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Identification, Characterization, and Regulation of a Novel Antifungal Chitosanase Gene (*cho*) in *Anabaena* spp.▽

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Two contrasting cyanobacterial species (*Anabaena fertilissima* and *Anabaena sphaerica*) were selected based on differences in antifungal behavior in order to study the mechanism for production of an antifungal enzyme and the genes responsible for this production. In *A. fertilissima*, chitosanase and antifungal activities were increased significantly under of growth-limiting conditions (8 of light and 16 h of darkness). The lack of such activities in *A. sphaerica* was associated with high levels of protein that accumulated during the stationary phase (at 28 days) under the same light conditions. The gene putatively responsible for chitosanase and antifungal activities was amplified using specific primers, and sequence analysis of the amplified products (1.086 and 1.101 kb in *A. sphaerica* and *A. fertilissima*, respectively) showed that they belong to the glycoside hydrolase 3 (GH3)-like family of *Anabaena variabilis* ATCC 29413. Pairwise alignment of the corresponding protein sequences identified a putative signal peptide (amino acids 1 to 23) and some amino acid changes in the sequence of *A. fertilissima* which may be responsible for functioning of the chitosanase and the observed antifungal activity. Hydrolysis of the chitosan oligosaccharide $(GlcN)_5$ to $(GlcN)_2$ and $(GlcN)_3$ confirmed the presence of chitosanase activity in *A. fertilissima*. Site-directed mutagenesis of the *A. fertilissima* chitosanase-encoding gene (*cho*) led to identification of catalytic residues (Glu-121 and Glu-141) important for the antifungal effect of the *cho* product. The level of expression of *cho* was monitored by quantitative real-time reverse transcription-PCR, which indicated that transcription of this gene is significantly enhanced under conditions that retard growth, such as a long dark period.

Cyanobacteria comprise a heterogeneous assemblage of photosynthetic prokaryotes having extraordinary biosynthetic potential and a repertoire of diverse metabolic activities. They are an important source of novel antifungal, antibacterial, and herbicidal or weedicidal compounds, which have been implicated in allelopathic interactions in water and soil (29). A majority of these metabolites are biologically active and are products of either nonribosomal polypeptide (NRP) or mixed polyketide-NRP biosynthetic pathways. The toxins produced by cyanobacteria are greatly influenced by various physiological and environmental factors, including light, temperature, nutrients, and pH (5).

The antifungal properties of cyanobacterial metabolites, most of which have not been exploited, have immense potential in agriculture for use against fungal plant pathogens. In bacteria, lytic enzymes, such as chitinases, chitosanases, proteases, and β-1,3-glucanases, are known to have key roles in biocontrol of various soilborne fungal pathogens. Chitin is a linear polymer of 1,4-linked *N*-acetyl-D-glucosamine (GlcNAc) residues, and the deacetylated derivative of chitin is chitosan. In contrast to chitin, chitosan has been found in very few organisms, but it has been found in members of the Zygomycota, such as *Mucor rouxii* (42), *Absidia coerulea* (22), and *Rhizopus oryzae* (8). The filamentous fungi *Lentinus edodes* and *Pleurotus sajo-caju* were investigated to determine their abilities to produce chitosan (30). Chitosan and chitosan-glucan complexes have been found in the mycelia of *Aspergillus niger*, *Humicola lutea*, and *Fusarium moniliforme* (39). Chitosanases (EC 3.2.1.132) produced by bacteria are classified into five glycoside hydrolase (GH) families (families 5, 8, 46, 75, and 80) (6, 10, 11, 12, 13). Families 5 and 8 are composed of enzymes that are hydrolytic with glycosides, and the family 46, 75, and 80 enzymes studied so far are chitosanases. This classification of chitosanases is based on amino acid sequence similarities of the catalytic domains. Recently, the family 46 chitosanase of *Amycolatopsis* sp. CsO-2 responsible for antifungal activity against *Rhizopus oryzae* was characterized (33). A comparison of the digestion of the cell wall of *Fusarium oxysporum* by a chitosanase and the digestion of the cell wall of *F. oxysporum* by a chitinase showed that *Bacillus pumilus* chitosanase is more effective than *Streptomyces griseus* chitinase (7). The *csnSM1* gene encoding an extracellular chitosanase has been identified in *Sphingobacterium multivorum*, which suppresses mycelial growth of *F. oxysporum* (21).

Our previous investigation revealed the activities of hydrolytic enzymes in several *Anabaena* strains and correlation of these activities with antifungal activity (31). In the present study, we characterized a putative antifungal gene (*cho*) encoding a chitosanase belonging to the glycosyl hydrolase 3-like family, and its regulation was studied under different environmental conditions.

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MATERIALS AND METHODS

Organism and growth conditions. Isolates belonging to two *Anabaena* species (*Anabaena fertilissima* RPAN1 and *Anabaena sphaerica* RPAN12) that have different antifungal activities (31) were used in this investigation; these isolates

FIG. 1. Positions of primers used in this study. A conserved primer region (Cho748F and Cho1692R) was used for amplification of a GH3-like gene encoding a chitosanase in *A. sphaerica* and *A. fertilissima*. The specific primer regions of ChoF1 and ChoR1 (used to isolate the putative gene encoding chitosanase) and ChoF3 and ChoF3 (used for qRT-PCR) are indicated. The conserved and designed primer regions shown are regions from the deduced nucleotide sequence (1,425 bp) of *A. fertilissima*, and the same primer regions are conserved in the deduced nucleotide sequence (1,410 bp) of *A. sphaerica*. Uppercase letters indicate the putative gene. The underlined sequence and X indicate the putative signal peptide and cleavage site, respectively. The start and stop codons are enclosed in boxes.

are part of the germplasm of *Anabaena* isolates from diverse agro-ecological areas of India (25). The phytopathogenic fungus *F. oxysporum*, which causes diseases of various crops (tomato, chili, pepper, brinzal, red gram, ginger, linseed, etc.), was used to evaluate antifungal activity and was obtained from the Indian Type Culture Collection, Division of Plant Pathology, IARI, New Delhi, India. Both *Anabaena* isolates were axenized using the method described by Rippka (32). They were grown and maintained in BG11 medium at 27 ± 1°C with a cycle consisting of 16 h of light and 8 h of darkness (16-h light/8-h dark cycle). The intensity of white light used was 50 to 55 μmol photons m^{-2} s^{-2} (38). The fungal strain was grown on potato dextrose agar and was maintained at 28 ± 2°C in a biological oxygen demand (BOD) incubator.

