Regulation of Chitinase Production by the 5'-Untranslated Region of the ybfM in Serratia marcescens 2170

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Serratia marcescens 2170 produces three chitinases and the chitin-binding protein CBP21, and efficiently degrades insoluble and crystalline chitin. The three chitinases and CBP21 are induced by S. marcescens chitinases, and the chitin-binding protein CBP21, and efficiently degrades insoluble and crystalline chitin of the N. chitinases and CBP21 are induced by degrades insoluble and crystalline chitin by S. marcescens chitinases. We have found that uptake of both (GlcNAc)2 and N-acetylglucosamine (GlcNAc) is important for the efficient utilization of (GlcNAc)2 because (GlcNAc)2 is less efficiently fermented by S. marcescens 2170 in the absence of chitobiase. In order to determine the mechanism of utilization of the degradation products of chitin by S. marcescens, chitobiase deficient transposon mutants were screened. A transposon present in chitobiase-deficient mutants was inserted into the ybfMN-ctb cluster. The mutants produced chitinases, except for TT327, in which a transposon was inserted into the 5'-untranslated region (5'-UTR) of ybfM. Ectopic expression of this region in TT327 restored chitinase production. These results indicate that the 5'-UTR of ybfM is important for chitinase induction in S. marcescens.

Key words: chitinase; 5'-untranslated region (5'-UTR); Serratia marcescens; chitobiase; chitobiase

Chitin, a β-1,4-linked polymer of N-acetylglucosamine (GlcNAc), is the second most abundant carbohydrate polymer in nature after cellulose. It exists predominantly in an insoluble crystalline form in the exoskeleton of crustaceans and arthropods and in the cell walls of fungi. Bacteria that produce chitin-degrading enzymes are the primary degraders of chitin. Chitin hydrolysis by chitinases is the most critical step in the degradation and utilization of chitin by bacteria. Production of multiple chitinases has been reported for many bacteria, including Bacillus circulans,1 Serratia marcescens,21 Aeromonas sp.,29 Alteromonas sp.,30 and Streptomyces.35,36 S. marcescens is an efficient biological degrader of chitin and one of the most extensively studied chitinolytic bacteria. S. marcescens 2170 produces three chitinases (ChiA, B, and C) and a chitin-binding protein (CBP21) in the culture medium when grown in the presence of chitin.7 Based on amino acid sequence similarity, chitinases are classified into glycoside hydrolase families 18 and 19.8,9 Bacterial family 18 chitinases can be further classified into subfamilies A, B, and C on the basis of amino acid sequence similarities of the catalytic domains.10 All the chitinases produced by S. marcescens belong to family 18. ChiA and ChiB belong to subfamily A, whereas ChiC1 belongs to subfamily B. A clear synergism in the hydrolysis of powdered chitin has been observed for a combination of these chitinases.11 CBP21 was recently found to cleave crystalline chitin by a mechanism that involves hydrolysis and oxidation.12 We found that ChiR, a LysR-type transcriptional activator, is essential for the production of all chitinases (ChiA, ChiB, and ChiC1) and CBP21 in S. marcescens 2170.13 N,N'-Diacetylchitobiose [(GlcNAc)2] is the major product of chitin hydrolysis by chitinases, and is the minimal substrate for chitinase and CBP21 induction in this bacterium.2,11 Uptake of (GlcNAc)2 is mediated by the phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS), and a (GlcNAc)2-specific enzyme II permease figures in the PTS system of S. marcescens 2170. Disruption of the (GlcNAc)2-specific enzyme II permease, encoded by the chb operon, drastically reduced the induction of chitinases and CBP21.14 S. marcescens 2170 also produces a 98.5-kDa chitobiase, which is mainly located in the periplasm, and hydrolyzes the β-1,4-glycosidic bond of (GlcNAc)2.15 A portion of (GlcNAc)2 enters the periplasm and is taken up into the cytoplasm by (GlcNAc)2-specific enzyme II permease. The rest of the (GlcNAc)2 is converted to GlcNAc by chitobiase, and GlcNAc is taken up via GlcNAc-specific PTS permease. We have found that uptake of both (GlcNAc)2 and GlcNAc is important for efficient utilization of (GlcNAc)2 in S. marcescens based on the observation that (GlcNAc)2 is less efficiently fermented by S. marcescens 2170 in the absence of chitobiase.15 Therefore, chitobiase is important for the efficient utilization of (GlcNAc)2. Both GlcNAc and (GlcNAc)2 induce chitobiase production, but (GlcNAc)2 is a more effective inducer than GlcNAc.15 In addition, chbC, a (GlcNAc)2-specific enzyme IIIC component of the PTS, is essential for the induction of chitobiase.14 Therefore,
uptake of (GlcNAc)₂ is important for the induction of chitinase as well as that of chitinases and CBP21. In this study, to determine the mechanism by which the degradation products of chitin are utilized by S. marcescens, chitinase-deficient transposon mutants were screened, and the phenotypes of the mutants were analyzed. The mutations were found to be located in the ybfMN-ctb cluster, encoding putative chitinoporin, putative lipoprotein, and chitinase. We discuss the importance of the 5′-UTR of ybfM in the induction of chitinase production in S. marcescens 2170.

