Involvement of the Multidomain Regulatory Protein XynR in Positive Control of Xylanase Gene Expression in the Ruminal Anaerobe Prevotella bryantii B14

Kohji Miyazaki,1 Hiroyuki Miyamoto,1 Derry K. Mercer,2* Tatsuaki Hirase,1 Jennifer C. Martin,2 Yoichi Kojima,3 and Harry J. Flint2

Laboratory of Animal Science, Kyoto Prefectural University, Shimogamo, Kyoto 606-8522, Japan,1 and Rowett Research Institute, Bucksburn, Aberdeen, AB21 9SB, United Kingdom2

Received 22 August 2002/Accepted 2 January 2003

The xylanase gene cluster from the rumen anaerobe Prevotella bryantii B14 was found to include a gene (xynR) that encodes a multidomain regulatory protein and is downstream from the xylanase and β-xylanase genes xynA and xynB. Additional genes identified upstream of xynA and xynB include xynD, which encodes an integral membrane protein that has homology with Nαsolute symporters; xynE, which is related to the genes encoding acylhydrolases and arylsterases; and xynF, which has homology with the genes encoding α-glucuronidases. XynR includes, in a single 833-aa polypeptide, a putative input domain unrelated to other database sequences, a likely transmembrane domain, histidine kinase motifs, response regulator sequences, and a C-terminal AraC-type helix-turn-helix DNA binding domain. Two transcripts (3.7 and 5.8 kb) were detected with a xynA probe, and the start site of the 3.7-kb transcript encoding xynABD was mapped to a position upstream of xynD. The DNA binding domain of XynR was purified after amplification and overexpression in Escherichia coli and was found to bind to a 141-bp DNA fragment from the region immediately upstream of xynD. In vitro transcription assays demonstrated that XynR stimulates transcription of the 3.7-kb transcript. We concluded that XynR acts as a positive regulator that activates expression of xynABD in P. bryantii B14. This is the first regulatory protein that demonstrates significant homology with the two-component regulatory protein superfamily and has been shown to be involved in the regulation of polysaccharide gene expression.

Recent studies examining 16S ribosomal DNA sequence diversity have confirmed that the genera Prevotella and Bacteroides are among the most numerous bacteria present in the anaerobic ecosystems of the rumen and hind gut, respectively (16, 24, 25). Certain species, including Prevotella ruminicola and Prevotella bryantii from the rumen and Bacteroides ovatus from the human colon, are capable of utilizing a particularly wide variety of diet-derived polysaccharides as growth substrates (5, 18, 19). Although not known to be capable of breaking down crystalline cellulose, these organisms are thought to play an important role in the utilization and conversion of plant cell wall polysaccharides in the gastrointestinal tract, and they appear to act in concert with cellulolytic species in the rumen system (4). Rather little is known, however, about the organization and regulation of the enzyme systems responsible for utilization of plant cell wall polysaccharides in Bacteroides and Prevotella species.

In the rumen species P. bryantii much of the xylan-degrading activity of cultures has been found to be cell associated, but this activity was not detected in assays performed with whole cells, suggesting a periplasmic, membrane, or intracellular location for at least some of the enzymes involved (13). Similar observations have been made for xylanolytic enzymes in human colonic Bacteroides spp. (20), while detailed analyses of starch-degrading enzyme systems in Bacteroides thetaiotaomicron have demonstrated the involvement of periplasmic hydrolytic enzymes and starch-binding proteins (1, 17).

Several components of the xylan utilization system of P. bryantii B14 have now been identified. The linked genes xynA and xynB code for a family 10 xylanase and an oxygen-sensitive family 43 enzyme that shows β-xylanase and exoxylanase activities, respectively (9, 10). Meanwhile, the unlinked gene xynC encodes a 66-kDa xylanase that has an unusual structure, in which the family 10 catalytic domain is interrupted by additional residues (6). Expression of xylanase activities in P. bryantii is known to be induced during growth on xylans (8–10, 13).