Biochemical characterization. Fourteen-, 28-, and 42-day-old cultures of both *Anabaena* species were grown independently under different light-dark conditions, including continuous light (CL), continuous darkness (CD), an 8-h light/16-h dark cycle, and a 16-h light/8-h dark cycle. The 16-h light/8-h dark cycle was treated as a control (C). For all treatments except the control, samples were collected at the end of the photoperiod; for the control, sampling was done at three critical time points, the middle of the 16-h light phase (C-MP), the end of the 16-h light phase (C-LP), and the end of the 8-h dark phase (C-DP). Extracellular filtrates of all samples were obtained by centrifugation at 4,000 × *g* for 5 min. Ten percent inocula of 20- to 25-day-old cultures were used for all experiments. The amount of protein in an extracellular filtrate was determined spectrophotometrically as described by Herbert et al. (14), using bovine serum albumin (BSA) as a standard. Chitosanase activity was analyzed by estimating the amount of reducing ends of sugars using the dinitrosalicylic acid method (24). The standard assay mixture was prepared by mixing of 0.5 ml of enzyme solution (extracellular filtrate) and 0.5 ml of glycol chitosan (1%, pH 6.0), which was incubated for 1 h at 37°C to allow completion of hydrolysis; 0.5 ml of dinitrosalicylic acid reagent was added, and the resulting mixture was boiled for 15 min, chilled, and centrifuged to isolate the insoluble chitosan. The resulting adducts of reducing sugars were analyzed and measured spectrophotometrically at 540 nm. One unit of chitosanase activity was defined as the amount of enzyme that produced 1 μmol of reducing sugars (expressed as glucosamine equivalents) per min under the assay conditions. The antifungal activity was evaluated by a disk diffusion assay using extracellular filtrates of *Anabaena* species (31). The fungus *F. oxysporum* was inoculated onto a potato dextrose agar plate and incubated for 3 to 4 days to obtain a uniform lawn. Sterilized 5-mm-diameter filter paper disks that were soaked in 50 μl of extracellular filtrates were placed on uniform fungal lawns, and zones of inhibition were measured after 3 to 4 days of incubation at 30°C (15).

DNA isolation and PCR amplification of chitosanase-encoding gene. Genomic DNA was extracted using an Ultraclean plant DNA isolation kit (MoBio Inc., Carlsbad, CA). The concentrations in the reaction mixture, primer sets, and thermal cycler conditions used for amplification of the gene coding for chitosanase were those described by Yun et al. (43). Specific primers ChoF1 (5′-ATG CCAGCATTGCAGAGAC-3′) and ChoR1 (5′-TTAAAAACAACAAGCGAT CGCC-3′) were designed to amplify the full-length chitosanase-encoding gene from both *Anabaena* species (Fig. 1).

Cloning and purification of chitosanase. Both amplified PCR products were purified using a Qiagen gel extraction kit (Qiagen) and were cloned into the pGEM-T Easy vector (Promega) and then transformed into *Escherichia coli* host strain DH5α. Automated DNA sequencing was performed using M13 primers (Axygen, India). After sequence analyses, both of the purified PCR products containing the chitosanase gene were subcloned into the pIVEX glutathione *S*-transferase (GST) fusion vector (Roche), and transformation was carried out using *E. coli* strain JM109(DE3) (Promega), which has an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible T7 polymerase gene for expression of the GST-chitosanase recombinant protein. Blunt-end cloning into the StuI site was performed according to the manufacturer's instructions. For this cloning, forward primer ChoF2-(P) (5′-ATGCCAGCATTGCAGAGACT-3′) was phosphorylated from the start codon, and reverse primer ChoR2-(5′-**CGAGCAG**CCCG GGTTAAAAACAACAAGC-3′) contained an XmaI recognition sequence (underlined) with 6 to 8 additional bases (bold type) after the stop codon. Sequencing was done using commercial primers. *E. coli* JM109 (DE3) cells harboring pIVEX-*cho* were induced to overexpress the chitosanase gene with 0.1 mM IPTG at the mid-exponential growth phase and incubated for 3 h at 37°C. Cells were harvested by centrifugation (6,000 × *g* for 15 min at 4°C), washed with 1× phosphate-buffered saline (PBS) (diluted 10× PBS, 1.4 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4; pH 7.3), and then resuspended in 1× PBS. The cells were disrupted by sonication, and a supernatant was obtained by centrifugation (13,000 × *g* for 30 min at 4°C). The crude extract (2 ml) was loaded onto a GSTrap FF column (Amersham Pharmacia Biotech), equilibrated with 1× PBS, and eluted with 10 mM reduced glutathione in 50 mM Tris-HCl (pH 7.5) at a flow rate of 1 ml/min. The eluted fractions were dialyzed overnight against factor Xa cleavage buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM $CaCl_2$; pH 7.5) and concentrated by using Amicon Ultra-4 filters (Millipore). The

