A Two-Component System Regulates the Expression of an ABC Transporter for Xylo-Oligosaccharides in *Geobacillus stearothermophilus*[▽]

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Geobacillus stearothermophilus T-6 utilizes an extensive and highly regulated hemicellulolytic system. The genes comprising the xylanolytic system are clustered in a 39.7-kb chromosomal segment. This segment contains a 6-kb transcriptional unit (*xynDCEFG*) coding for a potential two-component system (*xynDC*) and an ATP-binding cassette (ABC) transport system (*xynEFG*). The *xynD* promoter region contains a 16-bp inverted repeat resembling the operator site for the xylose repressor, XylR. XylR was found to bind specifically to this sequence, and binding was efficiently prevented in vitro in the presence of xylose. The ABC transport system was shown to comprise an operon of three genes (*xynEFG*) that is transcribed from its own promoter. The nonphosphorylated fused response regulator, His₆-XynC, bound to a 220-bp fragment corresponding to the *xynE* operator. DNase I footprinting analysis showed four protected zones that cover the –53 and the +34 regions and revealed direct repeat sequences of a GAAA-like motif. In vitro transcriptional assays and quantitative reverse transcription-PCR demonstrated that *xynE* transcription is activated 140-fold in the presence of 1.5 μ M XynC. The His₆-tagged sugar-binding lipoprotein (XynE) of the ABC transporter interacted with different xylosaccharides, as demonstrated by isothermal titration calorimetry. The change in the heat capacity of binding (ΔC_p) for XynE with xylotriose suggests a stacking interaction in the binding site that can be provided by a single Trp residue and a sugar moiety. Taken together, our data show that XynEFG constitutes an ABC transport system for xylo-oligosaccharides and that its transcription is negatively regulated by XylR and activated by the response regulator XynC, which is part of a two-component sensing system.

The natural degradation of the plant cell wall polysaccharides is a key step in the carbon cycle on Earth and is mediated mainly by microorganisms. The efficient breakdown of these polymers, cellulose and hemicellulose, is of great interest due to the need for renewable energy sources that contribute no net CO₂ emission to the atmosphere (18, 53). Hemicelluloses, which can comprise up to 35% of the total cell wall dry mass, are a heterogeneous group of branched and linear polysaccharides bound to the cellulose microfibrils in the plant cell wall via hydrogen bonds, cross-linking them into a robust network (15, 25). Plant cell wall-degrading microorganisms utilize an impressive variety of modular enzyme structures and different physiological strategies for the degradation of the plant cell wall. Anaerobic bacteria, such as *Clostridium* spp., have evolved unique multienzyme complexes, called cellulosomes, that integrate many cellulolytic and hemicellulolytic enzymes that mediate the attachment of the cell to the crystalline polymer and its controlled hydrolysis (2, 24, 65, 71). Aerobic fungi, such as *Trichoderma* and *Aspergillus*, secrete numerous cellulases and hemicellulases that work synergistically to completely degrade the polymers into mono- or disaccharides which may

be utilized by the surrounding microorganisms (14). Lastly, aerobic bacteria such as *Bacillus* and *Cellvibrio* (formerly *Pseudomonas fluorescens* subsp. *cellulosa*) secrete only a limited number of extracellular polysaccharide backbone-degrading enzymes that yield relatively large oligosaccharides. These oligosaccharides enter the cell via specific transporters, and their final breakdown is carried out by cell-associated or intracellular enzymes (5, 47, 73). This latter strategy has the advantage that the extracellular soluble products are not easily available to competing, nonhemicellulolytic microorganisms.

Although many hemicellulolytic enzymes have been studied extensively (70), very little is known about the sensing systems for the hemicellulolytic products and their uptake systems. Several microbial transport systems, including UhpT (81, 82), DcuB (88), UpgP (31, 48), and YxdLM (34), have been shown to be regulated by two-component systems (TCSs). This type of regulation enables the cell to respond to environmental or intracellular signals and to alter gene expression. TCSs comprise a sensor histidine protein kinase and a response regulator, usually found in separate proteins. The kinase, typically a membrane protein, becomes autophosphorylated at a conserved histidine residue using ATP as a substrate, in response to a specific signal. The phosphoryl group is then transferred to a conserved aspartate residue on the response regulator, altering its ability to bind target DNA sequences (75).

Geobacillus stearothermophilus T-6 is a soil bacterium that possesses a highly efficient and complete hemicellulolytic sys-

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[▽] Published ahead of print on 1 December 2006.

TABLE 1. Oligonucleotides used in this study

Primer	Sequence (5'–3') ^a	Application
xylR N-ter	CCAATTGCATATGCCATGGCTCACCATCATCACCATCAC AATA AACAGCTCGTCTTAAATTTG	Cloning into T7 expression vectors
xylR C-ter	ACGTGCTAAGCTGGATCCGCCTCTTTTCGCATGCGCT	
xynC N-ter	GTTAACGGAGGTGAAAAACCATGGATCATCACCATCACCATC ACGAGAAGACCATTTTAGTTG	
xynC C-ter	GAAAAGTACCAGTATTGGATCC TTAATTTTCTTTTGTGAC	
xynE N-ter	GTTCTCAAATCCATGGCTCATCATCATCATCTTCTTCTCGT CGAGCGATTCC	
xynE C-ter	GGCTGCAGGGATCCTTATTTGTGTGGCTTCCTCCGCC	
ABC C-ter1 HKCW2	CATAAACTTTACCCCTTCTCTATTCTTTG CAGCTACTCCATGATCCATTCTACCG	Determination of transcriptional start points
11R_TCS 21R_TCS	CTAATCAACCTTTGTTTATATTCTATACAAATCATTTCAGAG CTCTGAAATGATTTGTATAGAATATAAAACAAAGGTTGATTAG	DNA-binding and footprinting assays
ABC N-ter	CAGAGATTGCGGAAGAAGCTTGTTACAAAAC	
ABC C-ter	CATAAACTTTACCCCATGGCTATTCTTTG	
Primer1	TTAAAAAGAAAAGTAATCTAAAAATACTGGTACTTTTCTTAAT	
Primer2	TTATTAGGAAAAGTACCAGTATTTTAGATTACTTTTCTTTT	
INV2	CGTGATGTTTCATCTCCATTGG	In vitro transcription assays
IVT1 <i>xynA</i>	GTGCTGTCCGTTTTCACTG	
IVT2 <i>xynA</i>	GAAGCGAATATCCATGCC	
RT-PCR <i>1xynE</i>	GCAAGCAAAGAATAGAGAAGG	Real-time RT-PCR
RT-PCR <i>2xynE</i>	GAAGAACAACCAGACAGCACAATC	
RT-PCR <i>1xynA</i>	GCGGCAGTAGAACCTTA	
RT-PCR <i>2xynA</i>	AGGTTGAATGCTGATCG	

