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YvoA and CcpA Repress the Expression of *chiB* in *Bacillus thuringiensis*

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Bacillus thuringiensis **produces chitinases, which are involved in its antifungal activity and facilitate its insecticidal activity. In our recent work, we found that a 16-bp sequence, dre_{chiB} (AGACTTCGTGATGTCT), downstream of the minimal promoter region of the chitinase B gene (*chiB*) was a critical site for the inducible expression of *chiB* in *B. thuringiensis* Bti75. In this work, we show that a GntR family transcriptional regulator (named $YvoA_{Bt}$), which is homologous to YvoA of *Bacillus subtilis*, can specifically bind to the dre_{chiB} oligonucleotide sequences *in vitro* by using electrophoretic mobility shift assays (EMSAs) and isothermal titration calorimetry (ITC) assays. The results of quantitative real-time reverse transcription-PCR (qRT-PCR) and Western blotting indicated that deletion of *yvoA* caused an ~7.5-fold increase in the expression level of *chiB*. Furthermore, binding of purified $YvoA_{Bt}$ to its target DNA could be abolished by glucosamine-6-phosphate (GlcN-6-P). We also confirmed, in the presence of the phosphoprotein Hpr-Ser_{45}-P, that purified $CcpA_{Bt}$ bound specifically to the promoter of *chiB*, which contains the "cre_{chiB}" sequence (ATAAAGCGTTTACA). According to the results of qRT-PCR and Western blotting, deletion of *ccpA* resulted in a 39-fold increase in the *chiB* expression level, and glucose no longer influenced the expression of *chiB*. We confirm that *chiB* is negatively controlled by both $CcpA_{Bt}$ and $YvoA_{Bt}$ in Bti75.**

Chitinases (EC 3.2.1.14), which have the ability to digest chitin into *N*-acetylglucosamine, are produced by a wide range of organisms and can be potentially used in industry, medicines, scientific research, and agriculture (1–3). *Bacillus thuringiensis* (Bt) also produces chitinases (4–6). Several studies have reported that chitinases generated by Bt are involved in its antifungal activity and can enhance the insecticidal activity of Bt strains (7–10). Many studies have reported the expression and application of chitinases in microorganisms (11–15). However, reports on the regulation mechanism of chitinase genes are scarce.

The expression of the chitinase gene is controlled by the GntR family regulator DasR in *Streptomyces coelicolor* (16, 17). Actually, DasR is a global regulator that is involved in GlcNAc transport and metabolism, antibiotic synthesis (18), and morphological differentiation (16). Colson et al. previously identified a 16-bp consensus sequence (AGTGGTCTAGACCACT) in the promoters of chitin and GlcNAc metabolism-related genes in *Streptomycetes*, which was termed a DasR-responsive element (*dre*) (17). YvoA, the ortholog of DasR in *Bacillus subtilis*, is a bacterial repressor involved in GlcNAc transport and utilization; Titgemeyer and colleagues showed previously that YvoA binds specifically to a similar 16-bp consensus sequence (ATTGGTATAGACAACT) upstream of the *nagAB* and *nagP* genes (19, 20). In our recent work, we also found a 16-bp sequence, the dre_{chiB} sequence (AGACTTCGTGATGTCT), downstream of the minimal promoter region of the chitinase B gene (*chiB*) (Fig. 1A), which is a critical site for the inducible expression of *chiB* in *B. thuringiensis* Bti75 (21). Moreover, electrophoretic mobility shift assays (EMSAs) showed that some regulatory factors bind to the dre_{chiB} site in strain Bti75 cultured in the absence of the inducer. Thus, we hypothesized that YvoA of Bti75 is the regulator responsible for binding to dre_{chiB} to control the expression of *chiB*.

In addition, we also identified a 14-bp sequence that is similar to the catabolite response element (*cre*) consensus sequence inside the minimal promoter region. The *cre* consensus sequence is a 14-bp partially palindromic sequence that specifically interacts with CcpA (catabolite control protein A) in the metabolic process of carbon catabolite repression (CCR) (22). CCR is a general phenomenon whereby microbes adjust their expression of catabolic genes in response to the availability of rapidly metabolizable carbon sources (23–25). In low-GC Gram-positive bacteria such as *B. subtilis*, the key regulator of CCR is CcpA (a member of LacI/GalR family of bacterial regulatory proteins); with the help of Hpr phosphorylated at the Ser_{46} residue (Hpr-Ser_{46}-P), CcpA can specifically bind to target promoters at *cre* sites (23, 26, 27). Chitinase can hydrolyze chitin into GlcNAc, which can be utilized by microorganisms as a carbon and nitrogen source; therefore, it is unsurprising that it is also regulated by CCR. Until now, there has been no detailed study of the mechanism of regulation of chitinases by CCR in Bt.

In the present study, we show that the upstream region of the Bti75 *chiB* gene contains dre_{chiB} and cre_{chiB}, which are similar to the *cis*-acting elements *dre* and *cre* in other species. *In vitro* experiments indicated that the transcriptional regulators $YvoA_{Bt}$ and $CcpA_{Bt}$ can specifically bind to dre_{chiB} and cre_{chiB}, respectively. The results of quantitative real-time reverse transcription-PCR (qRT-

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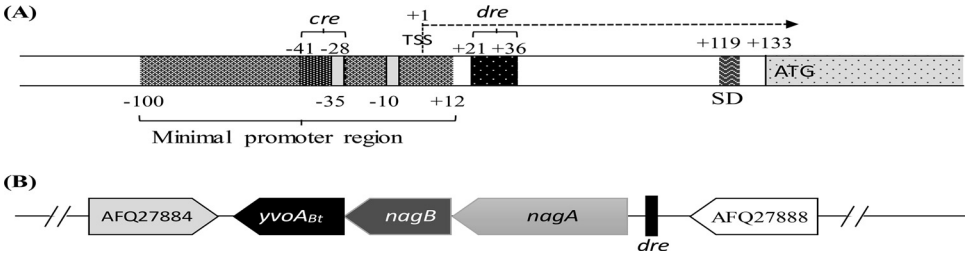

FIG 1 (A) Map of the genetic elements of the *chiB* upstream region (altered according to Fig. 4 in reference 21). The transcription start site (TSS) is indicated as +1. The locations of the *dre* site and *cre* site are labeled. Gray regions in the minimal promoter represent the −10 box and the −35 box. SD, Shine-Dalgarno sequence. (B) Synteny of the $yvoA_{Bt}$ locus in *Bacillus thuringiensis* HD-789. Gene organization was deduced from the complete genome sequences retrieved from the NCBI database. Black rectangles upstream of the *nagA* ortholog represent identified *dre*-like sequences.

PCR) and Western blot analyses confirmed that *chiB* is negatively regulated by $YvoA_{Bt}$ and $CcpA_{Bt}$ in Bti75.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Bti75, which is a highly efficiently inducible chitinase-producing strain, was previously isolated and maintained in our laboratory. Bti75 and its variants were cultured at 30°C with shaking at 200 rpm. *Escherichia coli* DH5α, for plasmid constructions, and *E. coli* BL21(DE3), for protein purification, were cultured at 37°C with shaking at 200 rpm. Luria-Bertani (LB) broth was used to grow *E. coli* as well as Bti75 and its mutants. For chitinase gene expression assays, S minimal medium [15 mM $(NH_4)_2SO_4$, 80 mM K_2HPO_4, 44 mM KH_2PO_4, 3.4 mM Na-citrate, 1 mM $MgSO_4 \cdot 7H_2O$ (pH 7.4)] (28) supplemented with 0.5% yeast extract powder, G medium (S medium plus 0.5% glucose), and C medium (S medium plus 0.5% colloidal chitin) were used. Colloidal chitin was prepared according to a previously described method (9). For strains with antibiotic resistance, appropriate antibiotics were added to the culture medium at the following concentrations: 5 μg ml^{-1} chloramphenicol (Cam), 50 μg ml^{-1} erythromycin (Erm), 50 μg ml^{-1} ampicillin (Amp), and 50 μg ml^{-1} kanamycin (Kan). The primers used in this study are listed in Table 1. Plasmids and strains used in this study are listed in Table 2.