TABLE 1. Synthetic oligonucleotide primers used for site-directed mutagenesis

Mutation	Oligonucleotide[a]		Change
	Direction	Sequence (5′ → 3′)	
E22D	F	5′ CTGGGTATTTCTGGTACTGA**C**TTGAGTGATGAAGATAAAC 3′	GAG → GAC
	R	5′ GTTTATCTTCATCACTCAA**G**TCAGTACCAGAAATACCCAG 3′	
L68F	F	5′ CATAGCCAAATACAATT**C**GAATATGCAGAACGCG 3′	TTG → TTC
	R	5′ CGCGTTCTGCATATTC**G**AATTGTATTTGGCTATG 3′	
E121D	F	5′ GAATTAAAATCTGA**C**GGCATCAACTTATC 3′	GAG → GAC
	R	5′ GATAAGTTGATGCC**G**TCAGATTTTAATTC 3′	
E141D	F	5′ CGCAAAATCCGGA**C**ATCGGTTCTCGC 3′	GAG → GAC
	R	5′ GCGAGAACCGAT**G**TCCGGATTTTGCG 3′	
L161F	F	5′ GGTGCGCGGGAATT**C**TATTATTTGGGACTG 3′	TTG → TTC
	R	5′ CAGTCCCAAATAATA**G**AATTCCCGCGCACC 3′	
Q211H	F	5′ GAACTTATCCCCTTCCA**C**AAAGCTTTGATTGAAG 3′	CAG → CAC
	R	5′ CTTCAATCAAAGCTTT**G**TGGAAGGGGATAAGTTC 3′	
Q221E	F	5′ GAAGAAGGGATTCCC**G**AGCTCATCATGACC 3′	CAG → GAG
	R	5′ GGTCATGATGAGCT**C**GGGAATCCCTTCTTC 3′	
Q244E	F	5′ GCTACCCTATCCCGC**G**AGCCCATCCTCAAAAC 3′	CAG → GAG
	R	5′ GTTTTGAGGATGGGCT**C**GCGGGATAGGGTAGC 3′	

[a] Bold type indicates mutant sites. F, forward; R, reverse.

purified fusion protein was digested with 80 U of factor Xa solution for 12 h at 5°C to remove the GST region, and then the digested fusion protein was loaded onto a GSTrap FF column chromatography as described above. The unbound fraction was collected and used as a purified enzyme. The purified Cho proteins from both *Anabaena* species were separated on 12.5% polyacrylamide gels using 0.1% sodium dodecyl sulfate (SDS) (17) and were visualized by staining with Coomassie brilliant blue (CBB) R-250.

Enzymatic hydrolysis of chitosan oligosaccharides. The degradation of the chitosan oligosaccharide $(GlcN)_5$ (a synthetic substrate purchased from Sigma Aldrich) by the purified Cho proteins of both *Anabaena* species was measured using high-performance liquid chromatography (HPLC). The $(GlcN)_5$ substrate was dissolved in 10 mM phosphate buffer (pH 7.0) to obtain a 15 mM solution. Purified Cho protein (0.5 to 1.5 μg) was added to the substrate solution for each enzymatic reaction, and then the reaction mixture was incubated at 30°C for 3 h. The reaction was stopped by boiling the mixture for 5 min. Reaction products were analyzed on a μBondapak NH_2 column (8 by 100 mm; Waters) with acetonitrile-water (65:35) as the solvent system using a 2414 differential refractive index detector (Waters) at room temperature and a flow rate of 2 ml/min. Degradation of $(GlcN)_5$ was evaluated by comparing peak areas in the HPLC profiles using standard curves obtained for pure saccharide solutions.

Site-directed mutagenesis. *E. coli* mutant *cho* clones were generated using the same expression vector (pIVEX GST fusion), and competent cells were generated to evaluate the functional sites responsible for antifungal activity using a QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. Eight amino acids (seven amino acids in the mature protein and one amino acid in the signal peptide) in the Cho protein of *A. fertilissima* were changed independently to other amino acids (GAG-to-GAC change for E22D, E121D, and E121D; TTG-to-TTC change for L68F and L161F; CAG-to-CAC change for Q211H; and CAG-to-GAG change for Q221E and Q244E). The oligonucleotide primers used for site-directed mutagenesis were designed with the Primer X software (Table 1). The mutant clones were sequenced to verify the desired mutations. All of the *E. coli* clones carrying different inserts, such as wild-type (as a positive control) and mutant forms of *A. fertilissima cho* and wild-type *A. sphaerica cho*, were independently induced overnight using 0.4 mM IPTG in LB broth. Simultaneously, an insert-free vector-transformed *E. coli* strain was also maintained, which served as a negative control. Antifungal activity (expressed as the size of the zone of inhibition) was measured by growing *E. coli* cultures overnight and using them directly for disk diffusion assays, as described above.

Real-time qRT-PCR. The levels of expression of *cho* with different times in the dark (14-h light/10-h dark, 12-h light/12-h dark, 10-h light/14-h dark, and 8-h light/16-h dark cycles) were measured to evaluate the environmental conditions required for maximum chitosanase production. Both *Anabaena* species were grown under the light-dark conditions described above. C-MP was used as a control for measuring the fold difference in expression as the length of the dark period in the light-dark cycle was increased. DNA-free intact RNA (10 μg) was isolated from a 28-day-old culture for each sample separately (Tri-reagent; Sigma) and then subjected to cDNA synthesis using a Stratagene High-Fidelity first-strand cDNA synthesis kit according to the manufacturer's instructions. A two-step reverse transcription-PCR (RT-PCR) was used to avoid the problem of primer dimer formation (4). Primers ChoF3 (5′-GAGAACCGATAATCGGAT TTTGC-3′) and ChoR3 (5′-CAGCCAAACCTCGTAAGGTACACC-3′) were designed for quantitative RT-PCR (qRT-PCR) (Fig. 1). qRT-PCR was carried out with a Corbett rotor gene 3000, using standard conditions with an annealing temperature of 55°C. The RT-PCR product was detected using SyBr green. The bacterial 16S rRNA gene was used as a reference gene for qRT-PCR.