^a Boldface bases indicate engineered restriction sites.

tem. The 30 genes comprising the system appear to be organized in at least nine transcriptional units within a 39.7-kb chromosomal segment. When grown in the presence of xylan, strain T-6 secretes a single extracellular endo 1,4- β xylanase (XynA) that hydrolyzes the polymer's main backbone, producing short modified oligoxylose units of two or more sugars in length. These modified xylosaccharides enter the cell by specialized ATP-binding cassette (ABC) sugar transporters (73), and they are further degraded to monomers by intracellular hydrolases, including a glycoside hydrolase family 10 (GH10) xylanase (77), two GH51 α -L-arabinofuranosidases (29), an α -glucuronidase (GH67) (23, 68, 89), three β -xylosidases (GH39, GH43, and GH52) (6, 8–11), and two xylan acetyl esterases (CE4).

In this study, we demonstrate that in *G. stearotheophilus* strain T-6, a two-component sensing system, encoded by *xynDC*, positively regulates the expression of an adjacent ABC transporter system (*xynEFG*) designed for the uptake of xylooligosaccharides.

MATERIALS AND METHODS

Bacterial strains and plasmids. *G. stearotheophilus* T-6 (NCIMB 40222) was isolated following an enrichment procedure for microbial strains capable of producing alkaline-tolerant, extracellular, thermostable xylanases (35, 72). *Escherichia coli* strains used were XL1-Blue (Stratagene) for general cloning and BL21(DE3) (Novagen, Madison, WI) for expression via the T7 RNA polymerase expression system with pET11d or pET9d (Novagen, Madison, WI).

Growth conditions. Growth medium for *G. stearotheophilus* was basic salt medium (BSM) supplemented with 0.5% glucose or xylose. BSM contained the following per liter: KH_2PO_4 , 0.4 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g; $(\text{NH}_4)_2\text{SO}_4$, 2 g; MOPS (*N*-morpholinepropanesulfonic acid) buffer, pH 7.0, 10.4 g; and trace elements solution, 4 ml. Trace elements solution contained the following per liter: $\text{CaCl}_2 \cdot$

$7\text{H}_2\text{O}$, 0.92 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1.51 g; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.148 g; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.105 g; and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.156 g. The pH of the trace elements solution was adjusted to 2.0 with sulfuric acid.

DNA and RNA isolation and manipulation. *G. stearotheophilus* T-6 genomic DNA was isolated by the procedure of Marmur (40) as outlined by Johnson (32). Plasmid DNA was purified using a QIAGEN plasmid kit (QIAGEN Inc., Chatsworth, CA). DNA was manipulated by standard procedures (58). The *xynDCEFG* coding sequence was revealed by chromosome walking, upstream of the previously reported *xynB2* gene (73), using the inverse PCR method (49). Total RNA was isolated with an RNeasy kit (QIAGEN) according to the supplied protocol.

DNA sequencing and analysis. DNA sequencing was performed on a Perkin-Elmer 377 automated sequencer (Weizmann Institute, Rehovot, Israel). Nucleotide and amino acid sequences were analyzed with either GeneRunner or the sequence analysis software package of the Genetics Computer Group, version 9 (University of Wisconsin, Madison, WI). Sequence homologies were searched with FASTA (50) or BLAST (3) algorithms. Protein sequences were analyzed with the Expert Protein Analysis System (ExPASy) proteomics server (<http://www.expasy.org>) of the Swiss Institute of Bioinformatics (SIB).

Cloning, production, and purification of *G. stearotheophilus* T-6 XylR. The *xylR* gene was cloned via PCR using *G. stearotheophilus* T-6 chromosomal DNA as a template. The primers (Table 1) were designed based on the homologous gene from *G. stearotheophilus* 10 (<http://www.genome.ou.edu/bstearo.html>). The PCR product was cloned into the pET11d expression vector and thereby fused to a His₆ tag at the N terminus of its coding sequence. Attempts to express the XylR protein in *E. coli* resulted in very low yields of soluble protein. To improve the solubility, growth was carried out at 18°C in the presence of 0.04 mM IPTG (isopropyl- β -D-thiogalactopyranoside) for 24 h. The XylR protein was purified from a crude cell extract by Ni²⁺-nitrilotriacetic acid (NTA) chromatography, yielding 1.5 mg purified enzyme from a 1-liter culture. The purified XylR protein was dialyzed overnight against 2 liters of buffer containing 50 mM Tris-HCl, pH 7.0, and 100 mM KCl, followed by the addition of EDTA and glycerol to final concentrations of 1 mM and 10%, respectively.

Cloning, production, and purification of His₆-tagged XynE and XynC. The *xynE* and *xynC* genes were cloned into pET11d and pET9d vectors (Novagen), respectively, yielding plasmids pET11d-*xynE* and pET9d-*xynC*. All the primers were designed to allow in-frame cloning of the genes into the T7 polymerase

expression vector by use of an NcoI restriction site at the 5' terminus and a BamHI restriction site at the 3' terminus (Table 1). The N-terminal primers contained six histidine codons to provide His₆-fused products. DNA sequencing verified the sequences of the cloned genes. *E. coli* BL21(DE3)(pET9d-*xynC*) cultures were grown overnight in Terrific broth (58) with kanamycin (25 µg ml⁻¹) and *E. coli* BL21(DE3)(pET11d-*xynE*) with ampicillin (100 µg ml⁻¹) without induction (1 × 500 ml in 2-liter shake flasks) at 37°C. The cultures were harvested, resuspended in 30 ml of buffer (10 mM imidazole, 50 mM Tris-HCl, 100 mM NaCl, pH 7.0), disrupted by two passages through a French press (Spectronic Instruments, Inc., Rochester, NY), and centrifuged (14,000 × g for 15 min) for the production of clear crude protein extracts.