We show here that regulation of the xynABD xylanase gene cluster in P. bryantii B14 involves a multidomain regulator that is related to two-component regulatory proteins encoded by a gene situated immediately downstream of xynB. Two-component regulator systems are involved in coordinating a wide range of bacterial responses to environmental changes, including chemotaxis and osmoregulation (15, 22). This is the first example of a regulatory protein of this type that is involved in regulating genes concerned with polysaccharide utilization, however, and one of the first instances of such regulation to be analyzed in the Bacteroides-Prevotella group of bacteria.

MATERIALS AND METHODS

Organisms, media, and growth conditions. P. bryantii B14 was grown anaerobically in RG medium containing (per liter) 150 ml of sterile rumen fluid, 0.9 g of K2HPO4, 0.9 g of KH2PO4, 1.8 g of (NH4)2SO4, 1.8 g of NaCl, 0.24 g of CaCl2, 0.24 g of MgSO4, 0.36 g of MnCl2, 0.05 g of CaCl2, 0.05 g of MgSO4, 0.05 g of FeCl3, 1.8 g of NaCl, 0.24 g of CaCl2, 0.24 g of MgSO4, 0.36 g of MnCl2, 0.05 g of CaCl2, 0.05 g of MgSO4, 0.05 g of FeCl3, and 2 g of glucose. Cultures were incubated at 39°C in 100-ml serum bottles for 20 h. Water-soluble xylan (WS-X) was prepared...
from oat spelt xylan by adding 10 g of oat spelt xylan to 90 ml of distilled water and shaking the preparation continuously at 39°C for 2 h. After centrifugation (5,000 × g, 30 min, 4°C), the supernatant was removed and transferred to a sterile 100-ml bottle to form a solution (WS-X). Escherichia coli clone 5/4a was grown in Luria-Bertani broth supplemented with 100 μg of ampicillin per ml. Medium components were purchased from Sigma-Aldrich, unless indicated otherwise.

**Total RNA extraction.** WS-X (0.05%, vol/vol) was added to mid-exponential-phase cells of P. bryantii B14 grown in RG medium and incubated for 30 min. After 30 min of incubation the total microbial RNA was extracted by using Sepazol-RNA I Super (Nacalai Tesque Inc., Kyoto, Japan) according to the manufacturer’s instructions. The total RNA was divided into aliquots and stored at −80°C until it was required for analysis.

**PCR.** For all PCRs including those used for sequencing purposes (see below) the following conditions were used. To a 100-μl (total volume) reaction mixture were added 100 ng of template DNA, 50 pmol of forward primer, 50 pmol of reverse primer, 1 μM deoxynucleoside triphosphates, 2.5 U of Taq polymerase (Toyobo Co. Ltd., Osaka, Japan), and 10 μl of 10× Taq buffer (Toyobo Co. Ltd.). The volume of the reaction mixture was adjusted to 100 μl with sterile distilled H2O. PCR amplification was carried out by using a Takara thermal cycler (Takara Bio Inc., Shiga, Japan) and the following cycle conditions: cycle 1, 94°C for 4 min; and cycles 2 to 31, 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s, with a final elongation step of 72°C for 8 min and a ramp rate of 3°C/s. All oligonucleotides were purchased from Nishinbo Industries, Inc., Tokyo, Japan.

**DNA sequencing and analysis.** The dideoxy chain termination procedure for DNA sequencing was carried out with *E. coli* clone 5/4a by using an ABI Prism BigDye terminator cycle sequencing Ready Reaction kit (Applied Biosystems, Warrington, United Kingdom) and an ABI Prism 377 DNA sequencer. The upstream 1,621 bp of the *xynR* gene and upstream sequences were not found in the *P. bryantii* B14 xylan utilization operon. Amino acid identities show the degrees of similarity with the three related genes. *xynR* (*P. bryantii* B14) and the gene indicated by the question mark (*P. bryantii* V975) show no sequence similarity. The *xynR* gene and upstream sequences were not found in *B. ovatus* 280) and sequenced as described above by using a combination of gene-specific primers and the M13-20 and Reverse primers. Sequencing primers were purchased from MWG-Biotech (UK), Ltd.

Computer-assisted DNA analysis was carried out by using online software available from the EMBL European Bioinformatics Institute website (http://www.ebi.ac.uk) or the EMBOSs sequence analysis package available through the Human Genome Mapping Project Resource Centre website (http://www.hgmp.mrc.ac.uk).

