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LysR-type Transcriptional Regulator ChiR Is Essential for Production of All Chitinases and a Chitin-Binding Protein, CBP21, in *Serratia marcescens* 2170

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To identify the genes required for chitinase production by *Serratia marcescens* 2170, various Tn*5* mutants somehow defective in chitinase production were isolated in a previous study. In order to identify the mutated gene in one of the chitinase-deficient mutants, N1, DNA regions flanking the Tn*5* insertion were cloned and sequenced. Sequence comparison showed that the mutation occurred in the ORF located between *chiB* and *cbp*, which encode chitinase B and chitin-binding protein CBP21, respectively. The ORF encodes a 313-amino acid polypeptide which has significant similarity with various LysR-type transcriptional regulators, and thus the gene was designated *chiR*. Targeted mutagenesis confirmed that disruption of the *chiR* gene results in the phenotype of N1. Gel mobility shift assays using partially purified ChiR protein demonstrated that this protein specifically binds to the intergenic region between *chiR* and *cbp*. These results strongly suggest that ChiR is a LysR-type transcriptional regulator which is essential for production of all chitinases and CBP21.

Key words: *Serratia marcescens*; LysR-type transcriptional regulator; chitinase; chitin binding protein

Serratia marcescens is an efficient biological degrader of chitin and one of the most extensively studied chitinolytic bacteria. The *chiA* and *chiB* genes encoding chitinases A and B of four *S. marcescens* strains, QMB1466, BJL200, KCTC2172, and 2170, have been cloned and sequenced.[1–7] Perrakis *et al.* reported the three-dimensional structure of chitinase A from one of the strains, QMB1466, several years ago.[8] In addition to *chiA* and *chiB*, the nucleotide sequence of a third chitinase gene, *chiC*, encoding chitinase C1 of strain 2170[9] and the corresponding chitinase gene encoding a 52-kDa chitinase of strain KCTC2172[10] have recently been reported.

To start studies on the chitinase system of *S. marcescens*, we chose strain 2170, since this strain is amenable to genetic analysis. When *S. marcescens* 2170 was grown in the presence of chitin as a sole carbon source, four chitinases, A, B, C1, and C2, and a chitin-binding protein of 21 kDa (CBP21) were detected in the culture supernatant as the major proteins.[7,11] CBP21 is a protein which binds to chitin but does not have hydrolyzing activity. This protein is produced only under the conditions under which chitinases are produced. Therefore, coordinate regulation of the expression of CBP21 with those of chitinases was suggested. The gene for CBP21 (*cbp*) was found in a region 1.5 kb downstream of the *chiB* gene. Chitin-binding proteins similar to CBP21 have been reported to be produced by many *Streptomyces* species.[12,13] Chitinase C1 consists of a catalytic domain, a fibronectin type III-like domain, and a C-terminal chitin-binding domain, and is the initial product of *chiC*. Chitinase C2 is a proteolytic derivative of chitinase C1 containing only the catalytic domain.

Production of multiple chitinases from different genes has been reported for many bacteria, including *Bacillus circulans*,[14] *S. marcescens*,[5–7] *Aeromonas* sp.,[16] *Alteromonas* sp.,[17] and *Streptomyces lividans*,[15] and efficient degradation of chitin is assumed to be achieved by the combined actions of the multiple chitinases. Most of the bacterial chitinases belong to family 18 in the classification of glycosyl hydrolases,[18] except for the family 19 chitinases commonly

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observed in *Streptomyces* species.[19] Bacterial family 18 chitinases are further classified into three subfamilies, A, B, and C, based on the amino acid sequence similarity of their catalytic domains, as we reported previously.[9] Chitinases produced by *S. marcescens* all belong to family 18, and chitinase C1 (and C2) belongs to subfamily B, whereas chitinases A and B belong to subfamily A. Coproduction of chitinases belonging to different subfamilies is also observed in several other chitinolytic bacteria, such as *B. circulans* WL-12,[14] *S. lividans*,[15] and *Aeromonas* sp.[16]

To identify the genes required for chitinase production by *S. marcescens* 2170, Tn*5* mutagenesis was done and various mutants somehow defective in chitinase production were isolated.[7] These mutants fall into five classes as judged by the appearance of clearing zones of colloidal chitin, namely, mutants with no clearing zones, large clearing zones, fuzzy clearing zones, delayed clearing zones, and small clearing zones. Among these classes, mutants with no clearing zones (N1–N6) seem to be most interesting since they may have the mutations in the genes essential for expression of all chitinase genes. Therefore, we first focused on the mutants of this class. In this report, we describe identification of the gene disrupted by Tn*5* insertion in mutant N1. This gene, designated *chiR*, encodes a LysR-type transcriptional activator essential for the expression of all chitinases and chitin-binding protein CBP21 of *S. marcescens* 2170.

Materials and Methods

Bacterial strains, plasmids, and culture conditions. *S. marcescens* 2170 was originally obtained from Prof. H. W. Ackermann (Department of Medical Biology, Faculty of Medicine, Laval University, Quebec, Canada). *S. marcescens* 2170 and its Tn*5* mutants (N1, N2, N3, N4, N5, and N6) were grown at 30°C with shaking in a yeast extract-supplemented minimal (YEM) medium containing 0.5% (wt/vol) concentrations of various carbon sources.[7] YEM agar plates containing 0.2% colloidal chitin (wt/vol) and 50 mg/ml of kanamycin were used to test chitinase production. *Escherichia coli* JM109 was used as the host organism and pUC119 as the vector for gene cloning. *E. coli* JM109 carrying pUC119 or its derivatives was grown in Luria-Bertani (LB) medium containing 100 μg/ml of ampicillin or 50 μg/ml of kanamycin. *E. coli* BL21(DE3) and pET-16b were used for overexpression of ChiR protein. *E. coli* strains S17-1 λpir and MC1061 λpir, and the plasmid pFS100 were used to create insertion mutations in the *chiR* gene.[20] *E. coli* S17-1 λpir and MC1061 λpir carrying pFS100 or its derivatives were grown at 30°C in LB medium containing 100 μg/ml ampicillin and/or 50 μg/ml kanamycin.