The His-tagged proteins were isolated using Ni²⁺-NTA resin (QIAGEN) at a ratio of 2 ml of resin per 50 ml of supernatant fluid. The resin was loaded onto a 0.8- by 4-cm plastic column (Bio-Rad) and was then washed with 2 column volumes of binding buffer (300 mM Tris-HCl buffer, pH 7.5, 300 mM KCl, and 20 mM imidazole). Proteins were eluted using elution buffer supplemented with 350 mM imidazole. The purified XynC protein was dialyzed and stored as described for XylR above. Samples (100 µl) of the purified proteins were stored at -80°C until further use.

Transcriptional analyses. Transcriptional analyses were performed on total RNA extracted from exponentially growing cells. Primer extension reactions were conducted as described previously (44) with avian myeloblastosis virus reverse transcriptase (Promega), 40 µg of total RNA, and the primers listed in Table 1. Northern blot analysis was conducted following the procedure described by Moran (44).

Mobility shift DNA-binding assays. Five unique radioactive DNA probes were used for gel retardation assays. For His₆-XylR, a 42-bp double-stranded DNA fragment containing the putative *xyl* operator (from position -25 to position +5 relative to the transcriptional start site of the *xynDC* operon) was prepared. The double-stranded probe was composed of two synthetic complementary oligonucleotides (Table 1) designed to have two noncomplementary T nucleotides at the 5' end for end labeling with Klenow fragment (Fermentas) in the presence of [α -³²P]dATP.

For His₆-XynC, three DNA fragments of 220, 140, and 121 bp corresponding, respectively, to positions -120 to +100, -120 to +20, and -21 to +100 with respect to the transcriptional start site of *xynE* were generated via PCR with primers listed in Table 1. These PCR products were purified with a Wizard SV gel PCR clean-up system (Promega) and end labeled using [γ -³²P]ATP and T4 polynucleotide kinase (New England Biolabs) as described by the manufacturer. A fourth probe was a 41-bp double-stranded DNA fragment containing a putative inverted repeat (from position -21 to position +20 relative to the transcriptional start site of the *xynE* operon). The double-stranded probe was composed of two synthetic complementary oligonucleotides (Table 1) designed to have two noncomplementary T nucleotides at the 5' end for end labeling with Klenow fragment (Fermentas) in the presence of [α -³²P]dATP.

The binding reaction mixture (30-µl total volume) contained 20 µl of solution comprised of 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 10% glycerol, 1 mM EDTA, 2 µg of salmon sperm DNA, 0.66 mM dithiothreitol, 33 µg of bovine serum albumin, 0.08 ng of labeled probe (about 50,000 cpm), and the indicated amount of protein. The binding mixture was incubated for 30 min at 45°C and then separated on a 6.6% nondenaturing polyacrylamide gel prepared in Tris-borate-EDTA buffer (58). Gels were dried under vacuum and exposed to a phosphorimager screen before analysis with a Fuji BAS2000 phosphorimager.

DNase I footprinting assay. The probes for DNase I footprinting assays were PCR products corresponding to positions -100 to +120 relative to the *xynE* transcriptional start site. Labeled DNA fragments were prepared via PCR using two primers, ABC N-ter and ABC C-ter, which were designed to contain HindIII and NcoI restriction sites, respectively (Table 1). The PCR product was purified from 4% MetaPhor agarose gel (Bio Science Rockland, Rockland, ME). Digestion with NcoI or HindIII permitted end labeling of the coding or noncoding strands, respectively. Labeled DNA fragments (400,000 cpm) were incubated for 30 min at 45°C in 30 µl of the binding reaction mixture described above with various concentrations of XynC. After adjusting the MgCl₂ and CaCl₂ concentrations to 16 mM and 5 mM, respectively, DNA-protein complexes were treated with 1.2 units of RQ1 DNase I (Promega) for 1 min at room temperature and the digest was stopped by addition of EDTA to 30 mM. The samples were purified using a QIAquick PCR purification kit (QIAGEN) and eluted with 30 µl of 10 mM Tris-HCl (pH 8.5) followed by 10 µl of formamide-loading buffer (95% [vol/vol] formamide, 20 mM EDTA, 0.05% [wt/vol] bromophenol blue, 0.05% [wt/vol] xylene cyanol FF). After being heated for 5 minutes at 90°C, the samples were applied to a denaturing 6% polyacrylamide gel containing 7 M urea. Sanger sequencing reaction products of the same fragments with the primers indicated above and α -³⁵S-labeled dATP were loaded on the same gel (Sequenase version

2.0 DNA sequencing kit; Amersham Biosciences). After electrophoresis, the gel was dried and visualized using a Fuji BAS2000 PhosphorImager.

Preparation of *G. stearothermophilus* T-6 cell extract. Cell extract from strain T-6 was prepared for the in vitro transcription reaction, which was followed by quantitative real-time PCR. Strain T-6 cell culture was grown at 55°C in Terrific broth medium (58) (0.5 liters medium in 2.5-liter baffled shake flask) to an optical density at 600 nm of 3.5. After growth, the culture was left on ice for 25 min and then harvested by centrifugation (10,000 × g, 10 min). The cell pellet was washed once with 500 ml of cold, diethyl pyrocarbonate-treated S-30 buffer (10 mM Tris-acetate, pH 7.4, 1 mM dithiothreitol, 14 mM magnesium acetate, 60 mM potassium acetate, and 7 mM 2-mercaptoethanol per liter of freshly added buffer). The washed pellet was resuspended in 10 ml of cold S-30 buffer, and the cells were broken by one passage through a French press (Spectronic Instruments, Inc., Rochester, NY). Following centrifugation (14,000 × g for 30 min), dithiothreitol was added to a final concentration of 1 mM to the clear supernatant. Aliquots (100 µl) were frozen immediately in liquid nitrogen and stored at -80°C for the in vitro transcription experiments.

