Structural basis for arabinoxylo-oligosaccharide capture by the probiotic *Bifidobacterium animalis* subsp. *lactis* Bl-04

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Summary

Glycan utilization plays a key role in modulating the composition of the gut microbiota, but molecular insight into oligosaccharide uptake by this microbial community is lacking. Arabinoxyllo-oligosaccharides (AXOS) are abundant in the diet, and are selectively fermented by probiotic bifidobacteria in the colon. Here we show how selectivity for AXOS uptake is established by the probiotic strain *Bifidobacterium animalis* subsp. *lactis* Bl-04. The binding protein BIAXBP, which is associated with an ATP-binding cassette (ABC) transporter that mediates the uptake of AXOS, displays an exceptionally broad specificity for arabinosyl-decorated and undecorated xylo-oligosaccharides, with preference for tri- and tetra-saccharides. Crystal structures of BIAXBP in complex with four different ligands revealed the basis for this versatility. Uniquely, the protein was able to recognize oligosaccharides in two opposite orientations, which facilitates the optimization of interactions with the various ligands. Broad substrate specificity was further enhanced by a spacious binding pocket accommodating decorations at different mainchain positions and conformational flexibility of a lid-like loop. Phylogenetic and genetic analyses show that BIAXBP is highly conserved within *Bifidobacterium*, but is lacking in other gut microbiota members. These data indicate niche adaptation within *Bifidobacterium* and highlight the metabolic syntrophy (cross-feeding) among the gut microbiota.

Introduction

The human gastrointestinal tract (GIT) hosts a highly diverse microbial community referred to as the gut microbiota (Eckburg et al., 2005) that comprises one of the most densely populated ecological niches in nature (Xu et al., 2007). Despite the dynamic nature and diversity of the gut microbiota, only four bacterial phyla dominate this community, with Firmicutes being the most abundant followed by Bacteroidetes, Actinobacteria and Proteobacteria (Arumugam et al., 2011). The *Bifidobacterium* genus is prevalent among gut Actinobacteria. *Bifidobacterium* is physiologically important as it harbours several probiotic (health promoting) strains able to improve bowel function, prevent or alleviate infectious diarrhoea, certain metabolic disorders and inflammatory conditions (e.g. Crohn’s disease) as well as confer host resistance against colonization of pathogens (Picard et al., 2005; Wallace et al., 2011). An important characteristic of probiotic bacteria is their ability to utilize a variety of oligosaccharides that are indigestible by the human host (Koropatkin et al., 2012).

Arabinoxylan, comprising a β-(1,4)-linked xylosyl backbone decorated with arabinosyl sidechains (Biely, 2012), is the second most abundant non-starch polysaccharide in cereal grains, mounting to 25–40% (w/w) of the dietary fibre content (Nyman et al., 1984). The only currently known gut xylanolytic taxa are *Bacteroides* and *Roseburia* strains that produce extracellular endo-xylanases able to hydrolyse the backbone of this polysaccharide. The action of these endo-xylanases, which are classified into glycoside hydrolase family 10 (GH10) according to the sequence and mechanism-based CAZy classification (http://www.cazy.org) produces both undecorated xylo-oligosaccharides (XOS) and arabinosyl decorated arabinoxyllo-oligosaccharides (AXOS) (see Fig. 1 for examples), which become accessible to cross-feeding species of secondary glycan degraders (Flint et al., 2007). Growth on XOS is limited to a few taxa among the gut microbiota, e.g. bifidobacteria and some *Lactobacillus brevis* strains (Crittenden et al., 2002), but several commensals and potential pathogens, e.g. members of the...
Clostridium and Escherichia genera are unable to utilize these oligosaccharides (Moura et al., 2007). Bifidobacteria have been shown to grow more efficiently on XOS and AXOS than on the monosaccharide xylose, (Palframan et al., 2003), indicative of a specific transport system for these glycans. Notably, the counts of Bifidobacterium animalis subsp. lactis Bl-04, although the gene encoding the ATP-binding protein (locus tag number: Balac_1610) is not a part of the locus as it was suggested to energize several glycan-specific ABC systems in this organism based on whole-genome microarray transcriptional analysis (Andersen et al., 2013).

Here, we describe the kinetics and energetics of the binding of arabinoxylan derived oligosaccharides to the SBP encoded in the AXOS locus in B. animalis subsp. lactis Bl-04. In addition, crystal structures of this binding protein in complex with undecorated (XOS) and arabinosyl decorated (AXOS) ligands are determined disclosing the molecular features of AXOS capture. These data bring novel insight into the molecular basis of AXOS uptake by probiotic bifidobacteria and advance the understanding of xylan metabolism and syntrophy (cross feeding) between different groups of the gut microbiota.

Results

Production and purification of recombinant xylo-oligosaccharide binding protein from B. animalis subsp. lactis (BlAXBP)

Recently, the ABC transport system that confers the uptake of xylo-oligosaccharides was tentatively identified by whole-genome microarrays transcriptional analysis (Andersen et al., 2013). The balac_0514 gene fragment encoding the mature solute-binding protein of this ABC transport system, designated as BlAXBP, was cloned into an expression vector for production in Escherichia coli. Recombinant BlAXBP was purified at yields of ∼2.5 mg·g⁻¹ cell wet weight.

Specificity and affinity of BlAXBP

Binding of BlAXBP to XOS with a degree of polymerization (DP) 2–6, and AXOS of DP 3–5 (Fig. 1) as well as to eight mono-, di- and oligosaccharides was analysed by surface plasmon resonance. Only XOS and AXOS resulted in significant response at the tested concentrations, which enabled discerning the kinetics of ligand binding (Fig. 2). BlAXBP displayed highest affinity towards xylotetraose with a $K_d = 45$ nM, followed closely by arabinoxylotriose.