Enzyme and protein assays. Chitinase activity was measured by a modification of Schales' procedure,[21] with colloidal chitin as the assay substrate. One unit of chitinase activity was defined as the amount of enzyme that produces 1 μmol of reducing sugar per min. Protein concentration was measured by the method of Lowry *et al.*[22] using bovine serum albumin as the standard.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 12.5% slab gels was conducted as described by Ames[23] with the buffer system of Laemmli,[24] and detection of chitinase activity after renaturation of enzymes were performed as described previously.[25]

Cloning of the flanking DNA regions of inserted Tn5. Chromosomal DNA of mutant N1 was extracted from the cells as described by Silhavy *et al.*,[26] partially digested with *Sau*3AI, and separated on a 0.7% agarose gel. The gel segment corresponding to the sizes between 7 and 10 kb was cut out, and DNA fragments in the gel were recovered by using GENECLEAN II (Bio 101, Inc., Vista, CA, U.S.A.). The DNA fragments were ligated to *Bam*HI-digested pUC119 and used to transform *E. coli* JM109 cells. The transformants carrying the plasmids containing various sizes of flanking DNA regions of the inserted Tn*5* together with the kanamycin resistance gene were selected on LB agar plates containing 50 μg/ml of kanamycin.

Nucleotide sequence determination and sequence analysis. Inserted DNA fragments in the plasmids were sequenced with an automated laser fluorescence sequencer (Model 4000L; LI-COR, Lincoln, NE, U.S.A.). Sequencing reactions were done by using the ThermoSequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech) according to the supplier's instructions with a double-stranded template. Nucleotide sequence data were analyzed using the GENETYX system (Software Kaihatsu Co., Tokyo, Japan). The deduced amino acid sequence was compared with those available in the translated GenBank, the SWISS-PROT protein sequence data bank, the National Biomedical Research Foundation protein data bank, and the DDBJ.

Southern hybridization. Southern hybridization was performed by using the AlkPhos Direct (Amersham Pharmacia Biotech) as described by the manufacturer. Chromosomal DNAs of the mutant strains of *S. marcescens* 2170 were extracted from the cells as described by Silhavy *et al.*,[26] and were digested by restriction enzymes. The Km^r region of Tn*5*, which was amplified by PCR with primers Tn*5*KmF (5′-AAAGCTTCACGCTGCCGCAA-3′) and Tn*5*KmR

(5′-AGCAGCTGAACCAACTCGC-3′), was used as a hybridization probe.

Construction of a chiR disrupted mutant. Truncated *chiR* corresponding to a 351-bp internal region of the gene was amplified by PCR with primers RDel-F (5′-TAAACCCTGACGCAGGA-3′) and RDel-R (5′-TCATACCACCAACTGAC-3′) and chromosomal DNA from *S. marcescens* 2170 as a template. The amplified fragment was ligated with *Eco*RV-cut pFS100 to generate plasmid pFSΔCHIR. The plasmid pFSΔCHIR was introduced into *E. coli* S17-1 λpir by electroporation and then transferred from that strain to *S. marcescens* 2170 by conjugation, as previously described.[27] Transconjugants were selected on LB medium containing 50 μg/ml of kanamycin.

Overproduction and preparation of ChiR protein. The coding region of *chiR* was amplified by PCR using primers ChiR-F (5′-AGGAATTCATATGACTAGATTATCCCTGG-3′ [the *Nde*I site is underlined]) and ChiR-R (5′-CGGGATCCATCAGTTGTGGCACCAC-3′ [the *Bam*HI site is underlined]). The amplified fragment was first cloned into the vector pUC119, excised as an *Nde*I-*Bam*HI fragment, and ligated with *Nde*I- and *Bam*HI-digested pET-16b to generate the plasmid pCHIR. For production of ChiR protein, *E. coli* BL21(DE3) harboring pCHIR was grown in 100 ml of LB medium containing 100 μg/ml ampicillin at 30°C. When the $OD_{600\,nm}$ reached 0.6, 1 mM (final concentration) of isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture and cultivation was continued for another 3 h. Then cells were collected by centrifugation, washed twice with a buffer [30 mM Tris-HCl (pH 7.9) and 30 mM NaCl], resuspended in 20 ml of the same buffer, and disrupted by sonication with a Tomy ultrasonic disruptor model UR-200P. The lysate was centrifuged at 8,000 × *g* for 20 min and the pellet containing insoluble ChiR was resuspended in 100 ml of 1 M sucrose and centrifuged. The pellet was resuspended in 100 ml of 2% Triton X-100 and 10 mM EDTA, stored at 4°C overnight, and centrifuged. The resulting pellet was resuspended in a solubilization buffer containing 8 M urea, 30 mM NaCl, and 30 mM Tris-HCl (pH 7.9) and incubated at 37°C for 1 h to dissolve the ChiR protein. The solution was centrifuged at 8,000 × *g* for 20 min to remove insoluble materials and dialyzed against a series of buffers with stepwise decreases in the urea concentration [30 mM Tris-HCl (pH 7.9) containing 30 mM NaCl plus 6,4, 2, and 0 M urea] for 8 h at each urea concentration. Then the ChiR protein solution was dialyzed against binding buffer containing 10 mM HEPES-KOH (pH 7.9), 50 mM KCl, 1 mM EDTA (pH 8.0), 5 mM $MgCl_2$, 10% glycerol, 0.5 mM dithiothreitol (DTT), and 0.5 μM (*p*-amidinophenyl)methanesulfonyl fluoride (APMSF). At this stage, the precipitate formed in the solution was removed by centrifugation. The final preparation was divided into portions and stored at −20°C.