Construction of *ccpA* and *yvoA* deletion mutants of Bti75. The plasmid used to generate strain Bti75Δ*yvoA* was constructed as follows: oli-

TABLE 1 Primers used in this study

Primer	Sequence (5′→3′)	Function
SalI-erm-F	CTGGTCGACGCAAACTTAAGAGTGTGT	*ccpA* and *yvoA* deletion
XbaI-erm-R	CGCTCTAGAGACCTCTTTAGCTTCTTG	*ccpA* and *yvoA* deletion
KpnI-CcpAup-F	TCAGGTACCTTAAACGAAAGAGGTCGTCTCG	*ccpA* deletion
XbaI-CcpAup-R	CGCTCTAGATTCATCTCATCGCACACTCCTT	*ccpA* deletion
SalI-CcpAdown-F	TCTGTCGACCGTATCCAATTTAGAGATTCAACG	*ccpA* delection
PstI-CcpAdown-R	CCGCTGCAGAGGTAAGCTATATACTAGGGAGGATT	*ccpA* deletion
PstI-YvoAup-F	GTCTGCAGCAAGCTTTCCGAAATGTA	*yvoA* deletion
SalI-YvoAup-R	GCGGTCGACATTCTCAGATGGGATTTTAT	*yvoA* deletion
XbaI-YvoAdown-F	CGCTCTAGACAGTGGAAATGAAAATGGAT	*yvoA* deletion
KpnI-YvoAdown-R	AATGGTACCCCCTCGTACTTAAATAGCCT	*yvoA* deletion
NcoI-CcpA-F	TCGCCATGGTAATGAACGTAACAATCTATGATGTAG	*ccpA* cloning
XhoI-CcpA-R	CGCCTCGAGTTTCGTTGAATCTCTAAATTGGAT	*ccpA* cloning
NcoI-Hpr-F	CTGCCATGGTCATGGAAAAAATCTTTAAAGTAACT	*hpr* cloning
XhoI-Hpr-R	CATCTCGAGTTCTCCTAATCCTTCGTTTTTCAT	*hpr* cloning
NcoI-Hprk-F	CGGCCATGGGTATGAAATGTTTTTTTCTATT	*hprK* cloning
XhoI-Hprk-R	ATCTCGAGTATCTCCTGATTCCCTAACTCAATCGC	*hprK* cloning
NcoI-YvoA-F	GCCCCATGGTGATGAACATCGACAAG	*yvoA* cloning
XhoI-YvoA-R	CTGCTCGAGTTTGTTACGTGCAATATTC	*yvoA* cloning
EcoRI-ChiB-F	ATATGAATTCATGAGGTCTCAAAAATTCACACTG	*chiB* cloning
XhoI-ChiB-R	ATCTCGAGGTTTTCGCTAATGACGGCATT	*chiB* cloning
P16SrRNA-RT-F	GCCGTAAACGATGAGTGCTAAGTG	16S rRNA RT-PCR
P16SrRNA-RT-R	TGAGTTTCAGTCTTGCGACCGTA	16S rRNA RT-PCR
PchiB-RT-F	GCCGCTGATGAAAAGACAAGA	*chiB* RT-PCR
PchiB-RT-R	TTCCCAGTCTAAATCTACGCCA	*chiB* RT-PCR
PchiB-F	CCTTTCGTTTTCATATATAGTTTGT	PchiB cloning
PchiB-R	CTAGATAAAATGATCAGACATCACG	PchiB cloning
Pcre-F	TTTTTCAACTTAATAAAGCGTTTACACTAAATCTTACATT	Pcre-CcpA EMSA
Pcre-R	AATGTAAGATTTAGTGTAAACGCTTTATTAAGTTGAAAAA	Pcre-CcpA EMSA
Pcre-R(B)	AATGTAAGATTTAGTGTAAACGCTTTATTAAGTTGAAAAA(5′ biotin)	Pcre-CcpA EMSA
Pdre-F	GCTCCCTTGTATAGACTTCGTGATGTCTGATCATTTTATC	Pdre-YvoA EMSA
Pdre-R	GATAAAATGATCAGACATCACGAAGTCTATACAAGGGAGC	Pdre-YvoA EMSA
Pdre-R(B)	GATAAAATGATCAGACATCACGAAGTCTATACAAGGGAGC(5′ biotin)	Pdre-YvoA EMSA

TABLE 2 Bacterial strains and plasmids used in this study

Plasmid or strain	Relevant characteristic(s)[a]	Source or reference
Plasmids		
pET-28a(+)	Expression vector; Kan^r; C/N-terminal His tag/thrombin/T7 tag, T7 *lac* promoter, T7 transcription start, F1 origin, *lacI*	Novagen
pET-CcpA	Kan^r; *ccpA* gene cloned into pET-28a(+), His tag binding C terminus of CcpA	This study
pET-YvoA	Kan^r; *yvoA* gene cloned into pET-28a(+), His tag binding C terminus of YvoA	This study
pET-Hpr	Kan^r; *hpr* gene cloned into pET-28a(+), His tag binding C terminus of Hpr	This study
pET-HprK	Kan^r; *hprK* gene cloned into pET-28a(+), His tag binding C terminus of HprK/P	This study
pET-ChiB	Kan^r; *chiB* gene cloned into pET-28a(+), His tag binding C terminus of ChiB	This study
pKSV7	Amp^r Cm^r; *Bacillus-E. coli* shuttle vector, temp sensitive	Laboratory collection
pKSV-e	Amp^r Em^r Cm^r; erythromycin gene of pHT315 with its promoter cloned into the pKSV7 SalI/XbaI site	This study
pKSV7-ue-CcpA	Amp^r Em^r Cm^r; 1,115-bp upstream fragment of *ccpA* cloned into the pKSV7-e KpnI/XbaI site	This study
pKSV7-ued-CcpA	Amp^r Em^r Cm^r; 1,072-bp downstream fragment of *ccpA* cloned into the pKSV7-ue SalI/PstI site	This study
pKSV7-ue-YvoA	Amp^r Em^r Cm^r; 1,042-bp upstream fragment of *yvoA* cloned into the pKSV7-e SphI/SalI site	This study
pKSV7-ued-YvoA	Amp^r Em^r Cm^r; 1,015-bp downstream fragment of *yvoA* cloned into the pKSV7-ue XbaI/KpnI site	This study
pHT315	*Bacillus-E. coli* shuttle vector; Amp^r Em^r	Pasteur Institute
Strains		
E. coli DH5α	F$^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44* λ$^-$ *thi-1 gyrA96 relA1*	Laboratory collection
E. coli BL21(DE3)		Invitrogen
Bti75	Efficient chitinase-producing strain	Laboratory collection
Bti75Δ*ccpA*	Bti75 Δ*ccpA*	This study
Bti75Δ*yvoA*	Bti75 Δ*yvoA*	This study

[a] Amp^r, ampicillin resistance; Em^r, erythromycin resistance; Cm^r, chloramphenicol resistance; Kan^r, kanamycin resistance.

gonucleotide primers SalI-erm-F and XbaI-erm-R were used to amplify the erythromycin resistance gene, *erm*, from pHT315. The PCR product was digested with SalI and XbaI and ligated into temperature-sensitive vector pKSV7 treated with the same enzymes to generate vector pKSV-e with erythromycin resistance. The upstream homologous fragment of *yvoA* was amplified by using primers PstI-YvoAup-F and SalI-YvoAup-R, and then ligated into pKSV-e after digestion with SphI and SalI, generating plasmid pKSV-ue-YvoA. Oligonucleotide primers XbaI-YvoAdown-F and KpnI-YvoAdown-R were used to amplify the downstream homologous fragment of *yvoA*, which was ligated into pKSV-ue-YvoA (digested by XbaI and KpnI), generating plasmid pKSV-ued-YvoA. Plasmid pKSV-ued-CcpA was constructed similarly to pKSV-ued-YvoA, using oligonucleotide primer pair KpnI-CcpAup-F and XbaI-CcpAup-R and primer pair SalI-CcpAdown-F, PstI-CcpAdown-R. The resultant plasmids were checked by restriction enzyme digestion and DNA sequencing.

Plasmids pKSV-ued-YvoA and pKSV-ued-CcpA were transformed into Bti75 by electroporation separately (29). When the correct transformant was obtained, we inoculated a single colony into LB medium containing Erm (50 μg ml^{-1}) and cultivated it at 42°C for 36 h. The transformant was plated onto LB agar plates containing Erm or Cam and cultivated at 30°C overnight. Colonies with resistance to Erm but not to Cam were considered possible candidates for Bti75Δ*yvoA* or Bti75Δ*ccpA*. The correct mutants were confirmed by PCR and DNA sequencing.

Protein expression and purification. For the expression of $YvoA_{Bt}$, the *yvoA* gene was amplified by PCR using oligonucleotide primers NcoI-YvoA-F and XhoI-YvoA-R. The fragment was digested with NcoI and XhoI and ligated into similarly digested pET28a(+) (Novagen, Germany), resulting in a His_6 fusion at the C terminus of YvoA (plasmid pET-YvoA). The plasmids used to express CcpA (plasmid pET-CcpA), Hpr (histidine-containing phosphocarrier protein) (plasmid pET-Hpr), HprK/P (Hpr kinase/phosphorylase) (plasmid pET-HprK), and ChiB (plasmid pET-ChiB) were constructed similarly to plasmid pET-YvoA, using oligonucleotide primer pairs NcoI-CcpA-F and XhoI-CcpA-R, NcoI-Hpr-F and XhoI-Hpr-R, NcoI-Hprk-F and XhoI-Hprk-R, and EcoRI-ChiB-F and XhoI-ChiB-R, respectively.

Plasmid pET-YvoA was then transformed into *E. coli* BL21(DE3) (Invitrogen, Carlsbad, CA, USA). When cells reached an optical density at 600 nm (OD_{600}) of ~0.6 to 0.9, they were induced by using 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). After ultrasonic disruption (400 W) of *E. coli* BL21(DE3), native $YvoA_{Bt}$ was purified by using its His tag and a nickel column (GE Healthcare, Piscataway, NJ). The eluate was dialyzed in buffer containing 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.5 mM EDTA, and 5% glycerol for ~18 h. The YvoA protein was stored at −20°C in the presence of 50% glycerol. A similar strategy was used to generate the $CcpA_{Bt}$, Hpr, HprK/P, and ChiB proteins.