In vitro transcription reaction. Template DNAs (800 bp) of the target gene (*xynE*) and of the reference (ref) gene (*xynA*, encoding T-6 extracellular xylanase) were amplified by PCR using specific primers (Table 1) and T-6 chromosomal DNA as the template. The in vitro transcription reaction mixture (33 µM of each nucleoside triphosphate, 4 µl of RNasin [Promega], 12 µl of commercial 5× RNA polymerase buffer [Fermentas]) to a final volume of 60 µl was kept on ice for 10 min. Fifteen microliters of fresh strain T-6 cell extract and the indicated amounts of the response regulator, His₆-XynC, were added. The reaction was initiated by the addition of 5 µl of template DNAs (1 µg/µl) of the target gene and the reference gene, and the mixture was incubated at 45°C for 50 min. Following incubation, the template DNA was digested with 25 units of RQ1 DNase I (Promega) for 25 min at 37°C. mRNA transcripts were purified with an RNeasy Mini kit (QIAGEN) as outlined by the manufacturer. The mRNA level transcribed in the in vitro transcription reactions was determined following reverse transcription and quantitative real-time PCR as described below.

RT and quantitative real-time PCR. Reverse transcription (RT) was performed following the manufacturer's protocol (ABgene, United Kingdom) by use of a first-strand synthesis kit and 2 µl of purified mRNA obtained from the in vitro transcription assays. In order to check for DNA contamination, control reactions were carried out in the absence of reverse transcriptase. Real-time RT-PCR primers were designed with the aid of the Primers3 program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) to generate amplicons ranging in size from 100 to 150 bp (Table 1). Gene quantification was performed with a Rotor-Gene 3000 (Corbett Research, Sydney, Australia) instrument. Each 25-µl reaction mixture included template cDNA, 180 nM of each primer, and Absolute 2× SYBR green mix (ABgene) containing SYBR green, deoxynucleoside triphosphates, MgCl₂, and Thermo-Start DNA polymerase. The activation (*n*-fold) of the target gene (*xynE*) was determined based on its real-time PCR efficiencies (*E*) and the crossing point difference (change in cycle threshold [ΔC_T]) for a treated sample versus a control sample (51) and calculated using the following equation: activation (*n*-fold) = $E_{(\text{target})}^{\Delta C_T_{\text{target}}(\text{control} - \text{treated})} / E_{(\text{ref})}^{\Delta C_T_{\text{ref}}(\text{control} - \text{treated})}$. For each gene, cDNA dilution curves were generated and used to calculate the individual real-time PCR efficiencies: $E = 10^{-1/\text{slope}}$. Typical efficiencies for *xynE* and *xynA* were 1.90 and 1.88, respectively. cDNA and RT negative controls were included in each run. Melt curves were analyzed to ensure specificity of primer annealing and lack of primer secondary structure.

Phosphorylation of His₆-XynC. Phosphorylation of His₆-XynC was accomplished by following the protocol of Lukat et al. (39). The XynC protein (6 mg) was incubated in 10 ml of a buffer containing 100 mM Tris-HCl (pH 7.0), 5 mM MgCl₂, 1 mM dithiothreitol, and 10 mM acetyl phosphate (Fluka).

Microcalorimetry titration studies. Titration calorimetry measurements were performed with a VP-ITC calorimeter (Microcal; Northampton, MA) as described by Wiseman et al. (85). Protein solutions for isothermal titration calorimetry (ITC) were dialyzed extensively overnight against buffer A (50 mM Tris-HCl, pH 7.0, 100 mM NaCl, 0.02% NaN₃). Ligand solutions of xylobiose (X₂), xylotriose (X₃), xylotetraose (X₄), xylopentaose (X₅), and xylohexaose (X₆) (Megazyme; Wicklow, Ireland) were prepared by dilution with the protein dialysis buffer. Aliquots (10 µl) of the ligand solution at 8.5 to 20 times the binding-site concentration were added to the reaction cell containing 1.41 ml of the 0.02 to 0.1 mM protein solution by the controlled action of a 250-µl rotating stirrer-syringe. The heat of dilution was determined to be negligible in separate titrations of the ligand into the buffer solution. Calorimetric data analysis was carried out with ORIGIN 5.0 software (MicroCal). Binding parameters, including binding stoichiometry (*n*), the binding constant (K_B [M⁻¹]), and the binding enthalpy (ΔH_B [kcal/mol of bound ligand]), were determined by fitting the experimental

binding isotherms. K_B was determined primarily by the slope of the isotherm at the equivalence point (17).

Nucleotide sequence accession number. The 39,692-bp sequence of the xylan utilization region and the sequence of the *xytR* gene from *G. stearothermophilus* T-6 have been deposited in the GenBank under accession numbers DQ868502 and DQ868501, respectively.

RESULTS

For the cloning of the xylan utilization-related genes from *G. stearothermophilus* T-6, we combined two main strategies: screening of genomic libraries for hemicellulolytic activities (73) and PCR-based chromosome walking. By using those methods, the entire xylan utilization cluster has been cloned and sequenced. The cluster is 39.7 kb long and contains 30 open reading frames; many of the products have been characterized structurally and biochemically (6–8, 10, 11, 23, 29, 66–69, 76, 77, 89). In this cluster, a putative TCS, *xynDC*, lies just upstream of a putative ABC transport system (*xynEFG*). Similar combinations of a TCS and an adjacent transport system that is regulated by the TCS have been described for several systems (31, 34, 88), suggesting that the putative TCS encoded by *xynDC* regulates the ABC transport system (*xynEFG*).