**Northern blotting.** Template DNA for probing was prepared by PCR with primers FW-*xynA* and RV-*xynB*, which contained a 1,721-bp fragment of both *xynA* and *xynB* (Fig. 1). The probe was labeled with 2 MBq of [α-32P]dCTP (3,000 Ci/mmol; American Radiolabeled Chemicals Inc., St. Louis, Mo.) by using a PrimeII random labeling kit (Stratagene, La Jolla, Calif.). Twenty micrograms of total RNA from *P. bryantii* B14, yeast RNA (Ambion Inc., Austin, Tex.), or mouse liver RNA (Ambion Inc.) was applied to a Northern blot. Northern blotting was performed as described by Sambrook et al. (20a), except that an ULTRAhyb solution (Ambion Inc.) was used in place of the prehybridization solution. Signals were detected by exposure to X-ray film (MXJB-1 medical X-ray film; Kodak, Rochester, N.Y.). The size of the target mRNA was estimated from the sizes of 16S and 23S rRNA, and the target RNA was detected by ethidium bromide staining before membrane transfer.

**Primer extension analysis.** A 0.2-ml portion of primer labeled at the 5′ end with fluorescein isothiocyanate, Trans-FTIC (Table 1), was mixed with 50 μg of total RNA and 1 μl of RNase inhibitor (Toyobo Co. Ltd.) in 20 μl of ReverseTracer buffer (Toyobo Co. Ltd.). The solution was incubated at 50°C for 1 h, and this was followed by hybridization at room temperature for 1.5 h. For reverse transcription, 1 μl of ReverseTracer Ace buffer (Toyobo Co. Ltd.) was added, and the mixture was incubated at 42°C for 1 h. After ethanol precipitation, the cDNA transcript was dissolved in a solution containing 10 μl of distilled water and 10 μl of stop solution supplied in a Thermo Sequence cycle sequencing kit (Shimadzu Corporation, Kyoto, Japan). The transcription initiation site was determined by examining a known sequence ladder.

**Production of the recombinant regulatory protein.** An 896-bp fragment of *xynR* was amplified by PCR by using the reg-upper primer (BamHI site attached) and the reg-lower primer (KpnI site attached) (Table 1) and then was digested with *BamHI* and *KpnI*. After purification, this fragment was ligated into the expression vector pQE-30 (Qiagen GmbH, Hilden, Germany). Expression and purification of the recombinant protein were performed by using a QIAexpressionist kit (Qiagen GmbH) according to the supplier’s instructions. The purity of the recombinant protein was checked by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis with a 12% polyacrylamide gel.

**Gel shift assay.** A DNA fragment containing the putative XynR binding site region was amplified by PCR with the gs1012 primer (*BamHI* site attached) and the gs901 primer (*BamHI* site attached) (Table 1), and this was followed by digestion with *BamHI* and *BamHI*. This fragment was radiolabeled with 1 MBq of [α-32P]dCTP (see above) by the action of DNA polymerase I for 30 min at