Phosphorylation of Hpr by HprK/P. To check whether HprK/P purified in this study could phosphorylate Hpr or not, phosphorylation assays were performed in the presence of 20 μM Hpr and 1 μM HprK/P in phosphorylation buffer (10 mM Tris-HCl [pH 7.0], 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 5% glycerol, 2 mM ATP, 20 mM fructose-1,6-bisphosphate [FBP]). The reactions were carried out at 37°C for 30 min, and the mixtures were then incubated for 5 min at 70°C to stop the reaction. Phosphorylation of Hpr was detected by both nondenaturing 12% PAGE and SDS-PAGE (30) (Fig. 2).

EMSAs. Protein-DNA interactions were evaluated by an electrophoretic mobility shift assay. Long DNA probes were amplified by PCR using

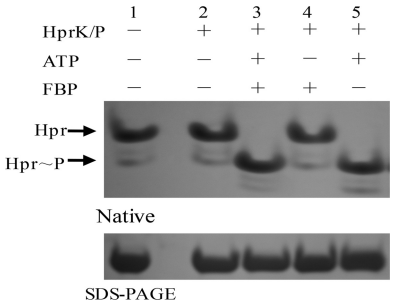

FIG 2 Hpr phosphorylation by HprK/P. Hpr-His_6 (20 μM) was mixed with HprK/P-His_6 (1 μM) in the presence of 2 mM ATP or 20 mM FBP. The reaction products were analyzed by both nondenaturing 12% PAGE and SDS-PAGE. Lane 1, Hpr; lane 2, Hpr and HprK/P; lane 3, Hpr, HprK/P, ATP, and FBP; lane 4, Hpr, HprK/P, and FBP; lane 5, Hpr, HprK/P, and ATP.

primers and the Bti75 genome as a template. Short DNA probes were generated by annealing primers in Tris-EDTA (TE) buffer. The reaction mixture was heated to 95°C for 5 min and then kept at room temperature for 40 min. The 5′ ends of several primers were labeled with biotin (listed in Table 1). The primers used to generate the probes were as follows: PchiB-F and PchiB-R were used to amplify a 158-bp fragment (named PchiB) of the *chiB* promoter that contained dre_{chiB} and cre_{chiB}, and Pdre-F and Pdre-R as well as Pcre-F and Pcre-R were used to generate 40-bp DNA fragments (Pdre and Pcre) that contained dre_{chiB} and cre_{chiB}, respectively.

The concentrations of probes and proteins used in this study are indicated in the corresponding figures and the legends to these figures. The reaction mixtures were incubated for 30 min at 37°C in reaction buffer containing 10 mM Tris-HCl (pH 7.0), 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM EDTA, and 5% glycerol. Nonspecific and specific competition assays were carried out in the presence of 0.5 $\mu g\ ml^{-1}$ sheared salmon sperm DNA and 100-fold, 150-fold, and 300-fold excesses of unlabeled fragments, respectively. After the reaction, the mixtures were separated with an 8% nondenaturing polyacrylamide gel in Tris-borate-EDTA (TBE) buffer. In the competition reaction, the probes were transferred onto a nylon membrane from the gels in TBE buffer. Finally, the nylon membrane was stained by using a biotin chromogenic detection kit (Thermo Fisher Scientific Inc.) to show the location of the labeled probes.

RNA extraction and quantitative real-time reverse transcription-PCR. Bacteria were grown at 30°C in culture medium for ~9 h to the logarithmic phase (OD_{600} of ~2.5), with shaking. Total RNA was extracted by using RNAiso Plus (TaKaRa, Dalian, China), according to the manufacturer's instructions. After the removal of genomic DNA by using RNase-free recombinant DNase I (TaKaRa), cDNA was synthesized from total RNA by using the PrimeScript RT reagent kit (Perfect Real Time; TaKaRa). Quantification of cDNA was carried out by using SYBR Premix Ex *Taq* (Perfect Real Time; TaKaRa), and real-time amplification of the PCR product was analyzed by using StepOne software (Applied Biosystems, Foster City, CA, USA), according to the supplier's instructions. The 16Sr RNA gene acted as the endogenous control. The relative amount of cDNA was calculated according to the $2^{-\Delta\Delta CT}$ method (31). The sequences of the primers for qRT-PCR are presented in Table 1.

Isothermal titration calorimetry. Isothermal titration calorimetry (ITC) measurements were carried out as described previously by Wang et al. (32). The experiments were performed on a MicroCal iTC200 isothermal titration calorimetry instrument (GE Healthcare) with a solution containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1 mM EDTA at 16°C. A 50 μM DNA probe (Pdre) was titrated into 10 μM $YvoA_{Bt}$. Both DNA probe and protein solutions were degassed by spinning at 15,000 × *g* for 15 min. The titration consisted of an initial injection of 0.4 μl, followed by 26 injections of 1.5 μl every 120 s at 16°C. To determine the baseline, the DNA probe was titrated into the same buffer without the protein under the same conditions. The titration data and binding plot after baseline subtraction were analyzed by using MicroCal Origin software.

Western blot assays. The purified ChiB protein was used for rabbit polyclonal antibody generation. Bti75 and Bti75Δ*yvoA* were grown in 100 ml S medium and C medium at 30°C for ~9 h, respectively. The two bacterial strains were collected by centrifugation at 7,000 × *g* for 15 min at 4°C and resuspended in 5 ml lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, and 1 mM PMSF [phenylmethanesulfonyl fluoride]). The same amount of bacteria was disrupted by sonication (400 W). Thirty-five microliters of the crude proteins was resolved by SDS-PAGE and transferred onto Immobilon-P membranes (Millipore, Billerica, MA). Anti-ChiB polyclonal antibodies and horseradish peroxidase-coupled goat anti-rabbit antibodies were used to detect the expression level of *chiB*. ChiB expressed from Bti75 and Bti75Δ*ccpA* was assessed similarly.

Computational prediction of CcpA- and YvoA-responsive elements in Bt. The PREDetector software program (33) was used to predict the positions of *cre*-like and *dre*-like sequences in *B. thuringiensis* subsp. *israelensis* strain HD-789 by using a list of *cre* targets in *B. subtilis* reported previously by Fujita (22) and three *dre* targets in Bti75 that we found in this work. The cutoff score was set at 8.0 to predict the *cre*-like sequence, and the score was set at 6.0 to predict the *dre*-like sequence, as fewer *dre* targets were used to generate the matrix.

RESULTS

YvoA and CcpA are presumed regulators of the expression of *chiB*. From BLASTP analysis, we found a GntR family transcriptional regulator (GenBank accession no. AFQ27885.1) in *B. thuringiensis* HD-789 that has 45% sequence identity and 67% similarity to the *B. subtilis* regulator YvoA ($YvoA_{Bs}$). Like YvoA in *B. subtilis*, this gene is located adjacent to *nagB*, which encodes the enzyme that catalyzes the deamination and isomerization of GlcNAc-6-P to Fru-6-P. Indeed, this gene is the third element of a tricistronic operon in which the first two positions are *nagA* (the GlcNAc-6-P deacetylase gene) and *nagB* (Fig. 1B). Thus, we named the gene $yvoA_{Bt}$ and speculated that its product may bind to the 16-bp locus upstream of *chiB* as a regulator.

At the same time, using the list of *cre* targets in *B. subtilis* reported by Fujita (22), we used the PREDetector software program (33) to identify an additional 14-bp sequence, cre_{chiB} (ATAAAGC GTTTACA), which is similar to the *cis*-acting element *cre*, in the promoter of *chiB* in the genomic sequence of *B. thuringiensis* HD-789 (see Data Set S1 in the supplemental material). The cre_{chiB} element overlaps the −35 box of the *chiB* promoter (Fig. 1A). The catabolite control protein CcpA is a pleiotropic regulator that mediates the global transcriptional response by binding *cre* elements site specifically. Thus, we hypothesized that *chiB* might also be regulated by $CcpA_{Bt}$ in Bti75.

YvoA can bind dre_{chiB} sequences *in vitro*. In this study, $YvoA_{Bt}$ of Bti75 was heterologously expressed in *E. coli*. To confirm the interaction between $YvoA_{Bt}$ and dre_{chiB}, we used unlabeled and biotin-labeled DNA fragments containing the dre_{chiB} site for EMSAs with purified $YvoA_{Bt}$. We incubated purified $YvoA_{Bt}$ with the 158-bp fragment of the *chiB* promoter PchiB (positions −106 to +52 relative to the transcription start site) and a 40-bp DNA fragment, Pdre (positions +9 to +48 relative to the transcription start site), which contains dre_{chiB} in the middle, for EMSAs. As shown in Fig. 3A, $YvoA_{Bt}$ retarded the migration of PchiB in a concentration-dependent manner.