Sequence analysis of the two-component system (*xynDC*) and the ABC transport system (*xynEFG*). The putative *xynD* gene product exhibits characteristic features of bacterial histidine kinase proteins, including two transmembrane (TM) helices (TM1 residues 15 to 35 and TM2 residues 255 to 306) flanking an extracellular domain (residues 36 to 254) and a conserved C-terminal cytoplasmic region containing the ATP-binding kinase domain. Downstream to *xynD* lies *xynC*, which encodes a protein with strong similarity to response regulators. As in the case for many response regulators, the *xynC* gene product has a predicted two-domain architecture, with an N-terminal signal receiver domain linked to a C-terminal effector domain (55). The N-terminal signal receiver domain (positioned at residues 5 to 122) shares homology with the CheY superfamily (83), whereas the C-terminal domain from position 157 to position 225 contains a putative helix-turn-helix motif that resembles the AraC-type DNA-binding domain (20). The neighboring genes, *xynEFG*, are likely to code for an ABC transport system. XynE appears to correspond to an extracellular sugar-binding protein, with characteristic features of signal peptides of bacterial lipoproteins. Its N-terminal region contains a putative cleavage site with an almost perfect match to the consensus cleavage site sequence for the lipoprotein signal peptidase II (Leu-Ser-Gly/Ala ↓ Cys) (84). Hydrophathy analyses for XynF and XynG show patterns of hydrophobic and hydrophilic regions that suggest that these proteins are likely to have membrane-spanning regions. Hydrophobic-moment analysis of XynF and XynG predict six transmembrane helices. The two proteins show homology to several integral cytoplasmic membrane proteins involved in sugar transport and contain a conserved hydrophilic segment with the consensus sequence, EAAX₃GX₉IXLP; this sequence is typical of integral membrane proteins from binding protein-dependent transport systems (27). On the basis of this signature, XynF and XynG can be assigned to the disaccharide subcluster of bacterial binding protein-dependent permeases. This subcluster contains proteins involved in the transport of short sugars and oligosaccharides. Thus, it is likely that the *xynEFG* cluster

FIG. 1. (A) Representative isothermal calorimetric titration curve of XynE with xylotriase. Titration calorimetry measurements were performed with a Microcal VP-ITC titration calorimeter (Microcal Inc., Northampton, MA). The top half shows the calorimetric titration of binding protein with ligand, and the lower half displays the integrated injection heats from the upper half, corrected for control dilution heats. The solid line is the curve of best fit that was used to derive binding parameters. (B) Changes in the binding enthalpies (ΔH_b) at different temperatures for interaction of xylotriase with XynE. The value of ΔC_p , $-158 \text{ cal mole}^{-1} \text{ K}^{-1}$, was derived from the slope of the linear correlation.

encodes three of the components of an ABC transport system for xylo-oligosaccharides. The gene for the ATP-binding protein is presumably located elsewhere on the chromosome.

The substrate-binding protein XynE binds xylo-oligosaccharides. Based on sequence homology, *xynE* encodes a substrate-binding protein. In gram-negative bacteria, substrate-binding proteins are located in the periplasmic space, whereas in gram-positive bacteria, they were found to be linked to the cytoplasmic membrane by a lipid anchor (22, 80). These proteins interact with various substrates, such as sugars, amino acids, oligopeptides, and vitamins. The ability of the XynE protein to

TABLE 2. Thermodynamic parameters of binding of xylo-oligosaccharides to XynE

Ligand	T (°C)	$K_B \times 10^6$ (M ⁻¹)	K_D (1/ K_B) (μ M)	ΔH_B (kcal/mol)	$T\Delta S_B$ (kcal/mol)	ΔG_B (kcal/mol)
X ₂	30	2.3 ± 0.5	0.44	-18.6 ± 0.2	-9.8	-8.8
X ₃	10	21 ± 4.3	0.05	-15.5 ± 0.2	-6.1	-9.4
	20	9 ± 1.2	0.11	-17.6 ± 0.1	-8.2	-9.4
	30	12 ± 2	0.08	-20.0 ± 0.2	-10.2	-9.8
	40	10 ± 1.3	0.1	-20.7 ± 0.2	-10.7	-10.0
	50	7.3 ± 0.8	0.14	-21.8 ± 0.1	-11.7	-10.1
X ₄	30	8.3 ± 1.2	0.12	-13.0 ± 0.1	-3.4	-9.6
X ₅	30	1.8 ± 0.2	0.56	-8.1 ± 0.1	0.6	-8.7
X ₆	30	0.7 ± 0.1	1.4	-8.8 ± 0.4	-0.7	-8.1

bind xylosaccharides was demonstrated using ITC (Fig. 1A). ITC provides a direct measure of binding enthalpy, ΔH_B , and allows the simultaneous determination of the binding parameters, which include the binding constant (K_B), entropy (ΔS_B), the free energy of binding (ΔG_B), and the binding stoichiometry (n). Thermodynamic binding parameters of xylo-oligosaccharides (X₂ to X₆) to XynE are summarized in Table 2, showing that all of the binding interactions were exothermic and enthalpy driven. The highest K_B values were gained with

xylotri-ose, suggesting that the binding protein XynE prefers trisaccharides. For other xylo-oligosaccharides, the order of binding affinities was as follows: X₂<X₃>X₄>X₅>X₆. Large negative heat capacity (ΔC_p) values are thought to be associated with hydrophobic stacking interactions and result from the dehydration of the aromatic side chains in the protein and the rings of the carbohydrate (38, 74, 78). From the thermodynamic parameters obtained at different temperatures, the heat capacity change ($\Delta C_p = \Delta H_B/\Delta T$, where H_B is binding enthalpy and T is temperature) for the binding reaction could be extracted. Plotting ΔH_B versus temperature for xylotri-ose gave a linear correlation, indicating that ΔC_p for binding is constant in the temperature range used (Fig. 1B). The yielded slope was $\Delta C_p = -158 \text{ cal mole}^{-1} \text{ K}^{-1}$, suggesting one stacking interaction in the binding site that can be provided by a single Trp residue interacting with a sugar ring (90).