**FIG. 1.** Diagram of the *P. bryantii* B14 xylan utilization operon. The available sequence of the related xylan utilization operon of *B. ovatus* V975 (26, 27) is shown beneath the equivalent genes in the *P. bryantii* B14 xylan utilization operon. Amino acid identities show the degrees of similarity for the three related genes. *xynR* (*P. bryantii* B14) and the gene indicated by the question mark (*B. ovatus* V975) show no sequence similarity. The recombinant protein fragment of XynR was synthesized from the PCR product obtained by using oligonucleotide primers reg-upper and reg-lower (Table 1). The probe used in Northern blotting was generated by PCR by using oligonucleotide primers FW-*xynA* and RV-*xynB* (Table 1). uORF, unidentified open reading frame.
37°C. The mixture contained 5 μl of the DNA polymerase I large fragment (New England Biolabs Inc., Beverly, Mass.), 2.5 mM of a deoxynucleoside triphosphate mixture (dATP, dGTP, and dTTP), and 1 MBq of [γ-32P]dCTP in 20 μl of DNA polymerase I buffer (New England Biolabs Inc., Beverly, Mass.). After the reaction was finished, unincorporated nucleotides were removed with QIAquick nucleotide removal kit (Qiagen GmbH). Competitor DNA was also prepared by PCR and was purified by PCR and was purified by QIAquick gel extraction kit (Qiagen GmbH). Then 0, 20, 40, or 80 ng of the recombinant regulator protein was added to 20 μl of binding buffer (40 mM Tris-HCl, 4 mM MgCl2, 100 mM NaCl, 2 mM sodium phosphate buffer (pH 6.8), it was resuspended in 25 ml of cold M-0 buffer (10 mM Tris-HCl [pH 7.9], 1 mM EDTA, 5 mM dithiothreitol). The suspension was sonicated with a UD-201 ultrasonic disrupter (Tomy Ltd., Saitama, Japan), and then 25 ml of cold M-1 buffer (50 mM Tris-HCl [pH 7.9], 4 mM MgCl2, 0.2 mM EDTA, 1 mM dithiothreitol, 25% sucrose, 50% glycerol) and 6.5 ml of saturated ammonium sulfate solution were added. After the preparation was left on ice for 20 min, ultracentrifugation (100,000 g for 3 h) was conducted. Then 5.9 g of ammonium sulfate and 0.01 ml of 1 M NaOH were added to the supernatant, and the solution was mixed continuously for 30 min. The solution was centrifuged (15,000 × g, 20 min), and the pellet was dissolved in 3 ml of cold M2 buffer [50 mM Tris-HCl (pH 7.9), 6 mM MgCl2, 40 mM (NH4)2SO4, 0.2 mM EDTA, 1 mM

Preparation of cell extracts. All procedures described below after cell growth were performed at 4°C. Cells in an 800-ml culture grown with pulse addition of WS-X (0.05%) until the mid-exponential phase were harvested by centrifugation (15,000 × g, 5 min). After the cell pellet was washed twice with cold 50 mM sodium phosphate buffer (pH 6.8), it was resuspended in 25 ml of cold M-0 buffer (10 mM Tris-HCl [pH 7.9], 1 mM EDTA, 5 mM dithiothreitol). The suspension was sonicated with a UD-201 ultrasonic disrupter (Tomy Ltd., Saitama, Japan), and then 25 ml of cold M-1 buffer (50 mM Tris-HCl [pH 7.9], 10 mM MgCl2, 2 mM dithiothreitol, 25% sucrose, 50% glycerol) and 6.5 ml of saturated ammonium sulfate solution were added. After the preparation was left on ice for 20 min, ultracentrifugation (100,000 × g, 3 h) was conducted. Then 5.9 g of ammonium sulfate and 0.01 ml of 1 M NaOH were added to the supernatant, and the solution was mixed continuously for 30 min. The solution was centrifuged (15,000 × g, 20 min), and the pellet was dissolved in 3 ml of cold M2 buffer [50 mM Tris-HCl (pH 7.9), 6 mM MgCl2, 40 mM (NH4)2SO4, 0.2 mM EDTA, 1 mM