To further investigate the specific interaction between $YvoA_{Bt}$ and dre_{chiB}, 0.5 ng μl^{-1} salmon sperm DNA (nonspecific competition) and a 100-fold excess of unlabeled Pdre (specific competition) were added to the mixture for the reaction of $YvoA_{Bt}$ with Pdre (biotin labeled). As shown in Fig. 4, $YvoA_{Bt}$ also retarded the migration of Pdre, while salmon sperm DNA could not compete away $YvoA_{Bt}$ from Pdre (biotin labeled) (Fig. 4A). On the other hand, when a 100-fold excess of unlabeled probe was present, the retarded band of biotin-labeled Pdre disappeared completely (Fig. 4C). The results from these experiments suggested that $YvoA_{Bt}$ binds to the dre_{chiB} sequence specifically to regulate the expression of *chiB*.

To study the interactions between $YvoA_{Bt}$ and Pdre quantitatively, we also used ITC to determine their binding affinity. As shown in Fig. 5, the binding of $YvoA_{Bt}$ to Pdre fitted well to a one-site binding model, with a calculated K_d (dissociation constant) value of ~0.46 μM (Fig. 5B), which was significantly different from that of the negative control (Fig. 5A). Therefore, our results suggested that $YvoA_{Bt}$ binds to Pdre specifically.

Hpr can be phosphorylated efficiently by Hprk/P. The Hpr

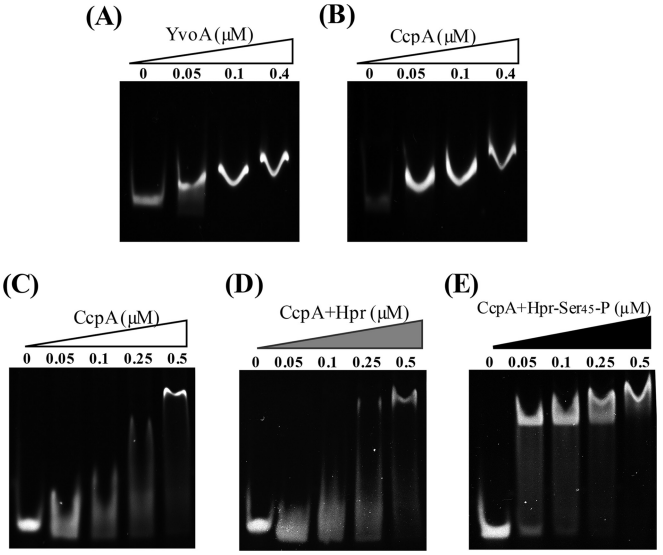

FIG 3 EMSAs to detect binding of $CcpA_{Bt}$ and $YvoA_{Bt}$ to their targets. DNA fragments were detected by staining. (The concentrations of $CcpA_{Bt}$ and $YvoA_{Bt}$ are indicated in the labels above each panel.) (A) Analysis of $YvoA_{Bt}$ binding to PchiB (158 bp). A total of 0.05 μM PchiB was mixed with various concentrations of $YvoA_{Bt}$. (B) Analysis of $CcpA_{Bt}$ binding to PchiB. A total of 0.05 μM PchiB was mixed with various concentrations of $CcpA_{Bt}$. (C to E) Analysis of $CcpA_{Bt}$ binding to Pcre (40 bp). (C) A total of 0.1 μM Pcre was mixed with various concentrations of $CcpA_{Bt}$. (D) A total of 0.1 μM was Pcre was mixed with various concentrations of $CcpA_{Bt}$ in the presence of 4 μM Hpr. (E) A total of 0.1 μM Pcre was mixed with various concentrations of $CcpA_{Bt}$ in the presence of 4 μM Hpr-Ser_{45}-P.

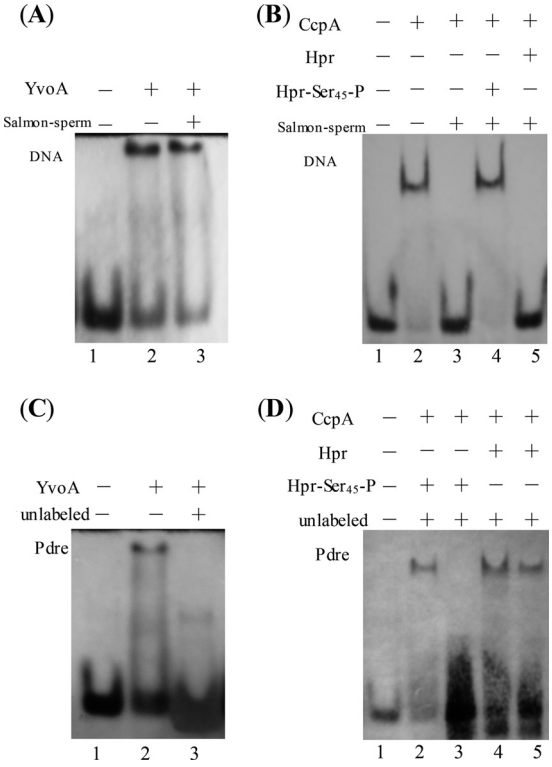

FIG 4 EMSAs to determine specific binding of $YvoA_{Bt}$ and $CcpA_{Bt}$ to dre_{chiB} and cre_{chiB}. DNA fragments were detected by using a biotin chromogenic reagent. (A and C) Nonspecific and specific competition assays with $YvoA_{Bt}$ (0.5 μM) and Pdre (0.1 μM) (40 bp). (A) Lane 1, Pdre (biotin-labeled DNA [Bio]); lane 2, Pdre (Bio) and $YvoA_{Bt}$; lane 3, Pdre (Bio), $YvoA_{Bt}$, and 0.5 ng μl^{-1} salmon sperm DNA. (C) Lane 1, Pdre (Bio); lane 2, Pdre (Bio) and $YvoA_{Bt}$; lane 3, Pdre (Bio), $YvoA_{Bt}$, and a 100-fold excess of unlabeled Pdre. (B and D) Nonspecific and specific competition assays with $CcpA_{Bt}$ (0.5 μM) and Pcre (0.1 μM). (B) Lane 1, Pcre (Bio); lane 2, Pcre (Bio) and $CcpA_{Bt}$; lane 3, Pcre (Bio), $CcpA_{Bt}$, and 0.5 ng μl^{-1} salmon sperm DNA; lanes 4 and 5, Pcre (Bio), $CcpA_{Bt}$, and 0.5 ng μl^{-1} salmon sperm DNA plus 2 μM Hpr and 2 μM Hpr-Ser_{45}-P, respectively. (D) Lane 1, Pcre (Bio); lanes 2 and 3, Pcre (Bio), $CcpA_{Bt}$, and Hpr-Ser_{45}-P plus 150- and 300-fold excesses of unlabeled Pcre, respectively; lanes 4 and 5, Pcre (Bio), $CcpA_{Bt}$, and Hpr plus 150- and 300-fold excesses of unlabeled Pcre, respectively.

and HprK/P proteins were cloned and purified similarly to $YvoA_{Bt}$. As shown in Fig. 2, we found that 1 μM HprK/P phosphorylated >20 μM Hpr at 37°C for 30 min in reaction buffer with ATP and FBP. Moreover, the same results were obtained by using reaction buffer with 2 mM ATP but without FBP (Fig. 2, lane 5), while HprK/P could not phosphorylate Hpr in reaction buffer without ATP (Fig. 2, lane 4).

CcpA can bind specifically to the *chiB* promoter with the assistance of Hpr-Ser_{45}-P. To confirm the interaction between $CcpA_{Bt}$ and cre_{chiB}, PchiB was tested with $CcpA_{Bt}$ alone in the reaction buffer. We found that $CcpA_{Bt}$ retarded the mobility of PchiB similarly to the interaction of $YvoA_{Bt}$ with PchiB (Fig. 3B). However, we observed a similar phenomenon (data not shown) when we used another DNA fragment (~150 bp) without obvious *cre* sites at a $CcpA_{Bt}$ concentration of 0.1 μM. These results raised the possibility that the DNA retardation observed was the result of nonspecific binding of $CcpA_{Bt}$ because of its intrinsic DNA binding nature.

To further investigate the specific interaction between $CcpA_{Bt}$ and cre_{chiB}, we synthesized a 40-bp DNA fragment, Pcre (positions −53 to −14 relative to the transcription start site), of the *chiB* promoter, which contains cre_{chiB} in the middle. Only when its concentration reached 0.5 μM could $CcpA_{Bt}$ obviously retard the movement of Pcre alone (Fig. 3C). Thus, we hypothesized that $CcpA_{Bt}$ alone would have a higher affinity for the long DNA fragment than the short one because of its intrinsic DNA binding nature. We then tried to determine whether Hpr or Hpr-Ser_{45}-P could further stimulate $CcpA_{Bt}$ binding to cre_{chiB}. Hpr and Hpr-Ser_{45}-P (2 μM each) were mixed in the mixture for the reaction of $CcpA_{Bt}$ with Pcre. We found that Hpr did not increase the affinity of $CcpA_{Bt}$ for the DNA fragments (Fig. 3D). In contrast, Hpr-Ser_{45}-P enhanced the affinity of $CcpA_{Bt}$ for the DNA fragments (Fig. 3E). We also further observed that even 10 nM $CcpA_{Bt}$ effectively retarded the movement of Pcre with the help of Hpr-Ser_{45}-P (data not shown), which proved that Hpr-Ser_{45}-P, rather than Hpr, significantly enhanced the affinity of $CcpA_{Bt}$ for Pcre. At the same time, we confirmed that Hpr-Ser_{45}-P or HprK/P itself could not retard the movement of PchiB (data not show).