Transcriptional analysis of the *xynDCEFG* cluster. The *xynDCEFG* genes are all transcribed in the same direction and potentially constitute a polycistronic operon. The intergenic spacer region between *xynD* and *xynC* is only 3 bp long and lacks any obvious rho-independent hairpin-like secondary structure, suggesting that the two genes are cotranscribed. The space between *xynC* and *xynE* is 110 bp long and also lacks any obvious

FIG. 2. Transcriptional analyses of the *xynDC*. (A) Determination of the *xynDC* transcript length using Northern blot analysis. Total RNA was extracted from mid-exponential-phase cultures of *G. stearothermophilus* T-6 grown in BSM supplemented with 0.5% glucose (lane 1) as the sole carbon source or with 0.5% xylose (lane 2). The RNA was subjected to electrophoresis, transferred to nitrocellulose (BA85; Schleicher & Schuell), and annealed to [³²P]DNA for *xynD*. (B) Mapping the 5' termini of the *xynDC* cluster by primer extension analysis. Total RNA was extracted as mentioned above. Extension products resulting from RNA obtained from cultures grown on 0.5% xylose are shown in lane 1 or with 0.5% glucose as the sole carbon source (lane 2). Dideoxynucleotide sequence reactions were carried out with the same primer used for the reverse transcriptase reactions. The position of the transcriptional start point is indicated with an asterisk on the inferred nontemplate strand sequence. (C) Sequence data for the regulatory region. The transcriptional start point (+1) is indicated by a vertical arrowhead. The -35 and -10 regions, the proposed ribosome-binding site (RBS), the initiating methionine codon, and the potential CREs are in boldface. The CRE sequence is TGT/AAANC GNTNA/TCA, where underlined letters represent the most critical bases, N is any base, and the vertical line denotes an axis of symmetry (41). The *xyI*R operator is indicated by horizontal arrowheads above the inverted repeat.

transcriptional terminator. However, this region does contain a potential promoter. Downstream from the last gene, *xynG*, there is a potential transcription terminator with a calculated ΔG of -4.2 kcal/mol followed by T nucleotides typical of a rho-independent terminator. To determine whether the *xynDCEFG* genes are cotranscribed, Northern blot analysis was performed. Total RNA was isolated from T-6 cultures grown in the presence or absence of xylose (a potential inducer) and annealed to a DNA probe for *xynD*. As shown in Fig. 2A, the probe hybridized to RNAs of many different sizes, with a maximum length of about 6 kb. This mRNA size is close to the calculated size (5.95 kb) of the presumed *xynDCEFG* transcript, suggesting that the five genes are cotranscribed from the *xynD* promoter. Hybridization to smaller RNA species is presumed to reflect, at least in part, the degradation of the 6-kb transcript. No hybridization was detected with mRNA from cultures grown without xylose, suggesting that xylose can function as a molecular inducer. Using primer extension analysis, the apparent transcriptional start point of the *xynD* promoter was identified and assigned to a T nucleotide 205 bases upstream from the ATG initiation codon of *xynD* (Fig. 2B). As expected, no extension product was detected with mRNA from glucose-grown cultures. The apparent -35 sequence, TTCTAA, differs from the *Bacillus subtilis* consensus sequence, TTGACA, at three positions and is separated by 17 bp from the potential -10 region, TCTATA, which differs by 3 nucleotides from the σ^A consensus sequence, TATAAT (26, 30, 45). An inverted repeat sequence (5'-TTTGTTTATATTCTATACAAA-3') resembling the *xylR* operator sites from *Bacillus* and other gram-positive bacteria (56) overlaps the -10 region, suggesting that the *xynD* promoter is negatively regulated by XylR (Fig. 2C). Two potential operator sites for catabolite-responsive regulation (catabolite-responsive elements [CREs]) are located between the promoter and the ATG initiation codon (41, 42) (Fig. 2C).

XylR binds the *xynD* promoter. We have recently cloned and sequenced the xylose repressor gene, *xylR*, from *G. stearothermophilus* T-6 via PCR based on the homologous gene from strain *G. stearothermophilus* 10 (37). The His₆-tagged gene product was expressed in *E. coli*, and the purified protein was used for gel mobility shift assays. The electrophoretic mobility of a 42-bp DNA fragment containing the putative *xynD* operator sequence was retarded when the fragment was incubated with 1 μ M His₆-XylR (Fig. 3A). To test whether xylose can act as the inducer of the *xynDC* operon, the binding of XylR to the *xyl* operator was assayed in the presence of D-xylose or D-arabinose. Binding was prevented in the presence of 10 mM xylose, while L-arabinose showed no effect (Fig. 3B). These results are consistent with the Northern blot analysis indicating that xylose can act as the molecular inducer of the *xynD* promoter.

Mapping the 5' end of *xynEFG*. As shown above, the *xynEFG* genes can be cotranscribed from the *xynD* promoter. However, the 110-bp spacer between *xynC* and *xynE* contains sequences that resemble potential promoter and regulatory elements as well. To test whether the *xynEFG* genes can also be transcribed from an additional promoter, primer extension analysis was performed. The apparent transcriptional start point corresponded to an A residue, which is 96 bp upstream of the translational initiation codon of *xynE* (Fig. 4A) and was detected only in xylose-grown cultures. The -35 sequence (TATCGA) differs from the *B. subtilis* consensus, TTGACA, by four nucleotides and is separated by

FIG. 3. Gel retardation of a 32 P-labeled *xynD* operator fragment by His₆-XylR. (A) All lanes contained about 0.08 ng of radioactively labeled DNA fragment containing the synthetic *xynD* operator. Lane 1 contained no protein; lanes 2 to 4 contained different concentrations of XylR. (B) Binding of XylR to the *xynD* operator in the presence of xylose (Xyl) or arabinose (Ara). All lanes contained 0.08 ng of radioactively labeled DNA and 1 μ M of the purified XylR. Lanes 1, 2, and 3 contained 5 mM, 10 mM, and 15 mM xylose, respectively; lane 4 contained 15 mM arabinose.