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arabinoxylobiose and xylotriose (Table 1, Fig. S1). The affinity dropped steeply for ligands smaller than a trisaccharide or larger than a pentasaccharide owing mainly to a slower association rate constant (kon) except for xylobiose where a faster dissociation rate constant (koff) also contributed to the lower affinity (Table 1, Fig. S1). Arabinosyl decorations were preferred at the non-reducing xyloxyl moiety (e.g. arabinoxylobiose and arabinoxylotriose), as judged by the higher affinity as compared to the corresponding unsubstituted ligands. By contrast, internal decorations (e.g. arabinoxylotetraose) resulted in reduced affinity compared to the undecorated ligand (Fig. 1, Table 1, Fig. S1).

The maximum binding affinity of BlAXBP for xylotetraose was at pH 6.5–7, but changed only modestly at pH 5–8 (Fig. S2A) consistent with pH in the gut ranging between 5.5 and 7.5 (Kleerebezem and Vaughan, 2009). The affinity of BlAXBP to xylotetraose decreased with increasing temperature in the interval 15–30°C mainly due to higher koff (Fig. S2B).

The thermodynamic parameters and binding stoichiometry for xylotriose and xylotetraose were determined using isothermal titration calorimetry (Fig. 3) and revealed a binding process driven by a favourable enthalphy change ΔH° = −165 kJ·mol⁻¹ which was compensated by a large unfavourable binding entropy TΔS° = −126 kJ·mol⁻¹ (Table 2). The affinity trend and the magnitude of the

**Table 1.** Kinetics of xylo-oligosaccharide binding to BlAXBP as measured by surface plasmon resonance.

<table>
<thead>
<tr>
<th>Ligand a</th>
<th>kon M⁻¹ s⁻¹</th>
<th>koff s⁻¹</th>
<th>Kd M⁻¹</th>
<th>χ²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylobiose</td>
<td>(5.7 ± 0.20) × 10⁴</td>
<td>(5.5 ± 0.08) × 10⁻¹</td>
<td>9.00 × 10⁻⁶</td>
<td>0.11</td>
</tr>
<tr>
<td>Xylotriose</td>
<td>(1.3 ± 0.02) × 10⁸</td>
<td>(1.3 ± 0.01) × 10⁻¹</td>
<td>1.02 × 10⁻⁷</td>
<td>0.10</td>
</tr>
<tr>
<td>Xylotetraose</td>
<td>(1.0 ± 0.001) × 10³</td>
<td>(4.6 ± 0.04) × 10⁻²</td>
<td>4.53 × 10⁻⁸</td>
<td>0.09</td>
</tr>
<tr>
<td>Xylopentaose</td>
<td>(9.0 ± 0.08) × 10³</td>
<td>(6.2 ± 0.03) × 10⁻²</td>
<td>6.57 × 10⁻⁷</td>
<td>0.17</td>
</tr>
<tr>
<td>Xylohexaose</td>
<td>(1.8 ± 0.01) × 10³</td>
<td>(4.4 ± 0.02) × 10⁻²</td>
<td>2.50 × 10⁻⁵</td>
<td>0.19</td>
</tr>
<tr>
<td>Arabinoxylobiose</td>
<td>(6.5 ± 0.13) × 10⁵</td>
<td>(5.2 ± 0.10) × 10⁻²</td>
<td>8.10 × 10⁻⁸</td>
<td>0.17</td>
</tr>
<tr>
<td>Arabinosylxylotriose</td>
<td>(1.1 ± 0.01) × 10⁶</td>
<td>(7.8 ± 0.04) × 10⁻²</td>
<td>7.08 × 10⁻⁸</td>
<td>0.14</td>
</tr>
<tr>
<td>Arabinosylxylotetraose</td>
<td>(9.0 ± 0.20) × 10⁵</td>
<td>(1.4 ± 0.03) × 10⁻¹</td>
<td>1.55 × 10⁻⁶</td>
<td>0.13</td>
</tr>
</tbody>
</table>

a. Chemical purity, composition and structure are described in Experimental procedures.
binding constants were in good agreement with SPR and fluorescence spectroscopy measurements (Tables 1 and 2, Fig. S3).

Overall three-dimensional structure

Crystal structures of BIAXBP in complex with several ligands were solved using the single-anomalous diffraction (SAD) method to a maximum resolution of 2.0 Å (Table 3). BIAXBP is classified into cluster B according to the structural alignment classification system and it shares the same overall fold as other solute binding proteins (Berntsson et al., 2010). Accordingly, BIAXBP comprises two domains of different size joined by a hinge region with the ligand binding site buried at the interface between the two domains (Fig. 4). The N-terminal domain (domain I) (1–143; 318–362) contains seven α-helices and five β-strands, two of which form a part of the hinge region and continue into the larger C-terminal domain (domain II) (147–314; 365–425). Domain II, which is stabilized by a disulphide bridge between Cys209 and Cys227, consists of 11 α-helices and four β-strands. The hinge region comprises two short β-strands arranged in an anti-parallel β-sheet spanning the two domains (144–146; 315–317) and the short loop 363–365 (Fig. 4).