TABLE 1. Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Position</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>FW-xynA</td>
<td>CAGCCTACGATGAAGGATG</td>
<td>5040-5022</td>
<td>Northern blotting</td>
</tr>
<tr>
<td>RV-xynB</td>
<td>GGAGGCTCAGCAAAGCTC</td>
<td>3319-3336</td>
<td>Northern blotting</td>
</tr>
<tr>
<td>Trans-FITC</td>
<td>CCGTAGCATCAAGTCCATAG</td>
<td>6379-6398</td>
<td>Primer extension analysis</td>
</tr>
<tr>
<td>reg-upper</td>
<td>CGGGATCCATGAGCATGATGCAATATC</td>
<td>1907-1927</td>
<td>Recombinant protein</td>
</tr>
<tr>
<td>reg-lower</td>
<td>GGGATCCCTTTCTGATGTCCTCGGATATAG</td>
<td>2782-2803</td>
<td>Recombinant protein</td>
</tr>
<tr>
<td>gs5102</td>
<td>CCATCGATTCCGAGGATGAGACCG</td>
<td>6661-6645</td>
<td>Gel shift assay</td>
</tr>
<tr>
<td>gs4961</td>
<td>CCGGATCCCTCTGATGTCCTCGGATATAG</td>
<td>6520-6536</td>
<td>Gel shift assay</td>
</tr>
<tr>
<td>Reg-UP</td>
<td>GCATTATGGAACATACGCG</td>
<td>1681-1700</td>
<td>Nuclease protection assay</td>
</tr>
<tr>
<td>Reg-DOWN</td>
<td>CTTGCGCAGTAAAGCAGAATGATAGG</td>
<td>2267-2290</td>
<td>Nuclease protection assay</td>
</tr>
<tr>
<td>IVT-F</td>
<td>CCAGAACCTACAGGCAAGGC</td>
<td>6030-6049</td>
<td>In vitro transcription</td>
</tr>
<tr>
<td>IVT-R</td>
<td>GCGTGCTAAAGCAGGAAGGAC</td>
<td>6829-6810</td>
<td>In vitro transcription</td>
</tr>
</tbody>
</table>

* a Restriction sites are indicated by boldface type.
* b FITC, fluorescein isothiocyanate.

37°C. The reaction mixture contained 5 U of the DNA polymerase I large fragment (New England Biolabs Inc., Beverly, Mass.). 2.5 mM of a deoxynucleoside triphosphate mixture (dATP, dGTP, and dTTP), and 1 MBq of [γ-32P]dCTP in 20 μl of DNA polymerase I buffer (New England Biolabs Inc., Beverly, Mass.). After the reaction was finished, unincorporated nucleotides were removed with QIAquick nucleotide removal kit (Qiagen GmbH). Competitor DNA was also prepared by PCR and was purified with a QIAquick gel extraction kit (Qiagen GmbH). Then 0, 20, 40, or 80 ng of the recombinant regulator protein was added to 20 μl of binding buffer (40 mM Tris-HCl, 4 mM MgCl2, 100 mM NaCl, 2 mM EDTA). 20% [vol/vol] glycerol, 2 μg of bovine serum albumin, 5 mM spermidine, 1 μg of competitor DNA) and incubated at 20°C for 15 min. One microliter of competitor DNA was added to the mixture, which was incubated for an additional 30 min. Polyclarlamide gel electrophoresis was performed with an 8% (wt/vol) polyacrylamide gel to determine the mobility of the labeled DNA. Signals were detected by exposure to X-ray film (MXJB-1 medical X-ray film; Kodak).

**TABLE 2. P. bryantii B14 xylan utilization operon**

<table>
<thead>
<tr>
<th>Open reading frame</th>
<th>Gene</th>
<th>Putative function</th>
<th>Closest database match</th>
<th>Open reading frame coordinates</th>
<th>Signal peptide</th>
<th>Stop codon</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>xynR</td>
<td>Two-component regulatory protein</td>
<td>28.4% identity (599 amino acids) to CyaC of Anabaena sp. strain PCC7120</td>
<td>1302-2806 (833)</td>
<td>?</td>
<td>TAA</td>
</tr>
<tr>
<td>2</td>
<td>xynB</td>
<td>β-Xylosidase/exoyylanase</td>
<td>67.8% identity (317 amino acids) to XyI of B. ovatus V975</td>
<td>3914-2955 (319)</td>
<td>?</td>
<td>TAA</td>
</tr>
<tr>
<td>3</td>
<td>xynA</td>
<td>Endo-1,4-β-xylanase</td>
<td>51.1% identity (350 amino acids) to XyII of B. ovatus V975</td>
<td>5106-3997 (369)</td>
<td>Yes</td>
<td>TAA</td>
</tr>
<tr>
<td>4</td>
<td>xynD</td>
<td>Sodium:solute symporter</td>
<td>71.0% identity to an unidentified open reading frame of B. ovatus V975</td>
<td>6535-5138 (465)</td>
<td>Integral membrane protein</td>
<td>TAA</td>
</tr>
<tr>
<td>5</td>
<td>xynE</td>
<td>Putative arylesterase/lipase acyl hydrolase (GDSL motif)</td>
<td>34.5% identity (383 amino acids) to Sce6.29 of Streptomyces coelicolor</td>
<td>7722-6538 (394)</td>
<td>Yes</td>
<td>TAA</td>
</tr>
<tr>
<td>6</td>
<td>xynF</td>
<td>α-Glucuronidase</td>
<td>38.7% identity (106 amino acids) to BH1061 of Bacillus halodurans</td>
<td>7987-8426 (&gt;147) (incomplete)</td>
<td>?</td>
<td>TAA</td>
</tr>
</tbody>
</table>