Bioinformatics and statistics. The data for protein, chitosanase, and antifungal activity determined in triplicate for all treatments and conditions were subjected to an analysis of variance (ANOVA) in accordance with the experimental design (completely randomized block design) using the MSTAT-C statistical package to quantify and evaluate the source of variation. BLASTN and BLASTP were used to identify nucleotide identity and amino acid sequence similarity, respectively (1). Open reading frames (ORFs) were identified using GENDB (23) and GENMARK (19). Nucleotide and amino acid sequences were aligned using Clustal W2 (18). A predicted signal peptide and cleavage site in the translated sequence were determined using Neural Network and hidden Markov model (HMM) algorithms (2). Ratios of relative *cho* expression were calculated using the method of Pfaffl (27). Statistical analyses to identify significant differences from the control were performed using REST software (28).

Nucleotide sequence accession numbers. The nucleotide sequences described in this study have been deposited in the GenBank database under accession numbers GQ241347 (*A. fertilissima*) and GU126473 (*A. sphaerica*).

RESULTS

Measurement of chitosanase and antifungal activities in different growth phases and under different light-dark conditions. *A. fertilissima* showed detectable chitosanase and antifungal activities after all growth periods (14, 28, and 42 days), and the maximum activities were observed at 28 days (Fig. 2A and B). In contrast, *A. sphaerica* did not show any chitosanase or antifungal activity (data not shown). Further, in 28-day-old cultures of *A. fertilissima*, the chitosanase activity was 19 and 95% greater in a C-DP culture and a culture grown with 8 of light and 16 h of darkness, respectively, than in a C-MP culture (Fig. 2A). The increase in the chitosanase activity was also associated with higher antifungal activity under similar treatment conditions, as determined by measuring zones of inhibition. The largest zone of inhibition (18 mm) was recorded for 28-day-old cultures grown with 8 h of light and 16 h of darkness. Nystatin (100 U) was used as a positive control, for which

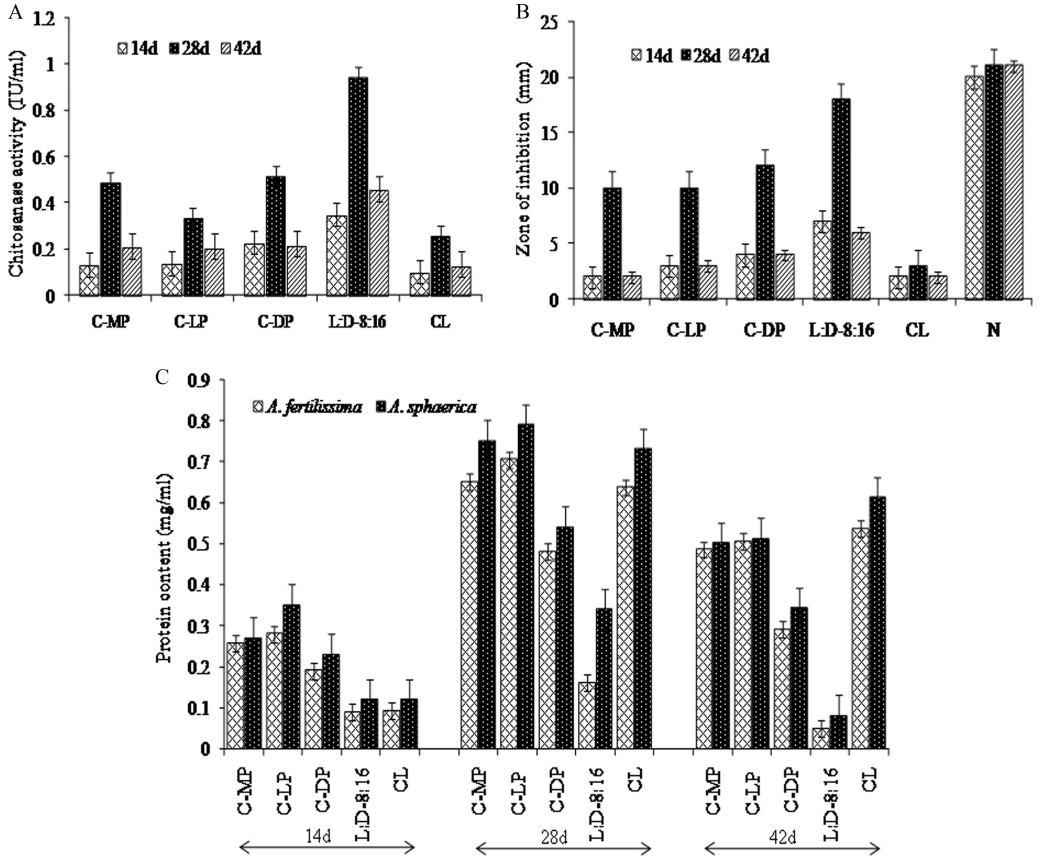

FIG. 2. Total protein contents and chitosanase and antifungal activities of 14-, 28-, and 42-day-old cultures incubated under different light-dark conditions. (A and B) Chitosanase activities and antifungal activities (expressed as the size of the zone of inhibition for *F. oxysporum*) in *A. fertilissima*. (C) Comparison of the total protein contents of *A. fertilissima* and *A. sphaerica*. C-MP (middle of the light phase), C-LP (end of the 16-h light phase), and C-DP (end of the 8-h dark phase) indicate three different controls for different time points during the 16-light/8-h dark cycle. CL indicates continuous light treatment, and N indicates treatment with nystatin (100 U) (positive control for antifungal activity). The differences between the means were statistically significant at a P value of <0.01 in a one-way ANOVA test. L:D-8:16, cycle consisting of 8 h of light and 16 h of darkness.