17 bp from the potential -10 region (AGTAAT), which differs from the σ^A consensus, TATAAT, by two nucleotides (45). The poor adherence of these putative promoter sequences to consensus sequences is typical of promoters that depend on a positive regulator. In addition, the promoter region contains an inverted repeat positioned at -17 to $+15$ with respect to the transcriptional start point and several direct repeats of the sequence GAAA (Fig. 4B). Interestingly, although the *xynE* promoter is induced by xylose (based on the primer extension analysis), the promoter region does not contain the typical XylR-binding motif, suggesting that transcription from this promoter is not controlled by XylR. However, the inverted and direct repeats identified in the promoter region suggest that an additional transcriptional regulator may function on the *xynE* promoter.

XynC binds to the *xynE* promoter region. To test whether the putative response regulator, XynC, can bind to the *xynE* promoter region, the *xynC* gene was fused to an His₆ sequence and the gene product was purified from *E. coli* by Ni²⁺-NTA chromatography. Based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis, the purity of the final protein solution was over 97%. A 220-bp DNA fragment corresponding to positions -120 to $+100$ with respect to the *xynE* transcriptional start site was used as a probe for testing XynC-DNA interaction. Gel mobility shift assays showed that the purified His₆-XynC protein can bind to the *xynE* promoter region; a nearly complete shift was seen in the presence of 0.7 μ M XynC (Fig. 5). This relatively high concentration of XynC may reflect the fact that the protein was not phosphorylated, as was observed for other response regulators (1). The binding seemed to be specific, since XynC did not shift an unrelated 200-bp fragment from the *xynA* promoter region (data not shown). To map more precisely the boundaries of the binding region, two DNA probes representing regions upstream and downstream of the apparent transcriptional start site were used. With these two probes, retardation was visible only at relatively high protein concentrations (4 μ M). In addition, XynC failed to

FIG. 4. Mapping the 5' termini of the *xynEFG* cluster by primer extension analysis. (A) Total RNA was extracted from mid-exponential-phase cultures of *G. stearothermophilus* T-6 grown in BSM supplemented with 0.5% xylose or with 0.5% glucose as the sole carbon source. Extension products resulted from RNA extracted from cultures grown on 0.5% xylose are shown in lane 1, and those from growth on 0.5% glucose are shown in lane 2. Dideoxynucleotide sequence reactions were carried out with the same primer used for the reverse transcriptase reactions. The position of the transcriptional start point is indicated with an asterisk on the inferred nontemplate strand sequence. (B) Sequence data for the regulatory region. The transcriptional start point (+1) is indicated by a vertical arrowhead. The -35 and -10 regions, the direct repeat sequences of GAAA, the proposed ribosome-binding site (RBS), and the initiating methionine codon are in boldface. An inverted repeat sequence is indicated by horizontal arrowheads.

bind to a 42-bp DNA probe that contained only the inverted repeat positioned at -17 to +15 with respect to the transcription start point. To delineate the sequence within the *xynE* promoter region that is bound by XynC, DNase I footprinting experiments were performed on the 220-bp fragment that was used in the gel shift experiments (Fig. 6A). Analysis of the coding strand footprint showed four protected regions at positions -53 to -46, -33 to -22, -12 to +12, and +34 to +38 (Fig. 6B and C). Inspection of these regions revealed that they all contain direct repeats of the sequence GAAA (Fig. 6B). XynC also caused certain sites to become hypersensitive to DNase I (Fig. 6A). Such hypersensitivity may indicate that binding of XynC induces DNA bending. To test whether phosphorylation of XynC promotes binding to DNA, we attempted to phosphorylate XynC in vitro with acetyl phosphate as a phosphate donor. Unfortunately, incubation of XynC with acetyl phosphate resulted in protein precipitation, presumably due to oligomerization. Indeed, native polyacrylamide gel electrophoresis analysis indicated that XynC treated with acetyl phosphate appears as a high-molecular-weight protein (data not

FIG. 5. Gel mobility shift assay for binding of XynC to the *xynE* promoter region. XynC and a radioactively labeled 220-bp DNA fragment containing the *xynE* promoter were incubated as described in Materials and Methods. Lane 1 contains no protein; lanes 2 to 6 contain increasing concentrations of purified XynC. The shifted band is indicated by arrow a, and free DNA by arrow b.

shown). This phenomenon was also observed for other response regulators (1).

XynC activates *xynE* transcription in vitro. The direct interaction of XynC with the *xynE* promoter suggests that XynC acts as a transcriptional regulatory protein. The effect of XynC on *xynE* transcription was tested in vitro and quantified by real-time RT-PCR. An in vitro transcriptional assay was performed by mixing nucleoside triphosphates, fresh cell extract of strain T-6 (as a source of RNA polymerase), purified XynC, and a DNA template corresponding to the promoter region and the coding sequence of the *xynE* gene. An unrelated gene (*xynA*) was used as a reference gene to take account of any experimental conditions that affect transcription efficiency. Real-time RT-PCR was carried out on the transcription products by use of primers that amplified internal regions within the *xynE* and *xynA* genes and gave efficiencies of about 90% for both genes. The transcript level of *xynE* increased 3- to 140-fold in the presence of XynC at 0.5 to 1.5 μ M, respectively (Table 3). As expected, the transcription level of the reference gene (*xynA*) was not influenced by the presence of XynC. These results indicate that XynC activates the transcription of *xynEFG*.

DISCUSSION

G. stearothermophilus T-6 possesses a complete system for degrading and utilizing hemicellulose. This system is highly regulated and is mediated via several mechanisms, including induction-repression, catabolite repression, transition phase regulation, and quorum sensing at low cell densities (21, 73). In this work we reveal that a regulatory TCS is involved in regulating an ABC transport system for xylo-oligosaccharides.