A DALI structure comparison search (Holm and Rosenström, 2010) against the Protein Data Bank (PDB), showed the closest structural homologue to be the recently published solute binding protein from the thermophile Caldanaerobius polysaccharolyticus (CpXBP1) which is specific to short xylo-oligosaccharides (Han et al., 2012) (PDB code 4g68; Z-score = 46.9; RMSD = 2.0 Å for 379 aligned Cα atoms and 23% sequence identity). Other high

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### Table 2. Thermodynamic parameters and binding constants obtained by ITC.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>ΔG° (kJ mol⁻¹)</th>
<th>ΔH° (kJ mol⁻¹)</th>
<th>TΔS° (kJ mol⁻¹ K⁻¹)</th>
<th>Kd (M⁻¹)</th>
<th>nd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylotriose⁹</td>
<td>−39.1</td>
<td>−165.5</td>
<td>−126.4</td>
<td>1.4 × 10⁻⁷</td>
<td>0.95</td>
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<tr>
<td>Xylotetraose⁹</td>
<td>−39.5</td>
<td>−163.2</td>
<td>−123.7</td>
<td>1.3 × 10⁻⁷</td>
<td>0.83</td>
</tr>
<tr>
<td>Xylotriose⁹</td>
<td>−39.9</td>
<td>ND</td>
<td>ND</td>
<td>1.0 × 10⁻⁷</td>
<td>0.75</td>
</tr>
<tr>
<td>Xylotetraose⁹</td>
<td>−42.0</td>
<td>ND</td>
<td>ND</td>
<td>4.5 × 10⁻⁸</td>
<td>0.70</td>
</tr>
<tr>
<td>Xylotriose⁹</td>
<td>−41.4</td>
<td>ND</td>
<td>ND</td>
<td>7.5 × 10⁻⁸</td>
<td>1.13</td>
</tr>
<tr>
<td>Xylotetraose⁹</td>
<td>−40.7</td>
<td>ND</td>
<td>ND</td>
<td>5.7 × 10⁻⁸</td>
<td>1.09</td>
</tr>
</tbody>
</table>

a. Determined by ITC.
b. Determined by SPR.
c. Determined by fluorescence emission spectroscopy.
d. Binding stoichiometry as measured by the different techniques. Binding data from SPR and fluorescence spectroscopy measurements are shown for comparison.

### Table 3. Data collection and refinement statistics of the complex structures of BIAXBP.

<table>
<thead>
<tr>
<th>Xylotetraose</th>
<th>Xylotriose</th>
<th>Arabinoxylobiose</th>
<th>Arabinoxylootetraose</th>
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<tbody>
<tr>
<td>Beamline</td>
<td>SLS X06SA</td>
<td>MAX-lab I911-3</td>
<td>MAX-lab I911-3</td>
</tr>
<tr>
<td>PDB ID</td>
<td>3zkk</td>
<td>4c1u</td>
<td>4c1t</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>0.979</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>45.8–2.2 (2.3–2.2)</td>
<td>28.2–2.0 (2.07–2.0)</td>
<td>32.2–2.39 (2.48–2.39)</td>
</tr>
<tr>
<td>Space group</td>
<td>P 2, 2, 2</td>
<td>P 2, 2, 2</td>
<td>P 2, 2, 2</td>
</tr>
<tr>
<td>Unit cell</td>
<td>68.0 124.0 61.3</td>
<td>67.5 96.7 56.3</td>
<td>65.4 96.1 56.7</td>
</tr>
<tr>
<td>Unique reflections (Å)</td>
<td>27 024 (2590)</td>
<td>20 785 (1861)</td>
<td>25 178 (2484)</td>
</tr>
<tr>
<td>Multiplicity⁴</td>
<td>6.6 (6.1)</td>
<td>7.3 (7.5)</td>
<td>4.1 (3.9)</td>
</tr>
<tr>
<td>Completeness (%)⁴</td>
<td>99.5 (97.5)</td>
<td>98.8 (90.1)</td>
<td>98.6 (99.7)</td>
</tr>
<tr>
<td>R-meas (%)⁴</td>
<td>5.6 (152.0)</td>
<td>5.9 (150.9)</td>
<td>7.7 (50.1)</td>
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<td>Mean I/σ(I)²</td>
<td>17.7 (2.12)</td>
<td>20.9 (2.24)</td>
<td>15.6 (3.46)</td>
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<tr>
<td>Wilson B-factor</td>
<td>55.6</td>
<td>62.3</td>
<td>23.7</td>
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<tr>
<td>R-factor⁶</td>
<td>0.20 (0.43)</td>
<td>0.19 (0.37)</td>
<td>0.15 (0.20)</td>
</tr>
<tr>
<td>R-free⁶</td>
<td>0.24 (0.44)</td>
<td>0.24 (0.44)</td>
<td>0.21 (0.25)</td>
</tr>
<tr>
<td>Number of atoms</td>
<td>3173</td>
<td>3164</td>
<td>6225</td>
</tr>
<tr>
<td>Macromolecules</td>
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<td>3002</td>
<td>3007</td>
</tr>
<tr>
<td>Ligands</td>
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<td>60</td>
<td>92</td>
</tr>
<tr>
<td>Water</td>
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<td>102</td>
<td>219</td>
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<tr>
<td>Protein residues</td>
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<td>396</td>
<td>396</td>
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<tr>
<td>RMS bonds (Å)</td>
<td>0.008</td>
<td>0.008</td>
<td>0.005</td>
</tr>
<tr>
<td>RMS angles (°)</td>
<td>1.30</td>
<td>1.28</td>
<td>0.76</td>
</tr>
<tr>
<td>Ramachandran favoured (%)</td>
<td>98</td>
<td>98</td>
<td>98</td>
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<tr>
<td>Ramachandran outliers (%)</td>
<td>0</td>
<td>0</td>
<td>0.25</td>
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<tr>
<td>Clash score</td>
<td>4.32</td>
<td>3.98</td>
<td>1.5</td>
</tr>
<tr>
<td>Average B-factor</td>
<td>32.1</td>
<td>30.3</td>
<td>27.5</td>
</tr>
<tr>
<td>Macromolecules</td>
<td>32.3</td>
<td>30.5</td>
<td>27.0</td>
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<tr>
<td>Ligands</td>
<td>25.3</td>
<td>31.0</td>
<td>34.3</td>
</tr>
<tr>
<td>Water</td>
<td>28.5</td>
<td>25.3</td>
<td>32.0</td>
</tr>
<tr>
<td>Average B-factor</td>
<td>43.6</td>
<td>43.7</td>
<td>43.7</td>
</tr>
<tr>
<td>Macromolecules</td>
<td>36.1</td>
<td>36.1</td>
<td>36.1</td>
</tr>
<tr>
<td>Ligands</td>
<td>41.9</td>
<td>41.9</td>
<td>41.9</td>
</tr>
</tbody>
</table>