ChiR protein thus obtained had a histidine tag consisting of 10 histidine residues at its N-terminus.

N-terminal amino acid sequence analysis of purified ChiR. Polyacrylamide gel-purified ChiR was electroblotted from the gel onto a polyvinylidene difluoride membrane, as described by Matsudaira[28] and the N-terminal amino acid sequence was determined with a protein sequencer PPSQ-21 (Shimadzu Scientific Instruments & Equipment, Tokyo, Japan).

Gel mobility shift assay. PCR, to amplify the fragments corresponding to the upstream regions of the *chiA*, *chiB*, *chiC* genes and the intergenic region between the *cbp* and *chiR* genes, was carried out using the following oligonucleotide primers and the chromosomal DNA of *S. marcescens* 2170 as the template: ChiA-F (5′-CTGAAGAGTGTGGTGCAAT-3′) and ChiA-R (5′-CTGATTCCTTTATTCCGAGAG-3′) for the upstream region of *chiA*, ChiB-F (5′-ATTAAGCCAACAGCGTCAG-3′) and ChiB-R (5′-GATGTTTTTCAATGGGGGA-3′) for that of *chiB*, ChiC-F (5′-ACATTCTGGCGGGCTTC-3′) and ChiC-R (5′-TTCATTAGGGGGGAGAGTT-3′) for that of *chiC*, and CBP-F (5′-ATGACACTAAATTCCCAAGGTG-3′) and CBP-R (5′-AAATAACCCTTCTTGTCGGCTT-3′) for the intergenic region between *cbp* and *chiR*.

The amplified fragments were end-labeled with [γ-32P]ATP (3,000 Ci/mmol) by using T4 polynucleotide kinase and purified using a QIAquick PCR Purification Kit (Qiagen). The standard protein-DNA binding reaction mixture contained 20,000 cpm of labeled DNA, 0.12 μg/μl of poly(dI-dC) (Amersham Pharmacia Biotech), and 250 ng of purified ChiR protein in 10 μl of binding buffer [10 mM HEPES-KOH (pH 7.9), 50 mM KCl, 1 mM EDTA (pH 8.0), 10% glycerol, 5 mM DTT, and 0.5 μM APMSF]. After incubation for 30 min at room temperature, the samples were put onto a nondenaturating 6% polyacrylamide gel and electrophoresed in 10 mM Tris-HCl (pH 7.8)-9.3 mM sodium acetate-0.28 mM EDTA to separate protein-DNA complexes from the unbound labeled DNA. The location of radioactivity was analyzed by autoradiography with Kodak X-Omat AR film (Eastman Kodak, New Haven, CT, U.S.A.).

Chemicals. Colloidal chitin was prepared from powdered chitin purchased from Funakoshi Chemical Co. (Tokyo) following the methods described by Jeuniaux.[29] The chitooligosaccharide mixture [$(GlcNAc)_{3-4}$] was obtained from Pias Co. (Osaka, Japan). Restriction enzymes and modification enzymes were purchased from Takara shuzo (Osaka,

Japan), Toyobo Biochemicals (Osaka, Japan), and New England Biolabs (Beverly, MA, U.S.A.).

Results

Phenotype of chitinase-deficient mutant N1

To identify the genes involved in chitin utilization in *S. marcescens* 2170 other than structural chitinase genes, Tn*5* mutagenesis was carried out and various mutants which have certain defects in chitinase production were isolated as described previously.[7] Among them, six mutants, N1 through N6, did not form any clearing zone on YEM agar plates containing colloidal chitin. On the other hand, other extracellular enzymes besides chitinases such as proteases, lipases, and nucleases, appeared to be produced normally as judged by the indicator plates to detect production of these enzymes.[7] Therefore, the loss of the ability to form clearing zones on colloidal chitin is not due to defects in the machinery for extracellular enzyme secretion.

The chitinase-deficient phenotype of mutant N1 was also studied in broth media. Mutant N1 and wild-type strain were cultivated in YEM liquid medium containing colloidal chitin, $(GlcNAc)_{3-4}$, or glycerol, and chitinase activity in the culture supernatant was examined. The growth of mutant N1 in the medium containing $(GlcNAc)_{3-4}$ or glycerol was similar to that of wild-type 2170; however, mutant N1 did not grow well in the medium containing colloidal chitin. Wild-type strain normally produced chitinase activity in the medium containing colloidal chitin or $(GlcNAc)_{3-4}$, but did not in the medium containing glycerol. On the other hand, no chitinase activity was detected in the culture supernatants of N1 grown in any of the tested media (Fig. 1). In addition, the chitinase activity of the cell-associated fraction of N1 was measured after disrupting cells grown in various media, but no activity was detected (data not shown).

Production of the chitin-binding protein CBP21 by N1 was also examined by analyzing proteins in the day-3 culture supernatant of N1 grown in the media containing different carbon sources, as shown in Fig. 2. A wild-type strain produced CBP21 normally together with chitinases A, B, and C1 in the medium containing either colloidal chitin or $(GlcNAc)_{3-4}$. On the other hand, neither CBP21 nor any of the chitinases was detected in the culture supernatant of mutant N1, indicating that mutant N1 has lost the ability to produce not only chitinases but also the chitin-binding protein CBP21. A protein band with similar size to chitinase C1 detected in lane 5 (N1) does not have chitinase activity and corresponds to a band observed in lane 2 (wild-type) which is slightly smaller than chitinase C1.

