacterial groups using non-invasive approaches like prebiotic therapy or diet modification.

Experimental procedures

Bacterial strains and culture conditions

Bacterial strains are listed in Table S1. *Bt* ATCC 29148 (VPI-5482) and genetic derivatives were grown in tryptone-yeast extract-glucose (TYG) medium (Holdeman *et al*., 1977), brain–heart infusion (BHI, Becton Dickinson) agar supplemented with 10% horse blood (Colorado Serum), or minimal medium (MM) supplemented with appropriate carbohydrates as previously described (Martens *et al*., 2008). Antibiotics were added as needed: gentamicin (200 μg ml^{-1}), erythromycin (25 μg ml^{-1}) and 5-fluoro-2′-deoxyuridine (200 μg ml^{-1}).

Cultures were grown at 37°C in an anaerobic chamber (Coy Manufacturing, Grass Lake, MI; 10% H_2, 5% CO_2 and 85% N_2) or by use of the reducing agent pyrogallol in alkaline solution (Itano and Neill, 1921). *Bt* gene deletion strains were constructed by allelic exchange using the plasmid pExchange-*tdk* as previously described (Koropatkin *et al*., 2008) and primers listed in Table S2.

Purification of SusD^{AP} and SusD^{PG}

SusD^{AP} was purified exactly as previously reported (Koropatkin *et al*., 2008). SusD^{PG} was purified using an identical strategy: nucleotides coding for amino acids 22–524 of the BT4670 ORF were amplified using primers listed in Table S2 and cloned into the expression vector pET28rTEV for purification by Ni-affinity chromatography. Purified forms of both proteins were used to raise polyclonal antisera in adult rabbits (Cocalico Biologicals, Reamstown, PA).

Antibody labelling, immunostaining, fluorescence microscopy and flow cytometry

To prepare for immunostaining, cells were grown as desired then fixed with formalin (4.5% formaldehyde in phosphate-buffered saline, PBS) for 1.5 h followed by three 10 min washings with PBS. Cells were blocked overnight at 4°C in blocking buffer (PBS, 0.02% NaN_3, 2% goat serum). Rabbit α-SusD^{AP} and rabbit α-SusD^{PG} antibodies were incubated with fixed *Bt* Δ*susD* and *Bt* Δ*BT4670*, respectively, to reduce non-specific binding. For time-dependent expression, *Bt* cells were incubated 2 h at room temperature in blocking buffer plus rabbit α-SusD^{AP} or rabbit α-SusD^{PG} antisera (1:500), washed three times with PBS, then incubated in blocking buffer with Alexa Fluor 488 goat α-rabbit IgG (Molecular Probes, 1:500) and washed another three times with PBS. Fluorescence intensity was determined by flow cytometry using a FACSCanto (BD Biosciences) and analysed by FacsDIVA v6 software (BD Biosciences). An initial gate was set to eliminate particles in sterile PBS prior to counting 30 000 events for each sample. A subsequent gate was set such that cells incubated with Alexa Fluor 488 goat α-rabbit IgG were considered positive for expressing SusD^{AP} or SusD^{PG} when their fluorescence intensity exceeded that of at least 99% of the control population (composed of cells that were incubated with the primary antibodies but not the secondary, fluorescent antibody). Expression was calculated as the percentage of positive counts per total counts in the initial gate. Flow cytometry was repeated with samples from three independent experiments, and fold change was determined relative to cells that were incubated in MM-glucose immediately prior to polysaccharide addition.