a. Values in the parenthesis are for the highest resolution shell.
b. Values in the parenthesis are before refinement.
score hits were carbohydrate-specific SBPs sharing low sequence identities (< 20%) and displaying diverse ligand specificities and taxonomic origin as compared to BlAXBP. The only characterized bifidobacterial SBP (lacto-N-biose SBP from *Bifidobacterium longum*, PDB code 2z8d) had a Z-score of 31.9 and sequence identity of 17% attesting the functional divergence of these taxonomically related SBPs.

**Ligand binding site**

The crystal structures of BlAXBP in complex with arabinoxylobiose, arabinoxylotriose, xylotriose and xylotetraose show well-defined densities for bound oligosaccharides in the binding site. The preferred ligand xylotetraose adopts a helical conformation close to the energetically favoured threefold symmetrical conformation observed in xylan (Nieduszynski and Marchessault, 1971) (Fig. 5A). The mainchain xylosyl units of the ligands are numbered starting with 1 for the terminal xylosyl moiety sandwiched between Trp195 and Trp384 of domain II (Fig. 5, see details below). The arabinosyl substitutions at positions 1 and 2 are denoted with 1’ or 2’ respectively (Fig. 5C and D). Unsubstituted XOS ligands were modelled with the non-reducing end at position 1 (Fig. 5A and B), although binding of these ligands in the opposite directionality (i.e. with the reducing end xylosyl at position 1) is equally feasible as the only difference between the oppositely oriented molecules is the position of the xylosyl ring oxygen (Fig. S4).

All the mainchain xylosyl moieties of xylotriose and xylotetraose are recognized by the protein. Xylosyl units 1 and 2 are bound by both polar interactions and aromatic stacking, whereas polar interactions shape the binding to the units at positions 3 and 4. The sugar units at positions 1 and 4 make most direct interactions, consistent with maximal affinity for xylotetraose, and tolerance for side-chain decorations at positions 1 and 2 (Fig. 5). In more detail: xylose unit 1 stacks onto Trp195 and Trp384 and additionally makes polar interactions with Asn72, His199 and Asp386 (Fig. 5A and B). Xylose unit 2 stacks onto Trp277 and makes polar contacts to Asn39, and Gln254, xylose unit 3 makes a hydrogen bond to Ser41 and Gln254 in the complex with xylotriose, while only a hydrogen bond to Gln254 and water mediated contacts to this unit are observed in the xylotetraose complex. Xylose unit 4 is engaged in contacts with Ala42, Gly45, Asp284, Lys283 and Phe350 (Fig. 5A).

Surprisingly, arabinoxylobiose and arabinoxylotriose bind with opposite directionalities. The binding pocket at position number 1 is occupied by the reducing end xylosyl of arabinobiose but by the non-reducing end xylosyl of arabinotriose (Fig. 5C and D). The arabinosyl moiety of arabinobiose at position 2 makes van der Waals’ contacts with Phe350 and is recognized by numerous polar contacts to Asn39, Ser144, Glu146, Thr318, Pro324 and Tyr346 (Fig. 6C). By contrast the arabinosyl moiety at position 1 in arabinoxylotriose is engaged in polar contacts with the backbone carbonyl oxygen of Asn72 and the amide nitrogen of Ala76 (Fig. 5D). The spacious binding pocket is filled with solvent molecules that mediate a network of hydrogen bonds, likely to make an important contribution to ligand binding (Fig. 5). Besides rearrangements in solvent, the main difference between the four complex structures is the conformational change in the loop region Ala40–Glu44 (lid loop) that blocks one end of the binding site in the xylotriose and arabinoxylotriose complexes, but is displaced outward upon binding the other two ligands with two mainchain hydrogen bonds between Ala42 and Gly45 and the xylosyl unit 4 in the xylotetraose complex (Fig. 5).