Location of Tn5 insertion in mutant N1

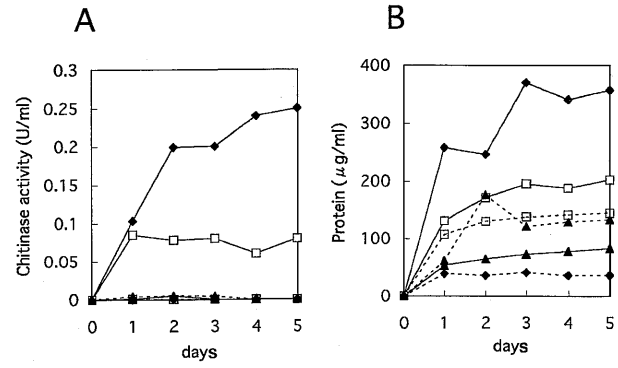

Fig. 1. Chitinase Activity (A) and Protein Concentration (B) in the Culture Supernatants of Mutant N1 and *S. marcescens* 2170.

Mutant N1 (dashed lines) and wild-type 2170 (solid lines) were grown in YEM medium containing 0.5% colloidal chitin (◆), $(GlcNAc)_{3-4}$ (□), or glycerol (▲). Chitinase activity in the culture supernatant was measured by a modification of Schales' procedure using colloidal chitin as an assay substrate. Protein concentration was measured by the method of Lowry *et al.* using bovine serum albumin as the standard.

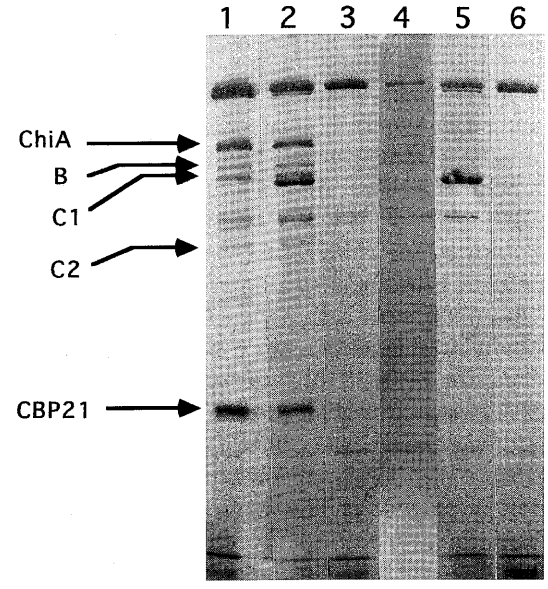

Fig. 2. SDS-PAGE Analysis of CBP21 in the Day-3 Culture Supernatant of Mutant N1 and *S. marcescens* 2170 Grown in Medium Containing Colloidal Chitin, $(GlcNAc)_{3-4}$, or Glycerol.

Proteins (50 μg in each lane) in the day-3 culture supernatant of *S. marcescens* 2170 (lanes 1 to 3) and mutant N1 (lanes 4 to 6) grown in the medium containing 0.5% colloidal chitin (lanes 1 and 4), $(GlcNAc)_{3-4}$ (lanes 2 and 5), or glycerol (lanes 3 and 6) were analyzed by SDS-PAGE.

To find the position of Tn*5* insertion in N1, we attempted to clone the flanking regions of the inserted Tn*5*. Approximately 39,000 clones of a genomic DNA library of mutant N1 were screened for the Tn*5* associated kanamycin resistance, and eight independent clones were identified. Plasmids were isolated from these Km-resistant clones and the nucleotide sequences of the inserted DNA regions were determined. The nucleotide sequence of a 1.5-kb region

around the Tn*5* insertion was finally obtained. This sequence was compared with those of the upstream and downstream regions of the chitinase genes of this bacterium previously determined by us. Surprisingly, it was found that the sequence of the flanking regions of Tn*5* coincided with the region between the *chiB* gene encoding chitinase B and the *cbp* gene encoding chitin-binding protein CBP21. The distance between the coding regions of *chiB* and *cbp* is 1434 bp and the position of Tn*5* insertion was 612 bp downstream of the termination codon of *chiB* and 819 bp upstream of the initiation codon of *cbp* (Fig. 3). In this region, an open reading frame (ORF) of 939 bp capable of encoding a polypeptide of 313 amino acids was identified (Fig. 4). The direction of transcription of the ORF is opposite to those of the *chiB* and *cbp* genes. The distances from the ORF to the coding regions of *cbp* and *chiB* are 404 bp and 91 bp, respectively. A possible ribosomal binding site and several potential promoter sequences were found upstream of the ORF. Downstream from the translation termination codon, a nucleotide sequence having the characteristics of a translation terminator was observed.

These results strongly suggest that the unability to produce chitinases and CBP21 protein phenotype of mutant N1 is due to the disruption by Tn*5* insertion of the identified new gene. Accordingly, we propose to name it as *chiR*, for chitinase regulation.

Analysis of the chiR gene product

The *chiR* gene encodes a polypeptide that consists of 313 amino acids with a calculated size of 35,046 Da. Proteins similar to the *chiR* gene product were searched for in protein data banks and significant similarities were found with various LysR-type transcriptional regulators (LTTRs), as shown in Fig. 5. LTTRs are the most common type of transcriptional regulators in prokaryotic organisms. The various LTTRs show a high degree of amino acid sequence similarity in their N-terminal domains, where the region containing a helix-turn-helix DNA-binding motif (Prosite signature PS00044) is located.[30] As shown in Fig. 5, a sequence that matches the LTTR helix-turn-helix motif was also observed in the N-terminal region of the deduced polypeptide of *chiR*. LTTRs are known to activate divergent transcription of the linked target genes or unlinked regulons encoding extremely diverse functions.[30] The *chiR* gene is located 404 bp upstream of the coding region of *cbp* and transcribed divergently from the *cbp* gene. Therefore, the target gene directly linked to *chiR* must be *cbp*.