Bacteroides thetaiotaomicron cells lacking all eight annotated (Xu *et al*., 2003) capsule polysaccharide synthesis (CPS) gene clusters (*Bt* ΔCPS) were used for dual immunostaining. *Bt* ΔCPS Δ*susD*^{AP} and *Bt* ΔCPS Δ*BT4670* cells were used to reduce non-specific antibody binding as described above. Rabbit α-SusD^{AP} and rabbit α-SusD^{PG} antibodies were then directly labelled with Alexa Fluor 488 and 594, respectively, using Invitrogen APEX antibody labelling kits. Immunostaining was performed with the primary antibodies as described above, and DAPI (2.5 μM) was added immediately prior to microscopy. Fluorescence microscopy was performed at the Center for Live Cell Imaging (CLCI) at the University of Michigan Medical School using an Olympus BX60 upright microscope. Fluorescence was observed using 100× UPlanApo (oil-immersion, NA = 1.35) objective and Olympus filter sets U-MWU2 (exBP330-386, emBA420, beam DM400) for DAPI, U-MWB2 (exBP460-490, emBA520IF, beam DM500) for Alexa Fluor 488, and 31004 (exD560/40x, D630/60m, beam 595DCLP) for Alexa Fluor 594 fluorescence. Images were collected using an Olympus DP70 CCD colour camera (RGB, 12-bits/channel). Fluorescence intensity analysis was performed with Metamorph Premier software (Molecular Devices). Individual cell fluorescence was assessed following background correction, in which the average background was subtracted from each set of images: DAPI, Alexa Fluor 488 and Alexa Fluor 594. Fluorescence of the two Alexa Fluor dyes was measured for only those cells that could be detected by DAPI staining. Fluorescence microscopy was repeated with samples from three independent experiments. The average autofluorescence associated with unlabelled cells was determined for each experiment and subtracted from the average fluorescence of each sample in Fig. 1A.

Glycan exposure and growth in polysaccharide mixture (PSM)

To prepare cells for exposure to glycans, *Bt* was grown to mid-exponential phase (A_{600} 0.6–0.8) in MM-glucose then washed 2× in MM prior to addition of the appropriate glycan(s), which were delivered as an equal volume of pre-reduced 1× minimal medium containing twice the final desired glycan concentration. For dose-dependent responses, *Bt* cells were grown for 30 min in MM-glucose plus 1.28×10^{-5}, 6.4×10^{-5}, 3.2×10^{-4}, 1.6×10^{-3}, 8×10^{-3}, 4×10^{-2}, 0.2, 1 and 5 mg ml^{-1} of the appropriate glycan. To monitor PUL expression over time in response to a single glycan, cells were grown in MM-glucose plus 5 mg ml^{-1} of the appropriate substrate. PUL expression over time was also determined during continuous exposure to a polysaccharide mixture (PSM) consisting of 0.05 or 0.5 mg ml^{-1} each of sugar beet arabinan, larch arabinogalactan, chondroitin sulphate from bovine trachea, dextran from *Leuconostoc mesenteroides*, heparin from porcine mucosa, levan from *Erwinia herbicola*, α-mannan from *Saccharomyces cerevisiae*, homogalacturonan from citrus peel, rhamnogalacturonan I from potato, and either amylopectin from maize or pectic galactan from potato, for a final total concentration of 0.5 or 5 mg ml^{-1} PSM. All substrates were from Sigma or Megazyme and identical to products listed previously (Martens *et al*., 2011). To monitor PUL expression over time in a mixture of 12 polysaccharides (PSM-12), mid-exponential-phase *Bt* was inoculated into MM plus 1.2 mg ml^{-1} this polysaccharide mixture [0.1 mg ml^{-1} of each of the 11 polysaccharides mentioned above plus mucin *O*-linked glycans purified from porcine gastric mucosa (Martens *et al*., 2008)].

Quantitative PCR (qPCR) of PUL genes

Total RNA was stabilized with RNAprotect and isolated with RNAeasy mini kit (Qiagen) according to the manufacturer's

instructions. DNA digestion was performed with TURBO™ DNase I (Ambion) followed by phenol (pH 4.3) and chloroform extractions and ethanol precipitation. Reverse transcription was performed with 1 μg of RNA using SuperScript III reverse transcriptase (Invitrogen) according to manufacturer's instructions. cDNA quantification was performed with a Mastercycler® ep realplex (Eppendorf), using KAPA SYBR® FAST qPCR Master Mix and 400 nM primers, except 62.5 nM primers for 16S rRNA, for 40 cycles of 95°C for 3 s, 55°C for 8 s, 72°C for 20 s, followed by a melting step to determine amplicon purity. All transcript levels were normalized based on 16S rRNA abundance. Primers used are listed in Table S2 and were directed against sentinel *susC*-like genes that we previously established to be the most-highly expressed in response to a given glycan and/or were associated with a growth defect when their associated regulator was genetically disrupted (Martens *et al*., 2008; 2011; Sonnenburg *et al*., 2010).

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Supporting information

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