* The accession numbers for the most homologues genes are as follows: P74982 (XynR gene), P49943 (XynB gene), P49942 (XynA gene), AAD20252 (XynD gene), O9KZK1 (XynE gene), and O9KE00 (XynF gene). The three B. ovatus V975 gene homologues all fall within the same operon and are transcribed in the same direction (26).
* The numbers in parentheses are the numbers of amino acids.
dithiothreitol, 15% (vol/vol) glycerol]. The solution was then dialyzed against M2 buffer for 6 h and kept frozen as a cell extract at −80°C until analysis.

**In vitro transcription assay.** Template DNA (799 bp) was amplified by PCR with the IVT-F and IVT-R primers (Table 1). The reaction mixture contained 16 nmol of each deoxynucleoside triphosphate and 2 μl of RNase inhibitor in 29 μl of RNA polymerase buffer and was kept on ice for 10 min. Then 10 μl of cell extract and 1 μl of recombinant regulatory protein (0, 400, or 800 ng/μl) were added to the reaction mixture. The reaction was started by addition of 2 μl of template DNA (1 μg/μl), and the mixture was incubated at 30°C for 45 min. After the incubation, the template DNA was digested with 20 U of RNase-free DNase (37°C, 15 min), and transcripts were purified with an RNAeasy kit (Qiagen GmbH). DIG-labeled probe synthesis was performed by using a DIG probe synthesis kit (Roche Diagnostics GmbH, Mannheim, Germany) by using the IVT-F and IVT-R primers (Table 1). Detection of DIG-labeled probe was performed with a nuclease protection assay (described below). The protected DNA probe (putative size, 523 bp) was electrophoresed on a 1% denatured agarose gel formed with a nuclease protection assay (described below). The protected DNA probe was transferred to a positively charged nylon membrane (Hybond-N+; Amersham Biosciences UK Ltd.). Chemiluminescent signals were detected by exposure to X-ray film (MXJB-1 medical X-ray film; Kodak).

**RESULTS**

Sequence analysis of regions flanking the xyn4B xylanase genes in *P. bryantii* B4. The sequences of two linked genes (synA and synB) that encode endoxylanase and β-xylosidase/exoylanase activities, respectively, were reported previously (9, 10). Sequencing of the regions upstream from synA and synB revealed three additional open reading frames, designated synD, synE, and synF (Fig. 1). The putative synD gene product has a structure characteristic of membrane-spanning proteins and has homology with Na symport-type transporters (15, 22). SynD shows 71% amino acid identity with the product of an unidentified open reading frame from *B. ovatus* V975 (27). SynE shows significant similarities to the products of **FIG. 2. Structure of the *P. bryantii* B4 two-component regulatory protein (XynR).** The linker domain contains a characteristic membrane-spanning motif (9 amino acids; DAS transmembrane prediction server [3]). The histidine kinase domain contains the characteristic H, N, G1, F, and G2 boxes characteristic of histidine kinases of two-component regulatory proteins. The response regulator domain contains the characteristic aspartate residue that is normally phosphorylated in these domains. The effector domain contains an HTH region characteristic of the AraC family of DNA binding proteins. aa, amino acids.
several genes recently identified from genome sequencing projects that are related to lipases and aryl esterases. XynF meanwhile exhibits similarities with α-glucuronidases (Table 2). The xynA, xynB, and xynD genes all show significant homology to members of a similar operon in B. ovatus V975 (Fig. 1).