Targeted mutagenesis of chiR

To confirm that the N1 phenotype is caused by the insertion of Tn*5* into the *chiR* gene, we first conducted complementation tests by introducing the plasmid carrying the intact *chiR* gene into mutant N1 by electroporation. However, the results were ambiguous. Some of the transformants formed clearing zones on the agar plates containing colloidal chitin, while others did not. In addition, the sizes and the restriction maps of the plasmids isolated from the transformants were different from those of the originally introduced plasmid.

Therefore, we attempted targeted mutagenesis of *chiR* to show that the mutation of *chiR* really causes

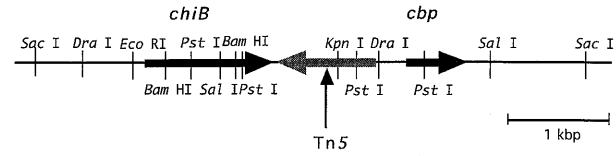

Fig. 3. The Position of Tn*5* Insertion in Mutant N1.

Horizontal arrows indicate positions and direction of transcription of the *chiB*, *cbp*, and *chiR* genes. The vertical arrow indicates the position of Tn*5* insertion which is 612 bp downstream of the termination codon of *chiB* and 819 bp upstream of the initiation codon of *cbp*.

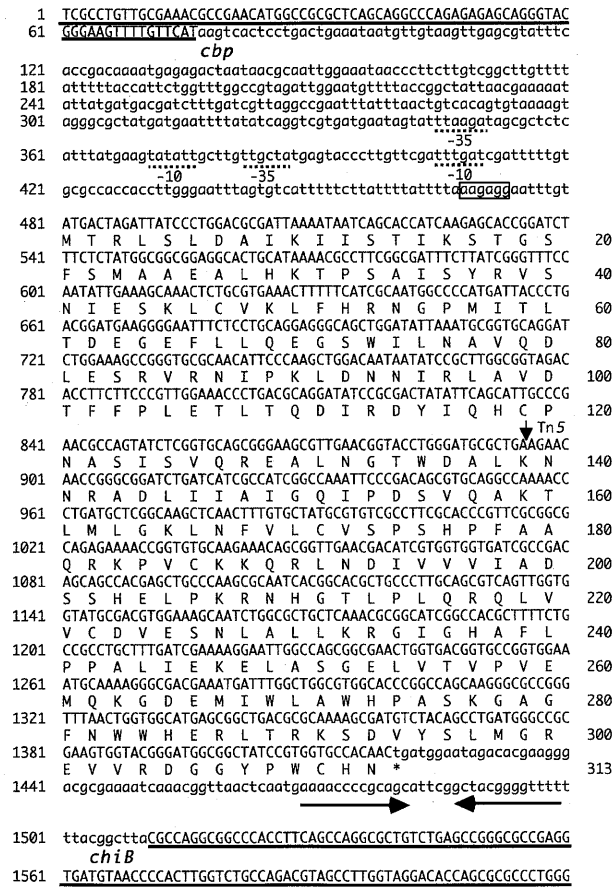

Fig. 4. Nucleotide Sequence of the *chiR* Gene and Deduced Amino Acid Sequence of the Gene Product.

Coding regions of the *cbp* and *chiB* genes are underlined. The "−10" and "−35" regions of the possible promoter sequences are dashed underlined. A tentative Shine-Dalgarno sequence is boxed. Horizontal arrows indicate an inverted repeat. The position of Tn*5* insertion is indicated by the vertical arrow.

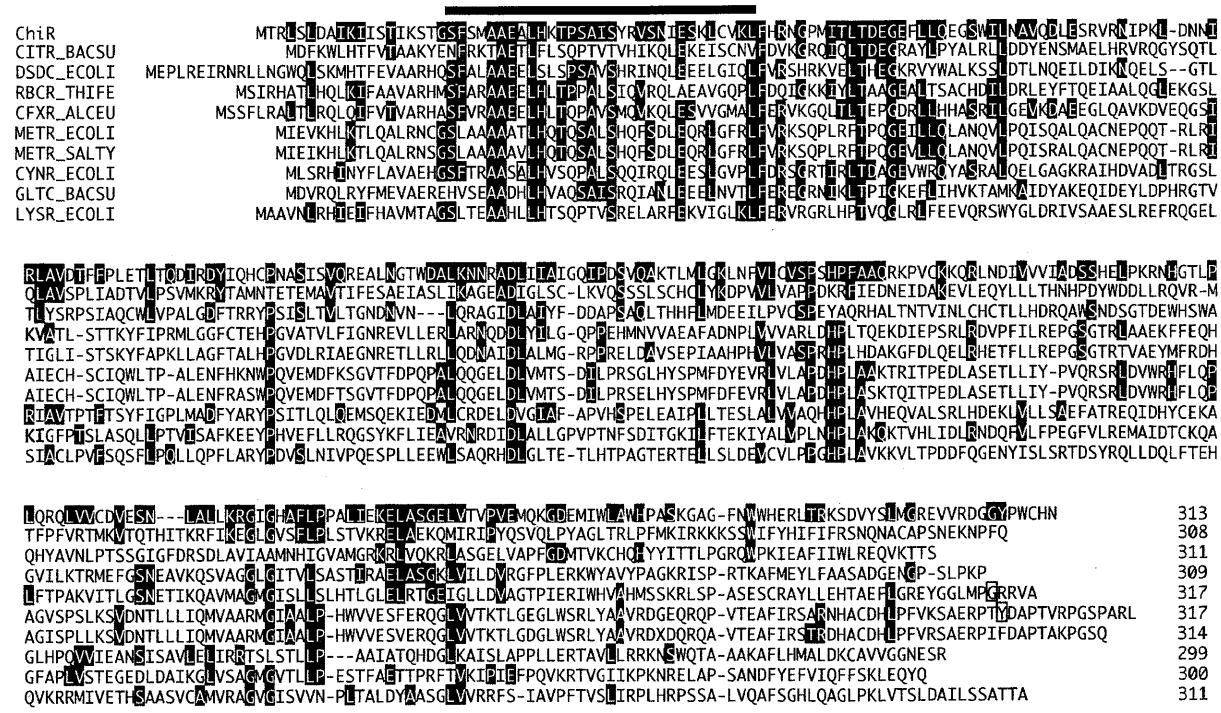

Fig. 5. Alignment of ChiR with LysR-type Transcriptional Activator.