the average zone of inhibition with *F. oxysporum* was 20 ± 1 mm wide (Fig. 2B). With the CL treatment, the chitosanase and antifungal activities were up to 48 and 70% greater, respectively, than the activities in the C-MP culture (Fig. 2A and B), while no growth was observed with the CD treatment (data not shown). In general, a strong correlation was observed between the chitosanase and antifungal activities; however, in some cases, increased chitosanase activity was not accompanied by increased antifungal activity. For instance, at 28 days, the chitosanase activity in the C-LP culture was 32% less than the chitosanase activity in the C-MP culture, but there was no significant difference in the antifungal activities. The comparative analysis of protein accumulation data showed that the protein content was only slightly greater for all treatments and controls in *A. sphaerica* than in *A. fertilissima*; the only exception was the treatment consisting of 8 h of light and 16 h of darkness for 28 days, where the protein content was 50% greater, which was a significant difference (Fig. 2C).

Isolation and sequence analysis of putative chitosanase-encoding (*cho*) gene. The *cho* gene was amplified from the two *Anabaena* species having different antifungal activities using chitosanase-specific primers (43) in order to characterize this gene, which is responsible for antifungal activity. Amplified PCR products that were 1,410 bp and 1,425 bp long were obtained from *A. sphaerica* and *A. fertilissima*, respectively. Sequence analysis revealed full-length genes consisting of 1,086 bp (*A. sphaerica*) and 1,101 bp (*A. fertilissima*), which were isolated by designing specific primers using the start and stop codons (Fig. 1). BLASTN analysis revealed 100% (*A. sphaerica*) and 97% (*A. fertilissima*) identity with a glycoside hydrolase family 3-like (GH3-like) gene of *A. variabilis* strain ATCC 29413. A pairwise amino acid sequence alignment revealed 5 insertions and 5 substitutions in the amino acid sequence of *A. fertilissima* compared to that of *A. sphaerica* (Fig. 3). Open reading frames encoding 362 and 367 amino acids with predicted molecular masses of 40 kDa and 40.6 kDa were observed in *A. sphaerica* and *A. fertilissima*, respectively. The N-terminal region of the deduced amino acid sequence of the *A. fertilissima* protein had a putative peptide signal that was 23 residues long, MPALQRLERFGIVLILGISGTEL*SDE DKRA (the asterisk indicates the postulated cleavage site), whereas neither a signal peptide nor a cleavage site was detected in the *A. sphaerica* protein (data not shown). The puta-

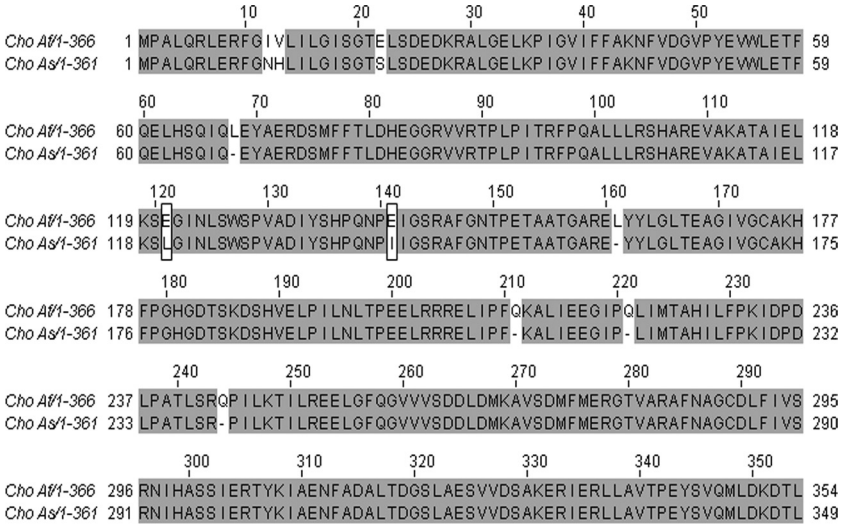

FIG. 3. Alignment of amino acid sequences of ChoAf (*A. fertilissima*) and ChoAs (*A. sphaerica*). Shading indicates conserved residues; a lack of shading indicates amino acid changes. Boxes indicate important amino acid changes (E-121 and E-141) in Cho of *A. fertilissima*.

tive gene identified was referred to as *cho*, which encodes chitosanase.

HPLC-based determination of the chitosanase activities of isolated Cho proteins. SDS-PAGE analysis revealed 40.6- and 40.0-kDa Cho proteins in *A. fertilissima* and *A. sphaerica*, respectively (Fig. 4). To evaluate the functional chitosanase, the purified Cho proteins were tested to determine their abilities to hydrolyze the chitosan oligosaccharide $(GlcN)_5$. The products of the enzymatic hydrolysis, $(GlcN)_2$ and $(GlcN)_3$, were quantified by HPLC. The loss of the $(GlcN)_5$ peak along with the simultaneous presence of $(GlcN)_2$- and $(GlcN)_3$-specific peaks clearly suggested that the Cho protein in *A. fertilissima* can hydrolyze this chitopentose. In contrast, only the $(GlcN)_5$-specific peak was found in *A. sphaerica*, which indicated that there was no chitosanase activity (Fig. 5).

Identification of catalytic residues of chitosanase responsible for antifungal activity. In order to study the amino acid residues essential for functioning of Cho in *A. fertilissima*, a few amino acid residues were changed to other residues by site-directed mutagenesis, and the *E. coli* clones generated were

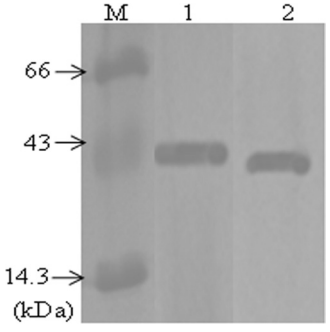

FIG. 4. Cho protein subjected to SDS-PAGE. Lane 1, purified Cho protein from *A. fertilissima*; lane 2, purified Cho protein from *A. sphaerica*; lane M, protein size markers. The sizes of the protein markers are indicated on the left. The gels were stained with Coomassie brilliant blue R-250.