To determine whether the binding of $CcpA_{Bt}$ to cre_{chiB} was specific or not, EMSAs were carried out in the presence of Hpr-Ser_{45}-P or Hpr with the addition of 0.5 ng μl^{-1} salmon sperm DNA (nonspecific competition) or 150- and 300-fold excesses of the same unlabeled DNA fragment of Pcre (specific competition). As shown in Fig. 4B, salmon sperm DNA easily competed away $CcpA_{Bt}$ from biotin-labeled Pcre without Hpr-Ser_{45}-P (Fig. 4B, lane 3). In contrast, $CcpA_{Bt}$ bound strongly to the labeled probe in the presence of Hpr-Ser_{45}-P mixed with a nonspecific fragment (Fig. 4B, lane 4). Moreover, Hpr could not assist $CcpA_{Bt}$ in bind-

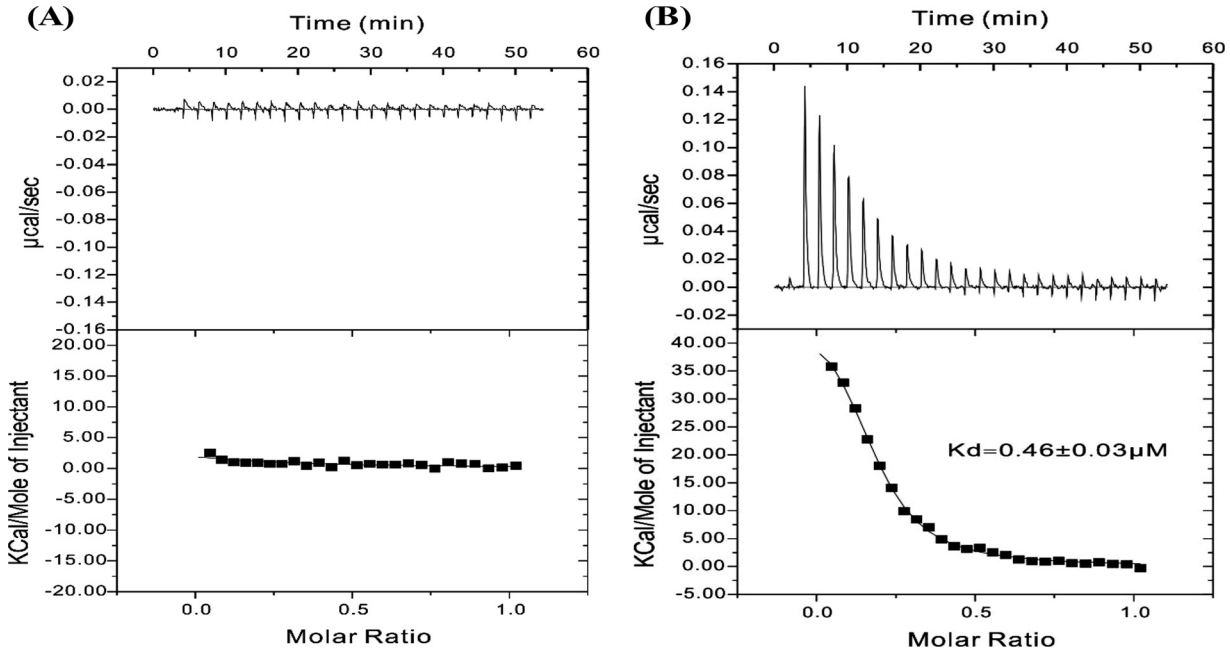

FIG 5 $YvoA_{Bt}$ DNA binding abilities measured by ITC. Shown are binding isotherms of 50 μM Pdre titrated with binding buffer (A) or binding buffer plus 10 μM $YvoA_{Bt}$ (B). For panel A, no binding was detected. For panel B, the data were fitted to a one-site binding model to give a K_d of ~0.46 μM.

ing to Pcre (Fig. 4B, lane 5). In the EMSA for specific competition, we found that when the amount of the specific fragment was increased 300-fold compared with the amount of the labeled probe, the retardation phenomenon in the presence of Hpr-Ser_{45}-P disappeared (Fig. 4D, lane 3). However, a retarded band was observed in the lane with Hpr (Fig. 4D, lane 5). Thus, according to the results of assays for specific and nonspecific competition, we confirmed that $CcpA_{Bt}$ specifically binds to the *cre* site of the *chiB* promoter in Bti75 with the help of Hpr-Ser_{45}-P, but Hpr did not have this function.

Both YvoA and CcpA can repress the expression of *chiB in vivo*. To study the role of $YvoA_{Bt}$ and $CcpA_{Bt}$ in the expression of *chiB in vivo*, Bti75 and Bti75Δ*yvoA* were cultured in S minimal medium and C medium (with colloidal chitin), respectively. As shown in Fig. 6A, we observed that the relative expression level of *chiB* in Bti75Δ*yvoA* was elevated ~7.5-fold compared to that in Bti75 in S medium. Moreover, the relative expression level of *chiB* in Bti75Δ*yvoA* in C medium was ~8-fold higher than that in Bti75 in S medium. At the same time, the relative expression level of *chiB* in Bti75 in C medium was elevated by ~4-fold compared with that in Bti75 in S medium but was also ~2-fold lower than that in Bti75Δ*yvoA* in S medium.

Strains Bti75 and Bti75Δ*ccpA* were cultured in S medium and G medium, respectively. The qRT-PCR results (Fig. 6B) showed no obvious difference in the relative expression levels of *chiB* in Bti75 and Bti75Δ*ccpA* in S medium. However, in G medium, the relative expression level of *chiB* in Bti75 was reduced by almost 39-fold compared with that in Bti75Δ*ccpA*. This indicated that $CcpA_{Bt}$ could severely repress the expression of *chiB* when the medium contains rapidly metabolizable carbon sources such as glucose. Taken together, the results suggested that $CcpA_{Bt}$ and $YvoA_{Bt}$ act as negative regulators of *chiB* in Bti75.

Western blotting indicates that YvoA and CcpA repress the expression of chitinase B. To detect the expression of *chiB* in Bti75 and its mutant strains at the protein level, Western blotting was performed. As shown in Fig. 7A, there was significantly more

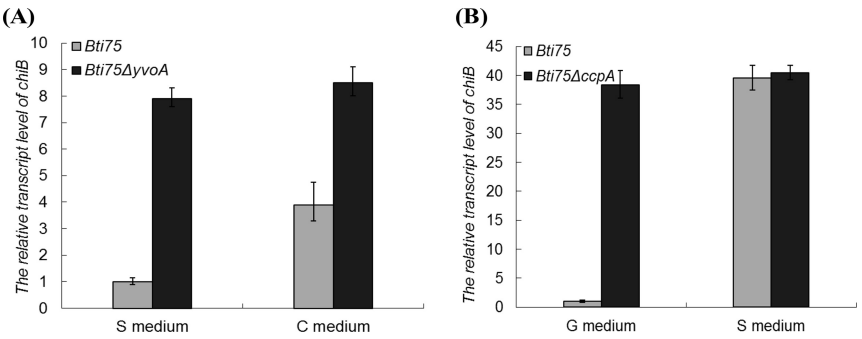

FIG 6 qRT-PCR analysis of the relative transcript levels of *chiB* genes of different strains in different media. (A) Relative transcript levels of the *chiB* genes of Bti75 and Bti75Δ*yvoA* in S medium (S minimal medium) and C medium (S medium plus 0.5% colloidal chitin). (B) Relative transcript levels of the *chiB* genes of Bti75 and Bti75Δ*ccpA* in G medium (S medium plus 0.5% glucose) and S medium.

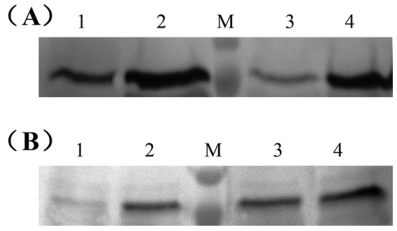

FIG 7 Western blot analysis to determine the expression levels of *chiB* in Bti75 and its mutants. (A) Expression levels of the *chiB* genes of Bti75 and Bti75Δ*yvoA* in C medium and S medium. Lanes 1 and 2, Bti75 and Bti75Δ*yvoA* in C medium, respectively; lanes 3 and 4, Bti75 and Bti75Δ*yvoA* in S medium, respectively. (B) Expression levels of the *chiB* genes of Bti75 and Bti75Δ*ccpA* in G medium and S medium. Lanes 1 and 2, Bti75 and Bti75Δ*ccpA* in G medium, respectively; lanes 3 and 4, Bti75 and Bti75Δ*ccpA* in S medium, respectively. M is the molecular mass marker, which shows molecular masses of 70 kDa and 100 kDa.