Amino acid residues identical to those of ChiR are indicated by black background. Solid bar indicates the regions corresponding to a helix-turn-helix motif. CITR_BACSU, *Bacillus subtilis* citrate synthase I repressor (P39127); DSDC_ECOLI, *E. coli* D-serine deaminase activator (P46068); RBCR_THIFE, *Thiobacillus ferrooxidans* rubisco operon transcriptional regulator (Q06610); CFXR_ALCEU, *Alcaligenes eutrophus* rubisco operon transcriptional regulator (P42722); METR_ECOLI, *E. coli* transcriptional activator protein MetR (P19797); METR_SALTY, *Salmonella typhimurium* transcriptional activator protein MetR (P05984); CYNR_ECOLI, *E. coli* cyn operon transcriptional activator (P27111); GLTC_BACSU, *B. subtilis* transcriptional regulatory protein GltC (P20668); LYSR_ECOLI, *E. coli* transcriptional activator protein LysR (P03030).

the N1 phenotype. A part of the *chiR* gene corresponding to the internal-351-bp region was amplified by PCR and cloned into *Eco*RV-cut pFS100 to generate plasmid pFSΔCHIR. pFS100 contains a kanamycin resistance (Km^r) cassette at the unique *Sal*I site of pGP704, a *pir*-dependent replication plasmid.[20,27] pFSΔCHIR was transferred from *E. coli* S17-1 λpir to *S. marcescens* 2170 by conjugation, and transconjugants were selected based on kanamycin resistance. Kanamycin-resistant transconjugants should contain the mobilized plasmid integrated into the chromosome by homologous recombination that occurred between the *chiR* gene and the plasmid, leading to two incomplete copies of the *chiR* gene. Integration of pFSΔCHIR into the chromosomal DNA of *S. marcescens* 2170 was confirmed by PCR. Production of chitinases and CBP21 by the mutant was examined by testing formation of clearing zones on agar plates containing colloidal chitin and analysis of proteins in the culture supernatant by SDS-PAGE. Like N1, the mutant did not produce any chitinases or CBP21, confirming that the phenotype of N1 was caused by the insertion of Tn*5* into the *chiR* gene.

ChiR protein binds to intergenic region between chiR and cbp

In order to investigate the interaction of ChiR with the upstream regions of *chiA*, *chiB*, *chiC*, and *cbp*, we overproduced ChiR protein and partially purified it. The ChiR overexpression plasmid pCHIR was constructed by introducing the coding region of *chiR* immediately downstream of the His·Tag coding sequence of the pET expression vector. When *E. coli* cells harboring the plasmid pCHIR were exposed to IPTG to induce T7 promoter transcription, production of a novel polypeptide was observed (Fig. 6). The apparent molecular mass of the overproduced polypeptide was 35 kDa, which corresponded to the molecular mass expected for the His-ChiR hybrid. N-terminal amino acid sequence analysis of the polypeptide confirmed that this protein was the ChiR protein with His·Tag at its N-terminus. Most of the 35-kDa polypeptide was found in the insoluble pellet, and thus differential centrifugation provided a significant purification step. The protein pellet obtained by centrifugation was solubilized in urea and was used for further experiments after removing urea by differential dialysis. Purification using His·Bind resin, which is very effective for purification of His-tagged protein, was not used because most of the purified protein became insoluble after dialysis of the pooled fraction.

To examine whether the partially purified ChiR protein was able to bind DNA containing the regula-

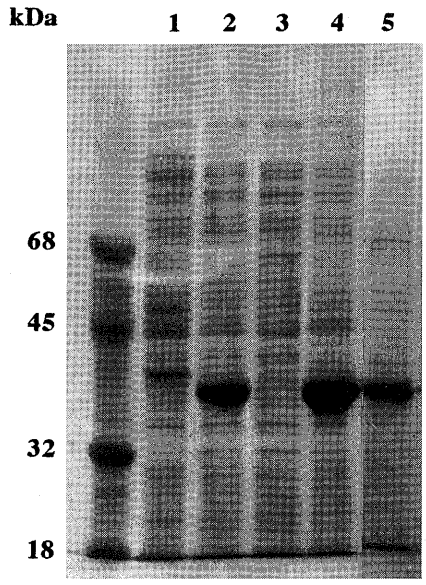

Fig. 6. Overproduction and Purification of ChiR.

E. coli BL21(DE3) cells carrying pCHIR, a *chiR* expression plasmid, were grown in the absence or presence of 1 mM IPTG. Cells were collected and disrupted by sonication, and soluble and insoluble fractions of the cell lysate were separated by centrifugation (see Materials and Methods). ChiR enrichment was monitored by SDS-PAGE and Coomassie blue staining. Lanes: 1, whole cell (no inducer); 2, whole cell with induction (1 mM IPTG for 3 h); 3, soluble fraction of cell lysate prepared from cells with induction; 4, insoluble fraction of cell lysate prepared from cells with induction; 5, partially purified ChiR.