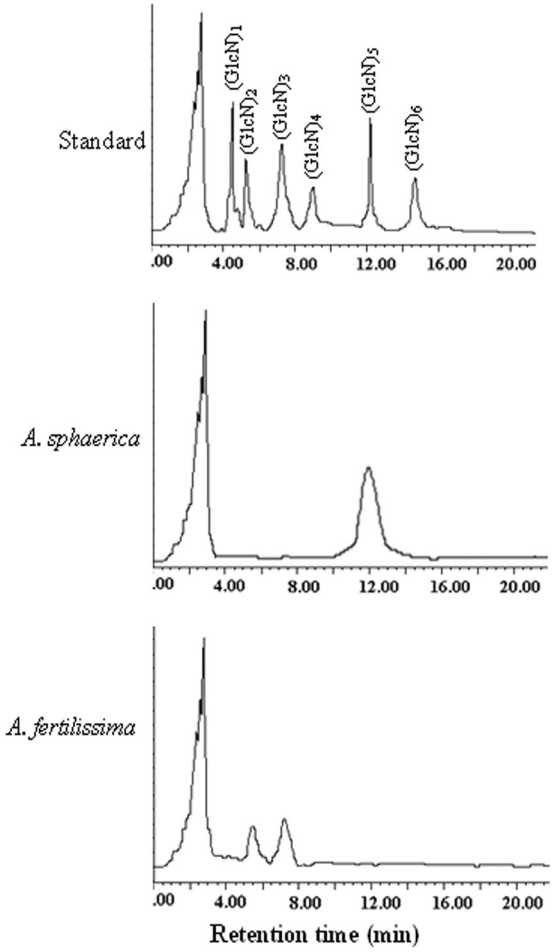

FIG. 5. High-performance liquid chromatography (HPLC) analysis results showing degradation of a synthetic chitopentamer by the Cho protein of *A. fertilissima*.

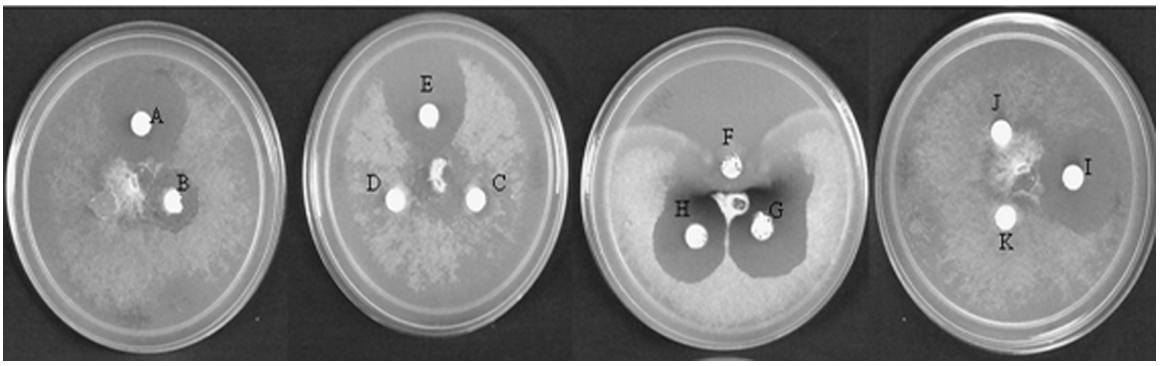

FIG. 6. Identification of catalytic residues of chitosanase of *A. fertilissima* by site-directed mutagenesis. *E. coli* clones harboring mutations in the *cho* gene of *A. fertilissima* were used. A, L68E mutant; B, E22D mutant; C, E121D mutant; D, E141D mutant; E, L161E mutant; F, Q211E mutant; G, Q221E mutant; H, Q244D mutant; I, wild-type *A. fertilissima cho* strain (positive control); J, wild-type *A. sphaerica cho* strain; K, insert-free vector-transformed *E. coli* (negative control).

tested to determine their antifungal activities. The results of the antifungal activity analysis (expressed as the size of the zone of inhibition) of *E. coli* mutant *cho* clones revealed that there was no zone of inhibition around colonies of the E121D and E141D mutants and that the zone of inhibition around colonies of the E22D mutant was somewhat reduced. The sizes of the inhibition zones for the other mutants (L68E, L161E, Q211E, Q221E, and Q244D) were similar to that for the *A. fertilissima* wild-type *cho* strain (positive control). No inhibition zone was observed for *E. coli* clones transformed with the insert-free vector (negative control) and the *A. sphaerica* wild-type *cho* strain (Fig. 6).

Expression profile for *cho* with increasing periods of darkness. The expression profile of *cho* was found to be dependent on the length of the dark period, and 0.15-, 0.25-, and 0.35-fold increases in expression were observed for the treatments consisting of 14 h of light and 10 h of darkness, 12 h of light and 12 h of darkness, and 10 of light and 14 h of darkness, respectively, compared to the control (C-MP). Further, the highest level of expression was observed for the treatment consisting of 8 h of light and 16 h of darkness, which indicated that these conditions are the most favorable conditions for expression of chitosanase and antifungal activities. The level of *cho* expression was almost unchanged in *A. sphaerica* when the time in the dark was increased (Fig. 7).