Materials and Methods

Bacterial strains, plasmids, and culture conditions. S. marcescens 2170 and mutants of it were grown at 33°C with aeration in a yeast extract supplemented minimal (YEM) medium containing various carbon sources. The yeast extract concentration in the medium was 0.025% or 0.050%. YEM agar plates containing 0.1% (GlcNAc)₂ were used for the DNA digested with NcoI and SphI. The chitinase activity of the mutants was extracted from the cells as described previously, and digested with YEM medium containing various concentrations of chitinase. The chitinase activity of the mutants was determined by the second PCR. The PCR fragments obtained by inverse PCR and by PCR, colony direct PCR were performed. In the first PCR, primers were used for transposon mutagenesis. The promoterless mini-Tn5 Sm/Sp and pUJ8 were used for transposon mutagenesis. 17) The promoterless mini-Tn5 Sm/Sp-lacZ, and plasmids pUTmini-Tn5 Sm/Sp-lacZ, and pACYC184 were used to introduce ybfM and its derivatives into S. marcescens. E. coli coli S17-1(pipir) carrying pUTmini-Tn5 Sm/Sp-lacZ, and pUJ8 were used for transposon mutagenesis. Growth was monitored by obtaining OD₅₅₀ values using a mini-photo 518 photometer (Taisei, Koshigaya, Japan). Antibiotics were used in the following concentrations: ampicillin, 100 μg mL⁻¹, chloramphenicol, 50 μg mL⁻¹, kanamycin, 50 μg mL⁻¹, tetracycline, 25 μg mL⁻¹, spectinomycin, 50–100 μg mL⁻¹.

Transposon mutagenesis and isolation of chitinase-deficient mutants. The promoterless trp-1-lacZ region of pUS8 was cloned into plasmid pUTMini-Tn5 Sm/Sp (Biemedal, Seville, Spain). 15) The resulting plasmid, pUTMini-Tn5 Sm/Sp-lacZ, was used in the transposon mutagenesis of S. marcescens 2170. First, mini-Tn5 Sm/Sp-lacZ was delivered to wild-type S. marcescens 2170 via conjugation with E. coli coli S17-1(pipir) carrying pUTMini-Tn5 Sm/Sp-lacZ. 14) Spectinomycin-resistant transconjugants were obtained by using LB plates containing spectinomycin and tetracycline, and these were transferred to master plates. Transposon mutants were grown for 48 h on a colloidal chitin agar plates containing spectinomycin. For screening for mutants with altered chitinase production, a low melting point agarose (1%) containing 0.1 mM 4-methylumbelliferyl N-acetyl-β-D-glucosaminide (4-MU-GlCNac) (Sigma, St. Louis, MO) was poured onto the colloidal chitin plates and incubated for approximately 10 min at room temperature. The chitinase activity of the mutant colonies was detected by the fluorescence of the released 4-MU under UV light. In this study, lacZ was not used in screening.