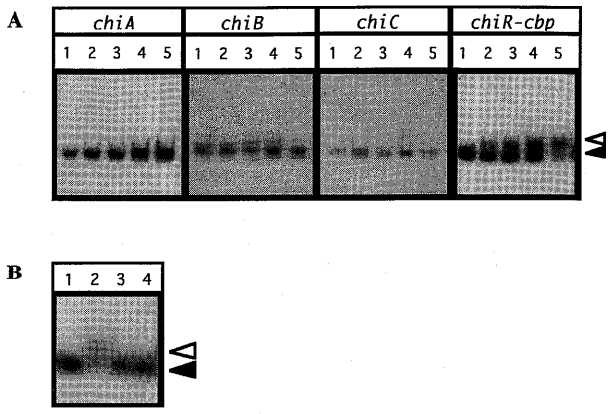

Fig. 7. Gel Mobility Shift Assays to Detect Possible Interaction of ChiR Protein with Intergenic Region of *chiR-cbp* and Promoter Regions of *chiA*, *chiB*, and *chiC*.

A) ^{32}P-labeled DNA probes containing either a promoter region of *chiA*, *chiB*, or *chiC*, or an intergenic region of *chiR-cbp* were incubated with various concentrations of partially purified ChiR protein. Lanes: 1, without protein addition; 2, with 250 ng of ChiR protein; 3, with 500 ng of ChiR protein; 4, with 1 μg of ChiR protein; 5, with 5 μg of ChiR protein. Protein-DNA complexes were separated from unbound probes by electrophoresis on a 6% native polyacrylamide gel. Open arrowheads indicate the position of protein-DNA complexes and solid arrowheads indicate position of unbound probe. B) Specific interaction of ChiR protein with the intergenic region of *chiR* and *cbp* was studied by using ^{32}P-labeled DNA probe containing *chiR-cbp* intergenic region. Lane 1, without protein addition; 2, with 250 ng of ChiR protein; 3, with 250 ng of ChiR protein and 1 μg of unlabeled DNA fragment containing *chiR-cbp* intergenic region as a specific competitor; 4, with whole cell extract (corresponding to 250 ng protein) prepared from *E. coli* BL21(DE3) cells carrying pCHIR grown no inducer.

tory regions of *chiA*, *chiB*, *chiC*, and the intergenic region between *cbp* and *chiR*, a gel mobility shift assay was performed. As shown in Fig. 7, addition of increasing amounts of ChiR protein resulted in a shift of the *cbp-chiR* DNA band to a position of slower mobility, and the amount of shifted complex was decreased when an excess of unlabeled *cbp-chiR* DNA was added as a competitor. On the other hand, *chiA*, *chiB*, and *chiC* DNAs did not give shifted bands in the presence of ChiR protein.

These results clearly demonstrated that ChiR protein is truly a DNA binding protein like the other LTTRs, and specifically binds to the intergenic region between *cbp* and *chiR*.

Discussion

S. marcescens 2170 releases a relatively limited number of proteins into the culture medium when grown in the presence of chitin.[7] The proteins detected in the culture supernatant include four chitinases: A, B, C1, and C2, and a 21-kDa chitin-binding protein (CBP21) lacking chitinase activity.[7,11] Production of CBP21 is only induced under the conditions under which chitinases are produced, suggesting coordinate regulation of the production of CBP21 and all chitinases.[11] The results obtained in this study show that the mutation of *chiR* stopped production of both CBP21 and all chitinases, and confirmed the coordinate regulation of the gene expression of chitinases and CBP21. However, important differences were observed in the relative locations of the CBP21 and chitinase genes to *chiR*. The *cbp* and *chiB* genes were found adjacent to the *chiR* gene. The *cbp* gene is upstream of the *chiR* gene and transcribed divergently, suggesting that the expression of *cbp* is directly regulated by ChiR, since most of the LTTRs activate divergent transcription of linked target genes. On the other hand, *chiB* is downstream of *chiR*, and the *chiA* and *chiC* genes do not seem to be in the region around the *chiB-chiR-cbp* region. The *chiA* gene is located 1.4 kb downstream of the ORF, which is similar to the *E. coli uhpC* gene encoding the regulatory protein of the sugar phosphate transport system (UHPC_ECOLI, P09836) and 0.6 kb upstream of the ORF similar to the *E. coli dppA* gene encoding periplasmic dipeptide transport protein (DPPA_ECOLI, P23847). *uhpC* and *dppA* are located at 82.9 min and 79.9 min of the *E. coli* chromosome, respectively. The *chiC* gene is between the ORF similar to the *E. coli nagD* and *asnB* genes encoding NagD protein (NAGD_ECOLI, P15302) and asparagine synthethase B (ASNB_ECOLI, P22106),

respectively. The *nagD* gene, a member of the *nagBACD* operon, and the *asnB* gene are both located at 15.1 min in the *E. coli* chromosome. Therefore, although it is not clear how similar the arrangement of the genes is in the *S. marcescens* and *E. coli* genomes, at least one can conclude that neither *chiA* nor *chiC* is linked to *chiR*.