DISCUSSION

Cyanobacteria are a special group of Gram-negative bacteria. They are phototrophic prokaryotes with a short generation time, can be easily handled, and have metabolic flexibility. These characteristics make them favorite model organisms for examining several metabolic processes in order to obtain a greater understanding of them (34). However, until now, no study had been conducted to reveal the antifungal activities of cyanobacteria at either the biochemical or molecular level. Toward this end, our previous investigation showed the potential role of hydrolytic enzymes in these organisms and demonstrated their role in biocidal activity against phytopathogenic fungi (31). In the current study, we used two contrasting species, *A. fertilissima* and *A. sphaerica*, which have different antifungal activities. Further, in order to determine the mechanism behind the different hydrolytic activities, the chitosanase and antifungal activities of these two *Anabaena* species in different growth phases (14, 28, and 42 days) were measured, and light-dark conditions with three different controls subjected to the normal photoperiod (16 h of light and 8 h of darkness) were examined for both *Anabaena* species. The time-dependent chitosanase and antifungal activities in three different controls (C-MP, C-LP, and C-DP) indicated that in *A. fertilissima* both of these activities were dependent on the dark phase and were maximal at the end of the 8-h dark phase in the C-DP culture. This shows that as the dark phase begins to increase, the corresponding chitosanase and antifungal activities also begin to increase. This was confirmed by evaluating both the chitosanase and antifungal activities with a long dark period (8 h of light and 16 h of darkness), and both of these activities were found to be maximal under these conditions and during the stationary phase (28 days). This finding is also supported by the observations of Volk (40), who showed that an antimicrobial compound accumulates during the stationary phase in

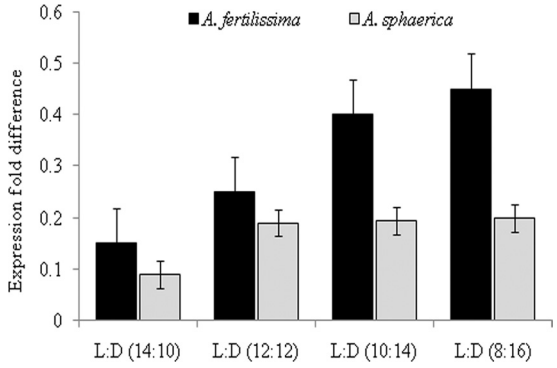

FIG. 7. Real-time expression profiles for *cho* from *A. fertilissima* and *A. sphaerica*. Expression was compared to that of the control (C-MP) (0 on the *y* axis) with different dark periods during the light-dark cycle. The bars indicate the means of three technical and three biological replicates. The differences in the means were found to be statistically significant at a P value of <0.01 by using a one-way ANOVA test. L:D (14:10), 14 h of light and 10 h of darkness; L:D (12:12), 12 h of light and 12 h of darkness; L:D (10:14), 10 h of light and 14 h of darkness; L:D (8:16), 8 h of light and 16 h of darkness.

Nostoc insulare. The production of this compound during the stationary phase indicated that its secretion somehow supported the last phase of growth. Sivonen (37) investigated the effect of light on the growth of and hepatotoxin production by *Oscillatoria agardhi* strains and found that two of the three toxic cultures produced high levels of toxins under low light intensity. Thus, these observations collectively suggested that the antifungal activity of *A. fertilissima* is associated with growth retardation. Large amounts of two exogenous organic carbon sources which prolong survival in the dark, fructose and glucose, are probably not present in this adverse environment. Thus, the biochemical nature of extracellular filtrates may change, and exogenous enzymes (like chitosanase) may be released so that the organism can survive under these stress conditions. The limited growth in the dark is supported by metabolism of photosynthetically derived glycogen produced and stored during the preceding light period. However, in some cases, such as 28 days for C-MP and C-LP cultures, a direct correlation between chitosanase and antifungal activities was not observed, which indicated that apart from chitosanase activity there are other factors which together regulate the antifungal activity. However, detailed investigation of this possibility is required to gain further insight. The higher level of protein in *A. sphaerica* than in *A. fertilissima* in the dark phase during the treatment consisting of 8 of light and 16 h of darkness (particularly at 28 days) suggested that an increase in the length of the dark phase leads to greater growth retardation, which acts as a physiological signal to increase the chitosanase and antifungal activities in *A. fertilissima*. The reduction in the photosynthetic efficiency in the dark phase might also be responsible for these activities. However, detailed investigations are required to determine other physiological conditions that regulate the chitosanase and antifungal activities.

The initial biochemical data suggested that the antifungal activity observed in *A. fertilissima* could be due to the chitosanase activity. This suggestion is supported by the findings of Marcotte et al. (20), who performed an X-ray analysis of an antifungal chitosanase from *Streptomyces* N174 and proved that it is responsible for the antifungal activity of this organism. Recently, Saito et al. (33) also characterized a chitosanase gene, *ctoA*, which is responsible for antifungal activity in *Amycolatopsis* sp. CsO-2. Yun et al. (43) described a set of specific primers to detect new strains for production of chitosanases similar to chitosanase A of *Mitsuaria chitosanitabida*. In the current study, we used the same set of primers to amplify a chitosanase-encoding gene from two *Anabaena* species. Analysis of the proteins encoded by the amplified PCR products showed that they were similar to glycoside hydrolase family 3-like (GH3-like) N-terminal domain proteins of *A. variabilis* strain ATCC 29413. The GH3 family N-terminal domain proteins (EC 3.2.1) include enzymes with known functions, such as beta-glucosidase (EC 3.2.1.21), beta-xylosidase (EC 3.2.1.37), *N*-acetyl-beta-glucosaminidase (EC 3.2.1.52), glucan beta-1,3-glucosidase (EC 3.2.1.58), cellodextrinase (EC 3.2.1.74), and exo-1,3-1,4-glucanase (EC 3.2.1). On the basis of these results, we hypothesized that the chitosanase-encoding gene (*cho*) that we amplified from the *Anabaena* species could encode a member of the GH3-like family. The corresponding amino acid sequences encoded by the amplified products from both species were not similar to bacterial family 46, 75, and 80 chitosanases but showed a high degree of similarity (33%) with the sequence of beta-*N*-hexosaminidase (PDB accession no. 3BMX) from *Bacillus subtilis* and 32% similarity with beta-*N*-hexosaminidase (PDB accession no. 1TR9) from *Vibrio cholerae*. Incidentally, these enzymes in both organisms also belong to the GH3 protein family (PFAM accession no. PF00933). Thus, our results showed that the primers designed for a GH80 family chitosanase by Yun et al. (43) with little degeneracy can be used to amplify a GH3-like chitosanase-encoding gene of *Anabaena* (Fig. 1). This conclusion is supported by a multiple-sequence alignment which revealed significant similarities between *cho* and reference sequences of *Flavobacterium* sp., *Herbaspirillum* sp., and *M. chitosanitabida*, which were amplified using the same *choA* primers (43) (data not shown). Moreover, the genome of *A. variabilis* strain ATCC 29423 has been sequenced (GenBank accession no. CP000117), and neither GH80 nor GH3 family proteins are present in this genus, as inferred from the NCBI database. Based on biochemical assays and molecular and bioinformatics analyses, we concluded that the *A. fertilissima cho* gene encodes a member of the GH3-like family and is responsible for the observed chitosanase and antifungal activities.