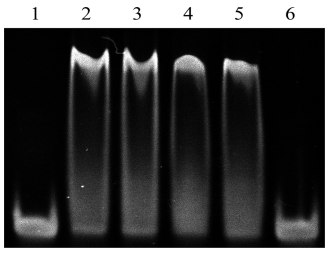

FIG 8 EMSAs to identify the inhibitor of binding of $YvoA_{Bt}$ to dre_{chiB}. Pdre (0.1 μM) was electrophoresed alone (lane 1) and after incubation with 0.4 μM $YvoA_{Bt}$ plus a final concentration of 100 mM Glc (lane 2), GlcNAc (lane 3), Glc-6-P (lane 4), GlcNAc-6-P (lane 5), or GlcN-6-P (lane 6).

ChiB in Bti75Δ*yvoA* than in the parental strain in both C medium and S medium, while the amounts of ChiB in Bti75Δ*yvoA* in C and S media were similar (Fig. 7A, lanes 2 and 4). For Bti75, there was a clear increase in the level of ChiB in C medium compared to that in S medium (Fig. 7A, lanes 1 and 3). All these results are consistent with the qRT-PCR results for Bti75 and Bti75Δ*yvoA* in C and S media.

On the other hand, as shown in Fig. 7B, there were roughly equal amounts of ChiB in Bti75 and Bti75Δ*ccpA* in C medium without glucose (lanes 3 and 4); however, in G medium, the level of the ChiB protein in Bti75 was almost undetectable (lane 1), and the amount of ChiB in Bti75Δ*ccpA* was similar to those in Bti75 and Bti75Δ*ccpA* in C medium (lane 2). These results were also consistent with the qRT-PCR results for Bti75 and Bti75Δ*ccpA* in G and C media. Thus, the Western blot results suggested that $YvoA_{Bt}$ and $CcpA_{Bt}$ repressed the expression of *chiB* in Bti75.

GlcN-6-P is an effector for YvoA. To investigate the possible effectors for $YvoA_{Bt}$, five different sugars (Glc, GlcNAc, Glc-6-P, GlcNAc-6-P, and GlcN-6-P) were incubated separately with Pdre and purified $YvoA_{Bt}$ for EMSAs; the final concentration of sugar was 100 mM. The EMSA results in Fig. 8 showed that only GlcN-6-P could abolish the DNA binding capability of $YvoA_{Bt}$; the other sugars had no effect on the binding of $YvoA_{Bt}$ to Pdre. This result is in agreement with data from effector analyses of YvoA in *B. subtilis* (19) and DasR in *S. coelicolor* (16).

DISCUSSION

We confirmed that *chiB* is negatively controlled by $YvoA_{Bt}$ and $CcpA_{Bt}$ in Bti75. There have been some reports about *cis*-acting elements and regulatory mechanisms of the genes related to chitin metabolism in *Streptomyces* and *Bacillus*. Titgemeyer and collaborators demonstrated DasR, which is a pleiotropic transcription factor, regulates chitin uptake and GlcNAc utilization (17), antibiotic synthesis (18), and morphological differentiation (16) in *S. coelicolor*. However, Bertram et al. found only two genes (*nagA* and *nagP*) that were directly repressed by YvoA. Thus, those authors thought that YvoA might be a less-prominent regulator than DasR and may control only the uptake and subsequent utilization of GlcNAc in *B. subtilis* (19). Consequently, they suggested that YvoA be renamed NagR.

In this work, we showed that *chiB* was negatively controlled by $YvoA_{Bt}$ in Bti75, based on the combination of *in silico*, *in vitro*, and *in vivo* data. Our results also confirmed the speculation of Xie et al. that a sequence-specific DNA binding factor of strain Bti75 could bind to the *dre* sequence for the inducible expression of *chiB* (21). Moreover, we found that *nagA* and *nagP* of Bti75 contain a 16-bp *dre*-like sequence within their upstream regions that is also directly repressed by $YvoA_{Bt}$ (data not shown). To identify further possible $YvoA_{Bt}$ binding sites in the genome of *B. thuringiensis* HD-789, we used the PREDetector software program (33) to identify corresponding16-bp *dre*-like sequence of *nagA*, *nagP*, and *chiB*. The data are listed in Data Set S2 in the supplemental material. Unlike NagR in *B. subtilis*, the genes controlled by $YvoA_{Bt}$ are not limited to *nagP* and the *nagAB-yvoA* operon. Besides *chiB*, the chitin binding protein gene also contains a *dre*-like site (AGTTG GCTAGTCATCT) within its upstream region. Furthermore, the results of *in vivo* and *in vitro* experiments indicated that the chitin binding protein gene was also negatively controlled by $YvoA_{Bt}$ (data not shown). The chitin binding protein is believed to facilitate microbial attachment to chitin and act synergistically with chitinases for chitin degradation. Thus, we predicted that $YvoA_{Bt}$ might be a more prominent regulator than $YvoA_{Bs}$. In addition to GlcNAc uptake and utilization, it also regulates genes involved in the chitin degradation pathway, such as the chitinase gene and the chitin binding protein gene. As to whether it can regulate other genes, further experiments are required.

Rigali et al. proved that GlcN-6-P was the inducing signal for DasR by using EMSAs (16). Resch et al. predicted that the effector of YvoA was GlcNAc-6-P, based on ITC data for *B. subtilis* (20). However, Bertram et al. incubated YvoA with dre_{nagA} in the presence of four different amino sugar compounds (GlcNAc, GlcNAc-6-P, GlcN-6-P, and GlcN) and found that only GlcN-6-P could abolish YvoA's binding to dre_{nagA} (19). Moreover, using DNase I footprinting, Gaugué et al. failed to detect any displacement of NagR from its *dre* binding sites by the amino sugar compounds that they used, whereas those authors state that under the same conditions, GlcN-6-P behaved as the inducing signal for the NagR homolog GamR (34). Recently, Fillenberg et al. produced crystallographic structures of NagR with the putative effector molecules GlcN-6-P and GlcNAc-6-P, implying that both of them are inducing signals for NagR in *B. subtilis* (35). However, we found that only GlcN-6-P could abolish the DNA binding capability of $YvoA_{Bt}$ among the five sugar compounds by EMSAs. Since $YvoA_{Bt}$ has ~45% sequence identity to $YvoA_{Bs}$, one could speculate that different inducing signals might be used. Besides this, the different methods and technologies that we used may yield inconsistent results. Elucidation of the inducing signal for $YvoA_{Bt}$ will require additional experiments.

Heravi et al. speculated that the chitinase gene (*chiS*) of *Bacillus pumilus* is under the control of CCR (36). Generally speaking, in low-GC Gram-positive bacteria such as *B. subtilis*, the key regulator for exerting CCR is CcpA. With the help of Hpr-Ser_{46}-P, CcpA can specifically bind to target promoters at *cre* sites (23, 26). The *cre* consensus sequence is a 14-bp *cis*-activating, partially palindromic sequence, TGWAARCGYTWNCW, in *B. subtilis* (23), which is similar to the *cre* sequences in other microorganisms. Moreover, CcpA may act as either a repressor or an activator, depending on the relative positions of the *cre* sequences in the genes (37). Hpr is a small phosphocarrier protein (~10 kDa), which is regarded as the central component of the PTS (phosphoenol pyruvate:carbohydrate phosphotransferase system), encoded by *pstH* (38). It can be phosphorylated on the His_{15} residue by enzyme I (EI) of the PTS during sugar uptake. On the other hand, Hpr-Ser_{46}-P is produced by ATP-dependent Hpr kinase/phosphatase (HprK/P) in response to high intracellular concentrations of glycolytic intermediates (26). However, Khan et al. observed that the conserved His_{15} and Ser_{46} residues of Hpr were shifted by one amino acid to positions 14 and 45, respectively, in *B. thuringiensis* subsp. *israelensis* (38). In this study, we also found a similar amino acid sequence of Hpr in Bti75. In addition, Reizer et al. showed that neither Hpr-His_{15}-P nor Hpr-(Ser_{46}-P)-(His_{15}-P) could bind CcpA to function in CCR (39). HprK/P is a bifunctional enzyme that presents kinase activity at high levels of ATP and FBP, whereas if the concentration of inorganic phosphate is high under starvation conditions, HprK/P will transform into a phosphorylase and dephosphorylate Hpr-Ser_{46}-P into Hpr (40).

In the present study, we found that $CcpA_{Bt}$ could bind to nucleotide sequences with increasing concentrations and had a higher affinity for long nucleotide sequences than for short ones. We proved that $CcpA_{Bt}$ binds nonspecifically at high concentrations. The results confirmed that only with the help of Hpr-Ser_{45}-P could $CcpA_{Bt}$ bind specifically to the *cre* site of the *chiB* promoter. Our findings are consistent with the generally accepted mode of binding of CcpA to *cre* sites. However, there are also several reports of specific DNA binding of CcpA to target sequences without cofactors (41–45), which is different from our data. Hammar et al. proposed that a transcription factor achieves specific binding to its targets by sliding along the DNA through nonspecific binding to specific binding sites (46). This hypothesis may explain the nonspecific binding of CcpA with the nucleotide sequence in some respects.