It is known that some LTTRs also activate unlinked regulons; however, other chitinase genes may not be regulated directly by ChiR. The LTTRs have been suggested to contact the α subunit of RNA polymerase and increase binding of the polymerase to the promoter.[31,32] Many of the LTTRs bind to the characteristic sequence T-N_{11}-A conserved upstream of the regulated promoter, which is usually a part of a dyadic sequence.[30,33-35] In the intergenic region of *chiR-cbp*, two T-N_{11}-A motifs that may interact with ChiR were observed (Fig. 8), but such motifs were not observed in the upstream regions of the *chiB*, *chiA*, and *chiC* coding sequences. We also searched for a consensus sequence which may interact with ChiR among the upstream regions of *chiA*, *chiB*, and *chiC* and the intergenic region of *chiR-cbp*, but no such sequence was identified except for the possible CRP (catabolite repressor protein) binding sequences shown in Fig. 9. These observations are consistent with the results of gel mobility shift assays that ChiR bound to the intergenic region of *chiR-cbp* but not to the upstream regions of the *chiA*, *chiB*, and *chiC* genes. This may suggest the presence of a regulatory cascade for chitinase gene expression, and that *chiR* is involved in an early stage in the cascade. In that case, there must be unidentified gene(s) which are regulated by ChiR. The existence of such genes is very likely because preliminary experiments indicated that the mutation(s) in some of the other chitinase-deficient mutants (mutants N1 through N5) are not in the same region as *chiR*. PCR using primers designed to amplify the entire *chiR* gene was carried out with mutants N1 through N5, and as a result, DNA fragments of the size corresponding to the entire *chiR* gene were amplified with N4 and N6, indicating that these mutants do not have a Tn*5* insertion in *chiR*. Therefore, identification of a Tn*5* insertion in these mutants may lead us to identify new gene(s) required for chitinase gene expression.

The presence of 12-bp direct repeat sequences either overlapping or close to promoter sequences of the chitinase genes of many *Streptomyces* species have been reported.[15,36-38] Ni and Westpheling demonstrated that the direct repeat sequences in the chitinase-63 promoter of *Streptomyces* direct both glucose repression and chitin induction. Saito *et al.* found a pair of direct repeat sequences in the promoter regions of six out of seven chitinase genes identified in the *Streptomyces coelicolor* chromosome.[37] Such repeated sequences were not observed in the promoter regions of the chitinase and *cbp*

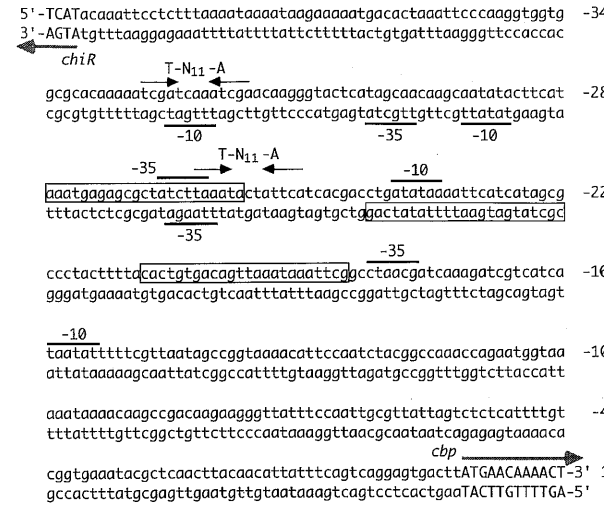

Fig. 8. The *chiR-cbp* Intergenic Region of *S. marcescens* 2170.

Numbers on the right indicate positions of the nucleotides with respect to the translation start site of *cbp*. The putative −10 and −35 elements of the promoter of *cbp* are overlined and those of *chiR* are underlined. The two inverted repeats corresponding to T-N_{11}-A motifs that are potential binding sites for a LysR-type transcriptional activator are shown by horizontal arrows. The possible CRP binding sites are boxed. The open reading frames of *cbp* and *chiR* are indicated by gray horizontal arrows.

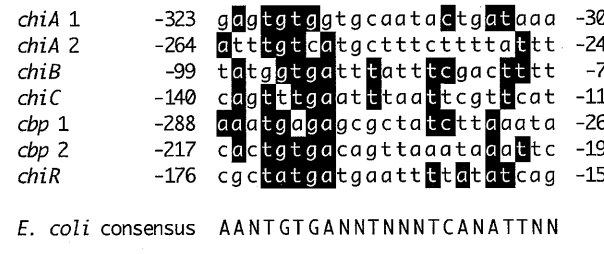

Fig. 9. The Possible CRP Binding Sites Observed in the Upstream Regions of Chitinase Genes and in the Intergenic Regions between *chiR* and *cbp*.

Numbers indicate the positions of the nucleotides with respect to the translation start sites of *chiA*, *chiB*, *chiC*, *cbp*, and *chiR*. Nucleotides identical to those of the consensus sequence of CRP binding sites in *E. coli* are indicated by black background.

genes of *S. marcescens*. Instead, possible CRP binding sequences were observed in the upstream regions of the known chitin-related genes, strongly suggesting that the expression of these genes is regulated by carbon catabolite repression in addition to the chitinase-specific regulation discussed above. In fact, chitinases and CBP21 are induced by chitin and chitooligosaccharides, and repressed by glucose. This situation is similar to that in many *Streptomyces* species; however, the mechanisms involved in induction and repression of chitinase genes and the genes for chitin-binding protein appear to be different between *Streptomyces* and *Serratia*. Differences are even more evident when we compare the induction and repression of chitinase production in *Alteromonas* sp.

strain O-7 with the regulation in *Serratia* and *Streptomyces*. Chitinases of *Serratia* and *Streptomyces* are induced by chitooligosaccharides, including $(GlcNAc)_2$, in addition to chitin, and GlcNAc represses expression of chitinase production. On the other hand, as demonstrated by Tsujibo *et al.*, not only GlcNAc but also $(GlcNAc)_2$ and $(GlcNAc)_3$ repressed the production of chitinases by *Alteromonas*, and glucose had no effect on chitinase production.[39] In addition, β-(1→6)-$(GlcNAc)_2$ induced a level of chitinase production similar to that induced by chitin in this bacterium.[39] These observations imply that there must be marked differences in the regulatory mechanisms of chitinase gene expression between *Alteromonas* and either *Serratia* or *Streptomyces*. Since *Alteromonas* sp. strain O-7 is a marine bacterium, it is possible that the aquatic environment requires mechanisms very different from those of soil chitinolytic bacteria. In any case, it is now becoming clear that there is a great diversity of the mechanisms for regulation of chitinase gene expression in various chitinolytic microorganisms.

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