**Xylo-oligosaccharide uptake by *B. animalis* subsp. *lactis* Bl-04**

To assay for XOS uptake, *B. animalis* subsp. *lactis* Bl-04 was grown in the presence of a XOS mixture and the xylose, xylobiose, xylotriose and xylotetraose concentrations were monitored in the culture supernatant throughout the exponential growth phase. The depletion of xylotriose and xylotetraose occurred in the mid-exponential phase, whereas xylobiose was still detected in the culture supernatant until the late log phase of growth suggesting that xylotriose and xylotetraose were internalized more efficiently than xylobiose (Fig. 6).
Fig. 5. Close-up of the BLAXBP binding site in complex with (A) xylotetraose, (B) xylotriose, (C) arabinoxylobiobiose and (D) arabinoxylotriose shown in two orientations differing by a 90° rotation along the x-axis. Difference electron density maps calculated with coefficient mF_{obs} - DF_{calc} and σA-weighted (contoured at 3σ) are shown as a light blue mesh. The lid loop that adopts different conformations depending on the ligand is depicted as a grey backbone trace of residues 40–44. Yellow dashes indicate polar interactions to protein atoms or water (red spheres). The main chain atoms are omitted for clarity unless they participate in polar interactions.

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Discussion

The preferential stimulation of distinct health-promoting groups from the gut microbiota by prebiotic oligosaccharides that are indigestible by the host is well established (Kolida and Gibson, 2011), and manipulation of the composition of the gut complex microbial community offers an attractive non-evasive strategy in treatment of pathogenic infections as well as metabolic and immune disorders. Molecular understanding of preferential microbial glycan utilization in the gut, however, remains elusive.

Transcriptional analyses have implicated a diversity of carbohydrate transport systems in uptake of oligosaccharides for intracellular degradation in probiotic bacteria (Andersen et al., 2012; 2013) and the occurrence of certain ATP-binding cassette (ABC) carbohydrate transporters together with high acetate production in bifidobacterial strains have been correlated to probiotic traits such as protection from pathogenic infection in mice (Fukuda et al., 2011). The present study reports the biochemical and structural features of the solute binding protein BlAXBP that mediates the capture of arabinoxylan oligomers within Bifidobacterium.

Capture and uptake of XOS and AXOS in bifidobacteria

The high affinity of BlAXBP to both undecorated and arabinosyl decorated xylo-oligosaccharides (Table 1, Fig. S1) is consistent with the growth of bifidobacteria on AXOS (van Laere et al., 1999). The degradation of AXOS occurs intracellularly and proceeds by the removal of arabinosyl and acetyl substituents by specific GH43 arabinofuranosidases (van den Broek et al., 2005) and acetyl esterases (Biely, 2012) respectively. This is followed by degradation of the xylo-oligosaccharide mainchain to xylose mainly by highly conserved GH43 β-xylosidases (>78% amino acid sequence identity between the most distant sequences with Bifidobacterium) that are invariantly encoded in the bifidobacterial AXOS utilization loci (Fig. 7). Thus, the presence of transport systems is a prerequisite for the utilization of these glycans, and bifidobacteria are the only members of the gut microbiota that are currently known to produce an ABC transport system to mediate AXOS uptake. The amino acid sequences of the AXOS-specific SBP share high sequence identity within Bifidobacterium (ligand binding residues virtually identical, Fig. S5), and segregate mainly based on taxonomic distance (Fig. S6), indicating functional conservation.

Intracellular GH43 β-xylosidases and the ABC transporter constitute the core of the AXOS utilization system within Bifidobacterium as highlighted by the consistent colocalization of their genes together with one or more GH43 arabinofuranosidase and esterase genes within the AXOS operon (Fig. 7). Larger divergence is noted in arabinofuranosidase sequences as well as in the composition of hydrolases and esterases encoded in the AXOS operon between the different Bifidobacterium groups (Fig. 7). In summary, a highly conserved SBP confers the capture of XOS and AXOS in Bifidobacterium despite considerable variations in xylanolytic enzymes in different bifidobacterial groups.

Structural features, kinetics and thermodynamics of XOS recognition

In this study we have employed surface plasmon resonance to discern the kinetics of binding, whereas insight into the binding energetics was provided by isothermal titration calorimetry. The use of independent biophysical techniques to measure binding corroborated the binding constants rendering the data more reliable. The differ-
ences in affinity of BIAXBP for various XOS ligands is determined largely by the association rate constant \( k_{\text{on}} \), while changes in the dissociation rate constant are modest except for xylobiose (Table 1, Fig. S1). The magnitude of the dissociation rate constant \( k_{\text{off}} \) reflects the short range interactions between the protein and the ligand, while \( k_{\text{on}} \) is influenced by diffusion, favourable electrostatics at the encounter complex distance (Sheinerman et al., 2000), and molecular rearrangements associated with the binding event. The highest affinity measured towards xylotetraose is in excellent agreement with the structural data revealing a binding site large enough to accommodate this ligand. Thus, fewer interactions are possible with xylobiose compared to xylotriose or xylotetraose, rationalizing its 16-fold faster \( k_{\text{off}} \) compared to xylotetraose (Table 1, Fig. S1). Unsubstituted ligands longer than xylotetraose are likely to protrude out of the binding site without forming additional protein contacts, consistent with the essentially invariant \( k_{\text{on}} \) for xylotetraose through xylohexaose. The highest affinity measured towards xylotetraose is in excellent agreement with the structural data revealing a binding site large enough to accommodate this ligand. Thus, fewer interactions are possible with xylobiose compared to xylotriose or xylotetraose, rationalizing its 16-fold faster \( k_{\text{on}} \) compared to xylotetraose (Table 1, Fig. S1). Unsubstituted ligands longer than xylotetraose are likely to protrude out of the binding site without forming additional protein contacts, consistent with the essentially invariant \( k_{\text{on}} \) for xylotetraose through xylohexaose. The high affinity for non-reducing end arabinosyl substituents in arabinoxylobiose and arabinoxylotriose (Table 1) is consistent with the contribution of the additional interactions between the arabinosyl side-chains and the protein to the binding energy (Fig. 5C and D). Internal arabinosyl decorations as in arabinoxylotetraose are accommodated but are less favoured as judged by the considerably slower association rate constant of arabinoxylotetraose as compared to xylo-tetraose. Similarly, the reduced affinity of xylobiose, xylo-pentoase and xylohexaose as compared to xylotetraose is mainly attributed to a reduction in \( k_{\text{on}} \), which suggests that binding of these ligands is associated with a slower transition from the open to the closed conformation of the SBP (Björkman and Mowbray, 1998). Thus the ligand preference of BIAXBP is fine-tuned mainly by the size of the association rate constant for optimal recognition of decorated and undecorated xylo-oligosaccharides with DP 3–4. Nonetheless, the lowest measured affinities for xylobiose and arabinoxylotetraose are in the micromolar range, suggesting that these ligands are likely to be transported and metabolized if they are available in sufficient concentrations. This is confirmed by the XOS uptake experiment, which showed that the preferred ligands xylotriose and xylotetraose are depleted before xylobiose (Fig. 7).