Downstream from xynB, on the opposite strand, is an open reading frame, designated xynR, which encodes a putative regulatory protein containing 833 amino acids. The C-terminal domain of XynR (residues 700 to 833) shows homology with helix-turn-helix (HTH) domains of the AraC family that are involved in DNA binding (2, 7). Residues 277 to 483 contain all five of the conserved motifs that are typically present in the histidine kinase portion of two-component regulators (12, 22) (Fig. 2). In between, residues 578 to 699 show homology with response regulator-type domains that are typically subject to phosphorylation (22, 23). The region preceding the histidine kinase domain includes a 9-amino-acid membrane-spanning motif (DAS transmembrane prediction server [3]) and represents a possible linker domain of the type that communicates conformational changes between the periplasm and the histidine kinase (14, 22). The N-terminal domain (residues 1 to 227) is unrelated to other database sequences. The XynR regulator from P. bryantii, however, differs from archetypal two-component regulatory proteins (12, 22) in that the sequences showing homology with sensor and response components are combined into a single polypeptide (Fig. 2).  

Ribosome binding sites from members of the Cytophaga-Flavobacterium-Bacteroides (CFB) phylum do not resemble those from E. coli or most other gram-negative bacteria. We were, however, able to identify a putative consensus sequence (A-[AT]-[AT]-[AT]-[AT]-[TC]-[TC]-[T]3 to 25 bp upstream of the start codon for all 11 of the known P. bryantii B4 catalytic genes (Fig. 3). The P. bryantii B4 two-component regulatory protein (xynR) and two B. ovatus V975 open reading frames also have similar upstream sequences. This sequence is, therefore, a possible candidate for a sequence that is involved in facilitating ribosome binding and initiation of translation.

**Transcription of xynABD.** Northern blotting analysis with a 1,721-bp fragment of xynA and xynB (Fig. 1) as the probe detected two transcripts (approximately 3.7 and 5.8 kb) in P. bryantii B4 (Fig. 4). Primer extension analysis (Fig. 5) with primer Trans-FITC (residues 6379 to 6398) revealed that the transcription start site (T at residue 6535) of the 3.5-kb transcript identified in Fig. 4 was located 16 bp upstream of the xynD start codon (residue 6535). Lanes A, G, C, and T contained sequencing ladders with the Trans-FITC primer. Lane S shows the transcription initiation site from P. bryantii B4 cells after pulse addition of 0.05% (vol/vol) WS-X.

**Binding of XynR to DNA upstream from xynD.** The coding sequences for the C-terminal domain of XynR (containing the HTH DNA binding domain [Fig. 2]) were amplified with primers reg-upper and reg-lower (Table 1 and Fig. 1) and cloned into the expression vector pQE-30. The His-tagged recombi-
nant protein was purified and tested for the ability to bind to a 141-bp DNA fragment containing the transcriptional start site identified for the \textit{xynABD} transcript by Northern blot analysis and primer extension analysis (Fig. 4 and 5). As the concentration of the recombinant XynR polypeptide was increased, there was a concomitant decrease in the amount of free DNA. Samples were electrophoresed on 8% (wt/vol) polyacrylamide gels.

An in vitro transcription assay (Fig. 7) revealed that the recombinant fragment of XynR binds to the transcription start site identified by primer extension analysis (Fig. 5). The nuclease protection assay demonstrated that the DNA sequence immediately upstream of the transcription initiation site was degraded, whereas the DNA sequence downstream of the XynR binding site was protected from degradation. Addition of increasing concentrations of recombinant XynR demonstrated that this protein activated transcription of the \textit{xynABD} transcript, thus confirming that XynR is a transcriptional activator.

A nuclease protection assay (Fig. 8) indicated that significantly larger amounts of \textit{xynR} mRNA were detected in the cells grown on xylan than in the cells grown on any other substrate tested. This demonstrated that expression or activity of \textit{xynR} was itself regulated in response to the growth substrate.