The presence of a *cho* gene encoding functional chitosanases in both *Anabaena* species was further confirmed by isolation of specific Cho proteins, followed by enzymatic hydrolysis of the chitosan oligosaccharide $(GlcN)_5$ (a synthetic substrate for chitosanase). The data obtained from HPLC analyses of the Cho protein from *A. fertilissima* revealed its ability to degrade $(GlcN)_5$ into $(GlcN)_2$ and $(GlcN)_3$. These results were similar to the results of previous investigations (35, 36). Thus, they provide direct evidence for the presence of an active chitosanase in *A. fertilissima* and further support our biochemical, molecular, and bioinformatics results.

Additionally, site-directed mutagenesis was also performed to identify the critical residues responsible for chitosanase and antifungal activities. *E. coli* mutant clones were constructed on the basis of an amino acid sequence comparison with Cho proteins of *Anabaena* species. In our study, recombinant *E. coli* cells carrying the wild-type or mutant forms of *cho* were directly spotted onto fungal lawns, and the antifungal activity was expressed as the size of the zone of inhibition. This method is a modification of a previously described method in which *E. coli* cells harboring a plasmid were spotted on agar plates containing a chitosan substrate, such as glycol or colloidal chitosan (16, 26). Although this method is suitable for screening *E. coli* clones for antifungal activity, the exact mechanism responsible for the release of a recombinant protein from the intact cells is still unknown. One probable explanation is the presence of a signal peptide that may allow secretion of chitosanase outside the periplasm of *E. coli*. Also, the *E. coli* cells may be lysed by *F. oxysporum* to release the proteins. However, further investigation is required to reveal the exact mechanism. The data obtained from site-directed mutagenesis revealed that Glu-121 and Glu-141 are essential for the antifungal activity of *A. fertilissima*. The absence of these residues in *A. sphaerica* supported this hypothesis (Fig. 3). The importance of glutamic and aspartic acid residues in regulation of the activity of chitinases and chitosanases was also proposed in previous reports (3, 9, 33, 41). The catalytic residues of *A. fertilissima* were also found to be similar to those of the previously char-

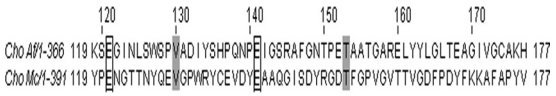

FIG. 8. Conserved catalytic amino acid residues of Cho in *M. chitosanitabidus* (ChoMc) (GenBank accession no. AB010493) and Cho of *A. fertilissima* (ChoAf). Shading indicates conserved amino acids, and the two conserved and catalytic amino acid residues (E121 and E141) are enclosed in boxes.

acterized chitosanase A (ChoA) of *Matsuebacter chitosanotabidus* 3001 (35) (Fig. 8); this further supported the hypothesis that the Glu-121 and Glu-141 residues are important in regulation of the chitosanase and antifungal activities.

Along the same lines, the expression profile of *cho* was also analyzed when cultures were subjected to increasing dark periods by using quantitative real-time RT-PCR (qRT-PCR), which revealed its environmental regulation as the time in the dark increased. As shown by the biochemical analysis, the chitosanase and antifungal activities were found to be increased with long periods of darkness (8 h of light and 16 h of darkness). This finding was validated by the finding that the level of expression of *cho* in *A. fertilissima* also increased significantly when a long dark phase was used. Thus, the increased expression of *cho* under conditions that imposed growth constraints further supported the hypothesis that the function of this gene is to encode a chitosanase that is responsible for antifungal activity. In contrast, in *A. sphaerica* the levels of expression of *cho* were almost the same under different light-dark conditions; this indicates that the regulatory mechanism which controls the expression and activity of chitosanase was not present. However, the level of expression of *cho* in all treatments was higher than that in the control (C-MP); irrespective of this, corresponding chitosanase and antifungal activities were not found. This further indicates that the lack of functional residues may be responsible for the absence of chitosanase and antifungal activities in *A. sphaerica*.

In conclusion, we characterized a novel *cho* gene encoding a chitosanase associated with the GH3-like family and showed that it is responsible for the antifungal activity in *A. fertilissima*. Two catalytic residues (Glu-121 and Glu-141) that play an essential role in the antifungal activity of Cho in *A. fertilissima* were also identified. A study conducted under different physiological conditions showed that the antifungal activity is enhanced significantly in an adverse environment. This study opens up new avenues to search for the detailed mechanism responsible for the antifungal activity in cyanobacteria.

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