The thermodynamic signatures of xylo-tetraose capture by BIAXBP were similar and revealed an enthalpically dominated binding, compensated by large entropic penalties in agreement with the structural data showing extensive direct and solvent mediated protein–ligand interactions (Fig. 5). The unfavourable entropy is likely to arise from the ordering of water molecules in the
binding pocket and the loss of conformational freedom of both the ligand and the protein sidechains and domains (Jelesarov and Bossardh, 1999) that possess rotational freedom along the hinge axis. The entropy of ordering the solvent molecules seems to be crucial in the entropic off-set of binding, as other glycan-specific solute binding proteins that lack ordered solvent in the binding pocket display significantly lower entropic compensation, e.g. the blood antigen binding protein from *Streptococcus pneumoniae* (Higgins et al., 2009), which has an entropic penalty of binding energy mounting to ca 10% ΔH as compared to 76% for *BlAXBP*). Conversely, the energetic fingerprint of *BlAXBP* is similar to the lacto-N-biose binding SBP from *B. longum* (Suzuki et al., 2008), which also employs ordered solvent molecules to mediate a hydrogen bonding network to the bound ligand.

*Structural plasticity of *BlAXBP*

Recently the xylo-oligosaccharide-specific binding protein from the thermophilic bacterium *C. polysaccharolyticus* has been structurally and biochemically characterized (Han et al., 2012), and in addition biochemical data are available for the XOS-specific SBP from *Streptomyces thermoviolaceus* (designated as BxIE) (Tsujibo et al., 2004). Binding of arabinosyl decorated ligands was not shown for any of these SBPs, which exhibit a different ligand preference with the highest affinity skewed towards smaller ligands, e.g. xylobiose and xylotriose than observed for *BlAXBP*. Also the kinetics of binding are different, as the magnitude of the dissociation rate constant governed the high affinity of BxIE towards xylobiose, while the association rate constant changes modulate the selection of *BlAXBP* towards xylotetraose. The most striking difference, however, between *BlAXBP* and the counterpart from *C. polysaccharolyticus* is the considerably higher structural plasticity of the bifidobacterial protein manifested in the ability to recognize xylo-oligosaccharides with diverse size and arabinosyl substitutions (Table 1, Fig. 5, Fig. S7). This striking plasticity is the result of different modes of binding of decorated and undecorated ligands, which is facilitated by the similar structure of xylo-oligosaccharides bound in opposite directionality and recognition of arabinosyl substituents at two different positions of ligands bound with different directionality (Fig. 5, Fig. S4). Additionally, *BlAXBP* possesses a more spacious pocket featuring internal cavities, which are filled with solvent molecules. These solvent molecules are rearranged based on bound ligand to mediate a hydrogen bonding network between the ligand and the protein. The spaciousness of the pocket in *BlAXBP* is effectuated by the lack of several bulky residues responsible for the narrow active site in the homologue from *C. polysaccharolyticus* (Fig. S8). A lid loop which is conformationally flexible to accommodate different ligands further contributes to the plasticity of the ligand binding site.

**Biological implications of the ligand preference of *BlAXBP*: xylos catabolism in the gut**

Plant cell wall polysaccharides, including arabinoxylan fibres that are abundant in human diet, provide the gut microbiota with an important metabolic resource. Members of the dominant *Bacteroides* genus and *Roseburia* species that possess modular GH10 endo-xylanases featuring catalytic modules appended to one or more xylan binding modules are reported as the prevalent xylanolytic taxa in the gut (Mirande et al., 2010). Xylan is captured and hydrolysed into large fragments that are subsequently transported via the SusC transport protein in *Bacteroides* (Dodd et al., 2011). The capture of xylan fragments in *Bacteroides* is effectuated by xylan binding domains, which typically display lower affinities on ligands smaller than xylohexaose (McCarty et al., 2006). Only xylan oligomers larger than a xylopentaose were reported to induce the xylanolytic system in *Bacteroides* (Mirande et al., 2010), which asserts the preference of *Bacteroides* for larger xylan fragments and suggests uptake of smaller ligands is less efficient. This specialization provides a possible rationale for the preference of bifidobacteria to exploit complementary substrate sizes, which avoids competition with dominant specialized bifidobacteria. Thus a gradient of increasing concentration of smaller xylo-oligosaccharides is likely to be formed downstream in the gut, providing a niche for bifidobacteria and other taxa with efficient XOS uptake and utilization systems. Only a few Firmicutes, e.g. *L. brevis* are reported to grow on XOS (Moura et al., 2007). The XOS utilization system in these organisms is much simpler as judged from the genetic loci encoding a transcriptional regulator, a GH43 putative β-xylanase and a major facilitator superfamily permease (e.g. *L. brevis* ATCC 367 and *Weissella koreensis* KACC 15510). The fine-tuning of XOS capture towards larger ligands than xylobiose, and towards arabinosyl decorated ligands may provide a competitive advantage for bifidobacteria over Firmicutes that are unable to utilize such ligands. This is consistent with the *in vitro* uptake experiments performed in this study, showing that the disaccharide xylobiose was taken up with lower efficiency than the tri- and tetrasaccharide counterparts.