XynEFG constitutes an ABC transporter for xylo-oligosaccharides. The *xynEFG* genes appear to encode an ABC transport system that belongs to the carbohydrate uptake transport-

$1/K_D$), values of 0.08 μM for X_3 and 1.4 μM for X_6 . Similar K_D values have been reported for other bacterial sugar-binding proteins by use of different methodologies, such as equilibrium dialysis (63), sugar uptake by whole cells (86), and surface plasmon resonance analysis (79). The xylobiose-binding lipoprotein (BxlE) from *Streptomyces thermoviolaceus* has a K_D of 0.008 μM (79), whereas the cellodextrin lipid-anchored binding protein from *Streptomyces reticuli* showed a K_D of 1.5 μM (63). A K_D value of 1.6 μM was obtained for trehalose/maltose with the binding protein from *Thermococcus litoralis* (28), and recently a K_D of 0.03 μM was measured for chitobiose with NgcE from *Streptomyces olivaceoviridis* (87). The ITC measurements of XynE and xylotriose allowed us to extract the heat capacity change of the interaction. Changes in the binding enthalpies (ΔH_B) at different temperatures yielded a slope of $\Delta C_p = -158 \text{ cal mole}^{-1} \text{ K}^{-1}$, suggesting that stacking interactions are involved in the binding of the sugar to the protein. The negative ΔC_p value can be provided by a single Trp residue (90), and indeed a conserved Trp residue (Trp285) is found in XynE. This residue corresponds to Trp230 in the maltodextrin-binding protein from *Pyrococcus furiosus*, and the crystal structure of this protein complexed with trehalose reveals that this aromatic residue is located in the binding site and interacts with the sugar ring (16). This conserved tryptophan residue is also found in the crystal structures of two other maltose-maltodextrin-binding proteins from *E. coli* and *Alicyclobacillus acidocaldarius* (60). In the *N*-acetyl-D-glucosamine-binding protein (NgcE), the corresponding tryptophan residue (Trp280) was shown to be essential for binding (57).

Like all membrane-spanning subunits of the ABC importers, the XynF and XynG proteins contain the EAA motif (EAAX₃GX₉IXLP) that is located ~100 residues from the C terminus (13, 59). In gram-negative bacteria, the ATP-binding protein gene is usually part of the ABC transporter operon, whereas in gram-positive bacteria the gene is often located elsewhere on the chromosome (52, 62, 73). It is likely that in this case a single ATP-binding protein can serve several ABC transport systems, as was demonstrated for *S. reticuli*, in which the ATP-binding protein (MsiK) is involved in the transport of both cellobiose and maltose (61, 62). Recently, we have cloned and sequenced the putative ATP-binding protein (XynK) from *G. stearothermophilus* T-6. This protein has a significant homology to MsiK, but it remains to be determined whether it is involved in the transport of xylo-oligosaccharides.

Regulation of the *xynDCEFG* transcript. The *xynDCEFG* transcriptional unit codes for a TCS and an ABC transport system. The *xynD* promoter contains a 16-bp inverted repeat that also appears in promoters of four other xylan utilization genes in strain T-6, including *xylM*, *xynA*, *xynX*, and *xylA*. DNA fragments containing this motif were shown recently to bind XylR by gel retardation assays (37). Similar XylR inverted repeat motifs were identified in other gram-positive bacteria (56). The regulatory region of *xynD* contains two potential CREs, suggesting that the transcriptional unit is also negatively regulated by the CcpA protein. The *ccpA* gene from strain T-6 was previously cloned and sequenced, and it showed 68% identity to *B. subtilis* CcpA (36). In *B. subtilis*, several TCSs are located near ABC transport systems on the chromosome but do not appear to be part of the same transcriptional unit (33).

In strain T-6, the L-arabinan utilization cluster also has a TCS located near an ABC transport system.

Positive regulation of *xynE* by XynC. As shown here, XynC binds to the regulatory region of *xynEFG* and activates transcription from the *xynE* promoter. DNase I footprint analysis with XynC identified four protected regions located between positions -53 and +34 with respect to the transcriptional start point (Fig. 6). Interestingly, the positions of the binding sites do not follow the classic pattern for transcriptional activators. In the usual case, the binding site is located upstream of the -35 region, allowing direct interaction of the activator with the carboxy-terminal domain of the RNA polymerase α subunit (4, 54). There are only a few examples of bacterial regulators that activate when bound to both upstream and downstream regions with respect to the transcriptional start point (46). However, it is also possible that the nonphosphorylated protein binds somewhat differently than the phosphorylated form.

A sensor/regulator mechanism controls xylo-oligosaccharide uptake. Based on their primary sequences, the *xynD* and *xynC* products appear to constitute a TCS. XynD would be classified as a class I histidine kinase (19). XynD has two putative transmembrane segments and a conserved C-terminal cytoplasmic region containing the ATP-binding kinase domain. As expected, the N-terminal sensing domain has no significant homology to any other sensor domains, reflecting the many different signals to which histidine kinases are responsive. Attempts to express the XynD sensor domain as a soluble protein were unsuccessful, preventing the determination of the chemical nature of the substrates that bind to XynD. However, it is likely that the XynD sensor domain binds to xylo-oligosaccharides, thus allowing the cell to respond to xylosaccharides in the local environment.

The genes for the ABC transport system (*xynEFG*) are regulated by both XylR and XynC. This type of regulation allows the cells to rapidly amplify the expression of the transport system and thus be able to utilize xylo-oligosaccharides efficiently when they are available. Such a sensing mechanism, used by a xylan-degrading microorganism, has been described only recently (43). The XynR protein from *Prevotella bryantii* B14 contains both a histidine kinase and response regulator domains. This protein was found to activate the expression of genes coding for a xylanase, β -xylosidase, and a putative symporter.

ACKNOWLEDGMENTS

This research was supported by the Israel Science Foundation (grant 1006/05 to Y.S.) and the United States-Israel Binational Science Foundation, Jerusalem, Israel (grants 93-171 and 96-178 to A.L.S. and Y.S.). Additional support was provided by the Otto Meyerhof Minerva Center for Biotechnology, established by the Minerva Foundation (Munich, Germany). Y.S. holds the Erwin and Rosl Pollak Chair in Biotechnology.

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