**DISCUSSION**

\textit{P. bryantii} B14 is a rumen anaerobe belonging to the gram-negative CFB phylum that is thought to make an important contribution to the utilization of xylans and other hemicellulosic polysaccharides in the rumen ecosystem. In the human colon, another member of this phylum, \textit{B. ovatus}, is also thought to play a significant role in xylan utilization. Strong parallels are evident between the xylan cluster studied here from \textit{P. bryantii} B14 and a shorter sequenced region reported from the human colonic strain \textit{B. ovatus} V975 (26, 27) (Fig. 1).
The products of the xynA, xynB, and xynD genes exhibit between 51 and 71% amino acid sequence identity with the products of xsa, xyII, and an unidentified open reading frame which occur in the same order in the B. ovatus chromosome. XynA and XyII are both endoβxylanases, while XynB and Xsa and are β-xilosidases (9). Our analysis suggests that XynD and its homologue in B. ovatus are membrane proteins that are likely to be involved in oligosaccharide transport. Previous knockout studies with B. ovatus V975 showed that disruption of the xynD homologue, in the Ö K mutant, severely reduced the growth rate on xylan compared with the growth rate of the wild-type strain (26). This effect was ascribed in part to the reduced expression of β-xylanase and xylanase activities due to a polar effect on the downstream xsa and xyII genes. Our results suggest that alterations in oligosaccharide transport could also have accounted for the reduced growth rate observed. This is consistent with observations described above which indicate that xynA, xynB, and xynD are likely to be transcribed as a single mRNA.

Previous work has demonstrated that xylanolytic activities are up-regulated in response to xylan as the growth substrate in P. bryantii B14 (8–11). The XynR regulatory gene product is therefore an obvious candidate for a regulatory protein that could be involved in this response. Our results show that the HTH-type DNA binding domain of this protein binds specifically to sequences within the xylanase gene cluster. This regulatory protein is of interest for several reasons. It is the first example of a regulator from a Prevotella strain that shows homology with two-component regulators described for other bacteria, and it is also the first example of a regulator of this type that has been found to govern expression of genes involved in polysaccharide utilization. Single polypeptides that combine multiple elements of these regulators are known to occur in other cases, including the ricC tetracycline resistance gene regulator in Bacteroides spp. (21). Such organization in the P. bryantii XynR regulatory protein is particularly intriguing, however. If we assume that an input domain responds directly to the availability of oligosaccharide signals, these signals must presumably be detected in the periplasm and the response must be transmitted to a cytoplasmically located DNA binding domain via a membrane-spanning portion of the protein. Alternatively, it is perhaps more likely that the regulator does not respond directly to substrate signals but is the final step in a more complex regulatory cascade. Indeed, this alternative is strongly suggested by the observation made in this study that xynR expression itself responds at a transcriptional level to the growth substrate.

The smaller transcript detected in this study with the 1,721-bp probe whose initiation site was located just upstream of xynD corresponds to the xynABD genes. The DNA binding domain of the XynR regulator was shown to bind a sequence immediately upstream of the xynD gene in a position that makes it very likely to regulate production of this transcript. Such regulation was confirmed by an in vitro transcription assay which showed that XynR acts as a positive regulator of xynABD transcription. It is not known whether additional binding sites for XynR occur upstream of xynE, but similar regulation of the larger 5.8-kb transcript might also occur.

The functions of xynE and xynF (encoding a 107-amino-acid partial sequence) have not been investigated experimentally, but the products of both these genes are possible candidates for enzymes involved in the debranching of xylan polysaccharides or oligosaccharides through the removal of esterified acetyl or phenolic acid groups and in the debranching of gluconic acid residues. The region downstream from xsa in B. ovatus (Fig. 1) does not contain any sequences homologous to P. bryantii xynR. Rather, this region includes a sequence that shows homology with the putative α-glucuronidase-encoding sequence of xynF (31.9% amino acid identity for a 119-amino-acid overlap [ClustalW]). Thus, the organization of the xylan utilization genes other than xynABD is clearly markedly different in B. ovatus than it is P. bryantii, and it remains to be established whether B. ovatus V975 also possesses a transcriptional regulator similar to XynR that governs xylanase gene expression.

ACKNOWLEDGMENTS

The Rowett Research Institute is supported by the Scottish Executive Rural Affairs Department.

Derry Mercer, Kohji Miyazaki, Hiroyuki Miyamoto, and Tatsuki Hirase contributed equally to this work.

REFERENCES