Niche adaptation is evident upon comparison of *BlAXBP* with the counterparts from *S. thermoviolaceus* isolated from decayed wood and *C. polysaccharolyticus* isolated from hot spring sediment. Both *S. thermoviolaceus* and *C. polysaccharolyticus* are capable of producing extracellular endoxylanases that generate mainly short XOS of DP 2–3 (McCarty and Williams, 1992; Han et al.,...
and their uptake proteins are optimized for this size of substrate. By contrast, *B. animalis* subsp. *lactis* and the majority of AXOS utilizing bifidobacterial strains rely on efficient capture to cross-feed from primary arabinoxylan degraders, e.g. *Roseburia* and *Bacteroides* in the densely populated colon.

The high specialization and complementary substrate preference evolved to avoid competition among dominant commensals are likely to be important niche adaptation mechanisms that establish metabolic syntrophy (cross-feeding) among the gut microbiota.

This study provides novel insight into the molecular features responsible for the impressive versatility of the first arabinoxyllo-oligosaccharide binding protein to be described and advances our understanding of the complex metabolic interplay and competition that play a key role in defining our gut microbiota.

**Experimental procedures**

**Chemicals**

All chemicals were of analytical grade. The following carbohydrates were used: XOS Longlive 95P (84% xylo-oligosaccharides composed of 43% xylobiose, 30% xylotriose, 10% xylotetraose, 17% DP≥xylohexaose, 13.5% other monosaccharides) described in Mäkeläinen et al. (2010). Xylo-oligosaccharides xylohexaose > 95%, xylo-pentaoctaose and xylohexaocta≥ 95% - were from Megazyme (Wicklow, Ireland). Arabinoxyllo-oligosaccharides, with DP 3–5 (95% pure) were a kind gift of Dr Barry McCleary (Mega-zyme): arabinoxyloligosaccharides: [α-L-Araf(1,3)]-β-D-Xylp(1,4)-D-Xylp; arabinoxyloligosaccharides: mixture of [α-L-Araf(1,2)]-β-D-Xylp(1,4)-β-D-Xylp(1,4)-D-Xylp; arabinobiose: 90% + β-D-Xylp(1,4)-β-D-Xylp(1,4) (10%); arabinoxyloligosaccharides: [β-D-Xylp(1,4)]-[α-L-Araf(1,3)]-β-D-Xylp(1,4)-D-Xylp (80%) + [α-L-Araf(1,4)]-[β-D-Xylp(1,4)-β-D-Xylp(1,4)-[β-D-Xylp(1,4)-D-Xylp (20%) (see Fig. 1 for the chemical structures of these ligands). Xylose, sucrose, cellulose, melibiose, isomaltose, raffinose, fructo-oligosaccharides from *Chromosomal DNA of* *B. animalis* subsp. *lactis* (kindly provided by DuPont Nutrition & Health, Madison, WI, USA) was isolated according to Fredslund et al. (2011) except that OD600 for induction was 7. Selenomethionine labelled BAXBP was produced in B834DE3 according to Studts and Fox (1999). Cell harvest, disruption and his-tag affinity purification was carried out according to Fredslund et al. (2011). Fractions containing BAXBP, identified by SDS-PAGE, were concen-trated, loaded onto a gel filtration Híload Superdex 200 16/60 column (GE Healthcare) and eluted with 10 mM MES 150 mM NaCl, pH 6.5 at 1 ml min⁻¹. BAXBP-containing fractions were pooled, concentrated, dialysed against 10 mM MES, pH 6.5, applied onto a 6 ml anion exchange Resource Q column (GE Healthcare) and eluted by a three step NaCl gradient yielding ~ 2.5 mg homogenous BAXBP per 1 g cell pellet. Chromato-graphic steps were performed using an ÄKTA-AVANT chromatograph (GE Healthcare) at 4°C. The His tag was removed using human thrombin (Calbiochem, Merck, Darmstadt, Germany) as recommended by the manufacturer. The protein concentration was determined by measuring A280 using a molar extinction coefficient ε280 = 78921 M⁻¹ cm⁻¹ determined experimentally by aid of amino acid analysis (Barkholt and Jensen, 1989), comparable to the calculated value of ε280 = 66140 M⁻¹ cm⁻¹.