In addition, we found that HprK/P phosphorylated Hpr efficiently in reaction buffer with 2 mM ATP in the absence of FBP, which is slightly different from data in previous reports (22, 23, 30). Thus, we predicted that ATP might be a key element for HprK/P to function. When the concentration of ATP reaches a certain threshold, HprK/P could act on Hpr. FBP may act as an auxiliary factor to lower the threshold concentration of ATP for HprK/P to function; thus, HprK/P can phosphorylate Hpr at a relatively low concentration of ATP. Of course, this hypothesis requires further experimental support.

We propose the following more detailed model for the regulation of the inducible expression of *chiB* in Bti75. (i) When the strain is cultured in the presence of both rapidly metabolizable carbon (such as glucose) and chitin or chitooligosaccharides that can be degraded into the inducer of YvoA, YvoA is displaced from the *dre* site of *chiB* by the inducer, while CcpA still binds to the *cre* site of *chiB* and blocks transcriptional elongation with the help of Hpr-Ser_{45}-P. (ii) When the culture contains rapidly metabolizable carbon sources but not the inducer, the expression of *chiB* is repressed by both $CcpA_{Bt}$ and $YvoA_{Bt}$. (iii) Transcription of *chiB* progresses when the strain is cultured in the presence of the inducer but not rapidly metabolizable carbon sources that displace $CcpA_{Bt}$ and $YvoA_{Bt}$ from the promoter. (iv) When the culture lacks both rapidly metabolizable carbon sources and an inducer, $CcpA_{Bt}$ dissociates from the promoter, but $YvoA_{Bt}$ remains at the *dre* site and continues to repress the expression of *chiB*.

TABLE 3 Genes that have *dre*-like and *cre*-like sequences in their promoters in Bti75

Gene(s)	*dre*-like sequence	*cre*-like sequence[a]
chiB	AGACATCACGAAGTCT	TGTAAACGCTTTAT
chiA	ATACATCTAGACAACT	–
nagP	ACACATCTATACAACT	AGAAAGCGTTTTCT
nagAB-yvoA	GCACGAGTAGTTGTCT	–
Chitin binding protein gene	AGTTGGCTAGTCATCT	–

[a] –, not found.

Many Bt strains can generate more than one chitinase (4–7). In our previous work, we also found another chitinase gene (*chiA*) in Bti75 (47). We found that there is also a 16-bp sequence (ATACATCTAGACAACT) (dre_{chiA}), which is similar to $dre_{Bacillus}$ downstream of the core promoter region of *chiA*. Also, disruption of this sequence resulted in the constitutive expression of *chiA*, and the site also appears in Data Set S2 in the supplemental material, so we speculate that $YvoA_{Bt}$ also participates in the regulation of *chiA* in Bti75. It is interesting to note that we did not find a *cre*-like sequence in the promoter of *chiA* according to Data Set S1 in the supplemental material. This suggests that different chitinase genes may have different regulatory mechanisms. At the same time, we also list other genes that we have proven or predicted to be regulated by $YvoA_{Bt}$ to show whether these genes have demonstrated or predicted *cre* sites according to Data Sets S1 and S2 in the supplemental material (Table 3). We found that some genes have both *dre*-like and *cre*-like sites in their promoters. This automatically raises the question of whether $YvoA_{Bt}$ and $CcpA_{Bt}$ are also involved in the regulation of other genes simultaneously. A definitive answer awaits additional experimental evidence.

This work demonstrates that *chiB* of *B. thuringiensis* is regulated by $YvoA_{Bt}$ and $CcpA_{Bt}$. Research on the regulatory mechanism of chitinase will permit better utilization of bacterial chitinase. Liu et al. demonstrated that the chitinase produced by *B. thuringiensis* improved its insecticidal activity (9). Furthermore, the roles of CcpA and YvoA have been studied in some detail in *B. subtilis* compared to *B. thuringiensis*. Therefore, the regulation mechanism of $CcpA_{Bt}$ and $YvoA_{Bt}$ in *B. thuringiensis* requires further investigation.

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REFERENCES

1. **Khoushab F, Yamabhai M.** 2010. Chitin research revisited. Mar Drugs **8:**1988–2012. http://dx.doi.org/10.3390/md8071988.

2. **Felse PA, Panda T.** 1999. Regulation and cloning of microbial chitinase genes. Appl Microbiol Biotechnol **51:**141–151. http://dx.doi.org/10.1007/s002530051374.
3. **Flach J, Pilet PE, Jollès P.** 1992. What's new in chitinase research? Experientia **48:**701–716. http://dx.doi.org/10.1007/BF02124285.
4. **Thamthiankul S, Suan-Ngay S, Tantimavanich S, Panbangred W.** 2001. Chitinase from *Bacillus thuringiensis* subsp. *pakistani*. Appl Microbiol Biotechnol **56:**395–401. http://dx.doi.org/10.1007/s002530100630.
5. **Driss F, Kallassy-Awad M, Zouari N, Jaoua S.** 2005. Molecular characterization of a novel chitinase from *Bacillus thuringiensis* subsp. *kurstaki*. J Appl Microbiol **99:**945–953. http://dx.doi.org/10.1111/j.1365-2672.2005.02639.x.
6. **Barboza-Corona JE, Reyes-Rios DM, Salcedo-Hernández R, Bideshi DK.** 2008. Molecular and biochemical characterization of an endochitinase (ChiA-HD73) from *Bacillus thuringiensis* subsp. *kurstaki* HD-73. Mol Biotechnol **39:**29–37. http://dx.doi.org/10.1007/s12033-007-9025-4.
7. **Gomaa EZ.** 2012. Chitinase production by *Bacillus thuringiensis* and *Bacillus licheniformis*: their potential in antifungal biocontrol. J Microbiol **50:**103–111. http://dx.doi.org/10.1007/s12275-012-1343-y.
8. **Xiao L, Liu C, Xie CC, Cai J, Chen YH.** 2012. The direct repeat sequence upstream of *Bacillus* chitinase genes is *cis*-acting elements that negatively regulate heterologous expression in *E. coli*. Enzyme Microb Technol **50:**280–286. http://dx.doi.org/10.1016/j.enzmictec.2012.02.001.
9. **Liu D, Cai J, Xie C, Liu C, Chen Y.** 2010. Purification and partial characterization of a 36-kDa chitinase from *Bacillus thuringiensis* subsp. *colmeri*, and its biocontrol potential. Enzyme Microb Technol **46:**252–256. http://dx.doi.org/10.1016/j.enzmictec.2009.10.007.
10. **Hu SB, Liu P, Ding XZ, Yan L, Sun YJ, Zhang YM, Li WP, Xia LQ.** 2009. Efficient constitutive expression of chitinase in the mother cell of *Bacillus thuringiensis* and its potential to enhance the toxicity of Cry1Ac protoxin. Appl Microbiol Biotechnol **82:**1157–1167. http://dx.doi.org/10.1007/s00253-009-1910-2.
11. **Bhattacharya D, Nagpure A, Gupta RK.** 2007. Bacterial chitinases: properties and potential. Crit Rev Biotechnol **27:**21–28. http://dx.doi.org/10.1080/07388550601168223.
12. **Babashpour S, Aminzadeh S, Farrokhi N, Karkhane A, Haghbeen K.** 2012. Characterization of a chitinase (Chit62) from *Serratia marcescens* B4A and its efficacy as a bioshield against plant fungal pathogens. Biochem Genet **50:**722–735. http://dx.doi.org/10.1007/s10528-012-9515-3.
13. **Chandrasekaran R, Revathi K, Nisha S, Kirubakaran SA, Sathish-Narayanan S, Senthil-Nathan S.** 2012. Physiological effect of chitinase purified from *Bacillus subtilis* against the tobacco cutworm *Spodoptera litura* Fab. Pestic Biochem Physiol **104:**65–71. http://dx.doi.org/10.1016/j.pestbp.2012.07.002.
14. **Ozgen A, Sezen K, Demir I, Demirbag Z, Nalcacioglu R.** 2013. Molecular characterization of chitinase genes from a local isolate of *Serratia marcescens* and their contribution to the insecticidal activity of *Bacillus thuringiensis* strains. Curr Microbiol **67:**499–504. http://dx.doi.org/10.1007/s00284-013-0395-5.
15. **Hu S, Zhang X, Li Y, Ding X, Hu X, Yang Q, Xia L.** 2013. Constructing *Bacillus thuringiensis* strain that co-expresses Cry2Aa and chitinase. Biotechnol Lett **35:**1045–1051. http://dx.doi.org/10.1007/s10529-013-1171-0.
16. **Rigali S, Nothaft H, Noens EE, Schlicht M, Colson S, Müller M, Joris B, Koerten HK, Hopwood DA, Titgemeyer F, van Wezel GP.** 2006. The sugar phosphotransferase system of *Streptomyces coelicolor* is regulated by the GntR-family regulator DasR and links *N*-acetylglucosamine metabolism to the control of development. Mol Microbiol **61:**1237–1251. http://dx.doi.org/10.1111/j.1365-2958.2006.05319.x.
17. **Colson S, Stephan J, Hertrich T, Saito A, van Wezel GP, Titgemeyer F, Rigali S.** 2007. Conserved *cis*-acting elements upstream of genes composing the chitinolytic system of streptomycetes are DasR-responsive elements. J Mol Microbiol Biotechnol **12:**60–66. http://dx.doi.org/10.1159/000096460.
18. **Rigali S, Titgemeyer F, Barends S, Mulder S, Thomae AW, Hopwood DA, van Wezel GP.** 2008. Feast or famine: the global regulator DasR links nutrient stress to antibiotic production by *Streptomyces*. EMBO Rep **9:**670–675. http://dx.doi.org/10.1038/embor.2008.83.
19. **Bertram R, Rigali S, Wood N, Lulko AT, Kuipers OP, Titgemeyer F.** 2011. Regulon of the *N*-acetylglucosamine utilization regulator NagR in *Bacillus subtilis*. J Bacteriol **193:**3525–3536. http://dx.doi.org/10.1128/JB.00264-11.
20. **Resch M, Schiltz E, Titgemeyer F, Muller YA.** 2010. Insight into the induction mechanism of the GntR/HutC bacterial transcription regulator YvoA. Nucleic Acids Res **38:**2485–2497. http://dx.doi.org/10.1093/nar/gkp1191.
21. **Xie CC, Shi J, Jia HY, Li PF, Luo Y, Cai J, Chen YH.** 2015. Characterization of regulatory regions involved in the inducible expression of *chiB* in *Bacillus thuringiensis*. Arch Microbiol **197:**53–63. http://dx.doi.org/10.1007/s00203-014-1054-3.
22. **Fujita Y.** 2009. Carbon catabolite control of the metabolic network in *Bacillus subtilis*. Biosci Biotechnol Biochem **73:**245–259. http://dx.doi.org/10.1271/bbb.80479.
23. **Stülke J, Hillen W.** 2000. Regulation of carbon catabolism in *Bacillus* species. Annu Rev Microbiol **54:**849–880. http://dx.doi.org/10.1146/annurev.micro.54.1.849.
24. **Brückner R, Titgemeyer F.** 2002. Carbon catabolite repression in bacteria: choice of the carbon source and autoregulatory limitation of sugar utilization. FEMS Microbiol Lett **209:**141–148. http://dx.doi.org/10.1111/j.1574-6968.2002.tb11123.x.
25. **Göerke B, Stuelke J.** 2008. Carbon catabolite repression in bacteria: many ways to make the most out of nutrients. Nat Rev Microbiol **6:**613–624. http://dx.doi.org/10.1038/nrmicro1932.
26. **Deutscher J, Francke C, Postma PW.** 2006. How phosphotransferase system-related protein phosphorylation regulates carbohydrate metabolism in bacteria. Microbiol Mol Biol Rev **70:**939–1031. http://dx.doi.org/10.1128/MMBR.00024-06.
27. **Schumacher MA, Allen GS, Diel M, Seidel G, Hillen W, Brennan RG.** 2004. Structural basis for allosteric control of the transcription regulator CcpA by the phosphoprotein HPr-Ser46-P. Cell **118:**731–741. http://dx.doi.org/10.1016/j.cell.2004.08.027.
28. **Kraus A, Hueck C, Gärtner D, Hillen W.** 1994. Catabolite repression of the *Bacillus subtilis xyl* operon involves a *cis* element functional in the context of an unrelated sequence, and glucose exerts additional *xylR*-dependent repression. J Bacteriol **176:**1738–1745.
29. **Lecadet MM, Chaufaux J, Ribier J, Lereclus D.** 1992. Construction of novel *Bacillus thuringiensis* strains with different insecticidal activities by transduction and transformation. Appl Environ Microbiol **58:**840–849.
30. **Kravanja M, Engelmann R, Dossonnet V, Blüggel M, Meyer HE, Frank R, Galinier A, Deutscher J, Schnell N, Hengstenberg G.** 1999. The *hprK* gene of *Enterococcus faecalis* encodes a novel bifunctional enzyme: the HPr kinase/phosphatase. Mol Microbiol **31:**59–66. http://dx.doi.org/10.1046/j.1365-2958.1999.01146.x.
31. **Livak KJ, Schmittgen TD.** 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods **25:**402–408. http://dx.doi.org/10.1006/meth.2001.1262.
32. **Wang Z, Yang X, Chu X, Zhang J, Zhou H, Shen Y, Long J.** 2012. The structural basis for the oligomerization of the N-terminal domain of SATB1. Nucleic Acids Res **40:**4193–4202. http://dx.doi.org/10.1093/nar/gkr1284.
33. **Hiard S, Marée R, Colson S, Hoskisson PA, Titgemeyer F, van Wezel GP, Joris B, Wehenkel L, Rigali S.** 2007. PREDetector: a new tool to identify regulatory elements in bacterial genomes. Biochem Biophys Res Commun **357:**861–864. http://dx.doi.org/10.1016/j.bbrc.2007.03.180.
34. **Gaugué I, Oberto J, Plumbridge J.** 2014. Regulation of amino sugar utilization in Bacillus subtilis by the GntR family regulators, NagR and GamR. Mol Microbiol **92:**100–115. http://dx.doi.org/10.1111/mmi.12544.
35. **Fillenberg SB, Grau FC, Seidel G, Muller YA.** 2015. Structural insight into operator *dre*-sites recognition and effector binding in the GntR/HutC transcription regulator NagR. Nucleic Acids Res **43:**1283–1296. http://dx.doi.org/10.1093/nar/gku1374.
36. **Heravi KM, Shali A, Naghibzadeh N, Ahmadian G.** 2014. Characterization of *cis*-acting elements residing in the chitinase promoter of *Bacillus pumilus* SG2. World J Microbiol Biotechnol **30:**1491–1499. http://dx.doi.org/10.1007/s11274-013-1569-9.
37. **Sonenshein AL.** 2007. Control of key metabolic intersections in *Bacillus subtilis*. Nat Rev Microbiol **5:**917–927. http://dx.doi.org/10.1038/nrmicro1772.
38. **Khan SR, Deutscher J, Vishwakarma RA, Monedero V, Bhatnagar NB.** 2001. The *ptsH* gene from *Bacillus thuringiensis israelensis*—characterization of a new phosphorylation site on the protein HPr. Eur J Biochem **268:**521–530. http://dx.doi.org/10.1046/j.1432-1327.2001.01878.x.
39. **Reizer J, Bergstedt U, Galinier A, Küester E, Saier MH, Jr, Hillen W, Steinmetz M, Deutscher J.** 1996. Catabolite repression resistance of *gnt*

operon expression in *Bacillus subtilis* conferred by mutation of His-15, the site of phosphoenolpyruvate-dependent phosphorylation of the phosphocarrier protein HPr. J Bacteriol **178:**5480–5486.

40. **Dossonnet V, Monedero V, Zagorec M, Galinier A, Pérez-Martínez G, Deutscher J.** 2000. Phosphorylation of HPr by the bifunctional HPr kinase/P-Ser-HPr phosphatase from *Lactobacillus casei* controls catabolite repression and inducer exclusion but not inducer expulsion. J Bacteriol **182:**2582–2590. http://dx.doi.org/10.1128/JB.182.9.2582-2590.2000.
41. **Moir-Blais TR, Grundy FJ, Henkin TM.** 2001. Transcriptional activation of the *Bacillus subtilis ackA* promoter requires sequences upstream of the CcpA binding site. J Bacteriol **183:**2389–2393. http://dx.doi.org/10.1128/JB.183.7.2389-2393.2001.
42. **Kim HJ, Roux A, Sonenshein AL.** 2002. Direct and indirect roles of CcpA in regulation of *Bacillus subtilis* Krebs cycle genes. Mol Microbiol **45:**179–190. http://dx.doi.org/10.1046/j.1365-2958.2002.03003.x.
43. **Puri-Taneja A, Schau M, Chen Y, Hulett FM.** 2007. Regulators of the *Bacillus subtilis cydABCD* operon: identification of a negative regulator, CcpA, and a positive regulator, ResD. J Bacteriol **189:**3348–3358. http://dx.doi.org/10.1128/JB.00050-07.
44. **Puri-Taneja A, Paul S, Chen Y, Hulett FM.** 2006. CcpA causes repression of the *phoPR* promoter through a novel transcription start site, $P_{(A6)}$. J Bacteriol **188:**1266–1278. http://dx.doi.org/10.1128/JB.188.4.1266-1278.2006.
45. **Ishii H, Tanaka T, Ogura M.** 2013. The *Bacillus subtilis* response regulator gene *degU* is positively regulated by CcpA and by catabolite-repressed synthesis of ClpC. J Bacteriol **195:**193–201. http://dx.doi.org/10.1128/JB.01881-12.
46. **Hammar P, Leroy P, Mahmutovic A, Marklund EG, Berg OG, Elf J.** 2012. The *lac* repressor displays facilitated diffusion in living cells. Science **336:**1595–1598. http://dx.doi.org/10.1126/science.1221648.
47. **Xie CC, Luo Y, Chen YH, Cai J.** 2012. Construction of a promoter-probe vector for *Bacillus thuringiensis*: the identification of *cis*-acting elements of the *chiA* locus. Curr Microbiol **64:**492–500. http://dx.doi.org/10.1007/s00284-012-0100-0.