**Surface plasmon resonance (SPR)**

Affinity of BAXBP for the different xylo-oligosaccharides was determined using a Biacore T100 (GE Healthcare). BAXBP, diluted into 10 mM sodium acetate pH 4.1 to 2.3 μM, was immobilized on a CMS sensor chip using a random amine coupling kit (GE Healthcare) to a density of 2900 response units (RU). Sensograms were recorded at 25°C in 20 mM phosphate/citrate buffer; 150 mM NaCl; pH 6.5, 0.005% (v/v) P20 surfactant (GE Healthcare) at a flow of 30 μl min⁻¹ with association and dissociation times of 90 s and 240 s respectively. Experiments were performed in triplicates in the range 3 nM–10 μM for xylotriolose–xylopentaoctaose, 5 nM–1 μM AXOS ligands and 0.5 μM–1 mM for xylobiose and xylotetraose, all dissolved in the same buffer as above. To investigate the ligand specificity of BAXBP, binding was tested towards
5 mM xylose, cellobiose, raffinose, sucrose, melibiose, isomaltose, FOS and GOS in the same buffer. The affinity of BIAXBP for xylotetraose was investigated at eight different pH values in the range pH 3–9, while dependence of binding on temperature was examined in the range 15–30°C. Data analysis was carried out using the Biacore T100 evaluation software, and equilibrium dissociation constants \( K_d \) were calculated by fitting a one site binding model to either the steady-state response data or the full sensograms to measure the binding kinetics. Stoichiometry \( (n) \) of BIAXBP binding was determined by the equation:

\[
 n = \frac{R_{\text{max}} \cdot MW_i}{MW_i \cdot R}
\]

where \( R_{\text{max}} \) (analyte maximum binding capacity) is obtained from the fits to the individual data sets and \( R \) is the attained immobilization level, \( MW_i \) and \( MW_o \) are the molecular masses of the immobilized protein and carbohydrate analyte respectively (Morton and Myszka, 1998). This stoichiometry is underestimated due to the inactivation of a certain proportion of the protein concomitant with the immobilization procedure.

**Isothermal titration calorimetry (ITC)**

Binding of xylotriose and xylotetraose to BIAXBP in 10 mM sodium phosphate pH 6.5 was measured at 25°C using an ITC200 microcalorimeter (MicroCal). BIAXBP (14 or 25 μM) in the sample cell (200 μl) was titrated by a first injection of 0.5 μl followed by 18 x 2 μl injections of carbohydrate ligand (140 or 250 μM) with 120 s between injections. Data were analysed using the MicroCal software (Wiseman et al., 1989).

**Fluorescence titration spectroscopy**

Intrinsic tryptophan emission was measured on a Spex Fluorolog322 fluorescence spectrometer (HORIBA Jobin Yvon, Irvine, CA, USA) in a 1 ml stirred quartz cuvette at 25°C. Purified BIAXBP diluted to 4 μM in 10 mM sodium phosphate pH 6.5 and pre-equilibrated for 5 min was titrated with 0.5 μl of 0.5 mM of xylotriose or xylotetraose. Instrument settings and data analysis were as previously reported (Erkens and Slotboom, 2010). The ligand-specific contribution to fluorescence quenching was corrected for in all measurements.

**Crystalization and structure determination**

Initial crystallization conditions (0.1 M Tris pH 7, 40% PEG-300 and 5% PEG-1000 at 278 K) was found using the Cryo crystalization screen (Emerald BioSystems, MA, USA), using a Mosquito® liquid handling robot (TTP Labtech, UK). BIAXBP and selenomethionine labelled BIAXBP (15 mg·ml⁻¹ in 10 mM MES pH 6.5 and 150 mM NaCl) was co-crystallized with xylo-oligosaccharides (1 mM) using vapour diffusion in either MES pH 6.5 and 150 mM NaCl) was co-crystallized with the sample cell (200 μl) was titrated by a first injection of 0.5 μl followed by 18 x 2 μl injections of carbohydrate ligand (140 or 250 μM) with 120 s between injections. Data were analysed using the MicroCal software (Wiseman et al., 1989).

**In vivo XOS uptake**

*Bifidobacterium animalis* subsp. *lactis* Bl-04 was grown at 37°C in LAB SEM media (Barrangou et al., 2003) supplemented with 1% (w/v) XOS (Longlive 95P) under anaerobic conditions. Growth was monitored by measuring \( A_{600} \). Samples were collected during the exponential growth phase and immediately chilled to 0°C. The supernatants after centrifugation were collected and sterile filtered before HPAEC-PAD analysis.

Xylo-oligosaccharides present in culture supernatants were analysed by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) ( Dionex, California, USA) using a CarboPac PA200 column and a mobile phase (0.35 ml min⁻¹) of constant 100 mM
NaOH and a gradient of sodium acetate 0–20 min of 0–160 mM and 20–25 min of 160–400 mM. Standards of xylose (Merck, Germany), xylobiose, xylotriose and xylotetraose (Megazyme, Wicklow, Ireland) were used to identify the peaks in the chromatograms using 10 μl injections. Samples were diluted in 10× in MQ water and subsequently 10× in 100 mM NaOH before analysis and compared with the standards.

Acknowledgements

This research was funded by a FeSu grant from the Danish Strategic Research Council to the project ‘Gene discovery and molecular interactions in pre/probiotics systems. Focus on carbohydrate prebiotics’, an instrument grant from the Danish Council for Independent Research | Natural Sciences for Biacore T100; a PhD stipend from the Technical University of Denmark (to M.E.); A.V.-Z. and D.J.S. were supported by the European Union (European Research Council starting grant to D.J.S. and EDICT programme) and the Netherlands Organisation for Scientific Research (NWO). Mette Pries and the Strategic Research Council to the project ‘Gene discovery and molecular interactions in pre/probiotics systems. Focus on carbohydrate prebiotics’. We are grateful to Dr Sampo Lahtinen (DuPont Nutrition and Health, Kantvik, Finland) and thanked for amino acid analysis. We are grateful to Dr Barry McCleary (Megazyme) for kind gifts of GOS and AXOS samples respectively.

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**Supporting information**

Additional supporting information may be found in the online version of this article at the publisher's web-site.