Identification of transposon insertion sites. Transposon insertion sites in the genomes of the transposon mutants were determined by inverse PCR or arbitrarily primed PCR. For inverse PCR, chromosomal DNA of the mutants was extracted from the cells as described previously, 14) and digested with NcoI, SphI, or EcoRV. NcoI does not digest mini-Tn5 Sm/Sp-lacZ, and SphI and EcoRV digest at least one position in this plasmid. The digested DNA was self-ligated and amplified by PCR using primer sets mtTsp-F (5′-GGCCGAAGTTAATCGGCACATC-3′) and mtTsp-R (5′-TTTCCCAGTCACGACGTTG-3′), or mtTsp-F and mtTsp-R (5′-GTGAGTGATTTTGCTGACACCC-3′), or mtTsp-F and lacZ-R (5′-CTGCACACCATACACAGCC-3′), for the DNA digested with NcoI, SphI, or EcoRV. In arbitrarily primed PCR, colony direct PCR was performed. In the first PCR, primers mtTsp-F and R1B1 (5′-GGCCACCCGTGCGTGACTGACNNNNNNNNGATAT-3′), and the transposon flanking regions were amplified using primers mtTsp-F and mtTsp-R (5′-GGCCGAAGTTAATCGGCACATC-3′), or mtTsp-F and lacZ-R (5′-CTGCACACCATACACAGCC-3′) in the second PCR. The PCR fragments obtained by inverse PCR and by arbitrarily primed PCR were sequenced.

5′ RACE. S. marcescens 2170 was grown at 33°C with aeration in a YEM medium containing 0.1% (GlcNAc)₂ to the mid-exponential phase. Bacterial culture was mixed with 2 vol. of RNPreactect Bacterial Reagent (Qiagen, Hilden, Germany) and incubated for 5 min at room temperature. Total cellular RNA was then prepared and treated with DNase I using a MasterPure RNA Purification Kit (Epicentre, Madison, WI). 5′-RACE assay was carried out essentially as described previously. 16) An RNA sample was treated with tobacco acid pyrophosphatase (TAP, Epicentre, Madison, WI) to convert 5′ triphosphates to monophosphates. The RNA adapter (5′-RACE adapter, 5′-GAUAUGCGCGAAAUCCGUUGAAACGACACACACGACGACG-3′) was ligated to the 5′-end of RNA by means of T4 RNA ligase. cDNA was obtained by the TerscriptRM RT-PCR System (Invitrogen, Carlsbad, CA) and primer ybfM3R (5′-AGCGCAG-CCGTTTACGTGACGAC-3′). The cDNA product was amplified by PCR with primers, 5′-adapter (5′-GGCCGAATTCCTGCAGTGAATAG-3′) and ybfM-5′-PCR (5′-GGCTTCTTACGTGACGAC-3′). The PCR product was separated on agarose gel and purified, and cloned using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Four plasmids prepared from each of the transformants were sequenced.

Cloning of the ybfM gene. Plasmid pACyCylbM containing ybfM, including the regions up to 328 bp upstream and 74 bp downstream of the ybfM ORF, was constructed by amplifying ybfM of S. marcescens 2170 with primers ybfM-F (5′-GGCTGAAACATTATCCTGCT-3′) and ybfM-R (5′-GGCCCAATCATCAGCATGATT-3′), and was ligated into the EcoRV site of pACYC184. Because the amplified region included the promoter sequence of ybfM, ybfM was expressed by its own promoter. The growth rates of E. coli and S. marcescens harboring pACyCylbM were slightly lower than the strains harboring pACYC184, presumably because the excess production of YbfM was toxic to these strains. Plasmid pACYCylbM was digested with BamHI, and the DNA fragment that contained ybfM was cloned into the BamHI site of low copy number plasmid pTH18skr to generate pTHylbM. The orientation of ybfM in pTHylbM was opposite to that of the lac promoter of the vector. Hence, ybfM was expressed by its own promoter in this plasmid.

Construction of plasmids containing various upstream regions of ybfM. To construct plasmids containing various upstream regions of ybfM, the promoterless trp-1-lacZ region of pUS8 was cloned into plasmid pTH18skr to generate pTH18skr. An EcoRI-SphI fragment containing the 48 bp fragment of the transposon insertion site in the genome of S. marcescens 2170 was subcloned into EcoRI/SphI digested pTH18skr to generate pTH18skr. To construct the plasmid, plasmids pTH18skr and ybfM-5′ (5′-GGATCTTACAGTATTGCTGACGGG-3′) were digested with BamHI, and the DNA fragment that contained ybfM was cloned into the BamHI site of low copy number plasmid pTH18skr to generate pTH18skr. The orientation of ybfM in pTHylbM was opposite to that of the lac promoter of the vector. Hence, ybfM was expressed by its own promoter in this plasmid.
pkrTer – 137 + 117-R and pkrTer – 75 + 117-R was cloned in the orientation opposite to the lac promoter of the vector.

**Enzyme assays.** Chitinase activity was measured by a modification of the Schales’ procedure with colloidal chitin as substrate, and 1 U of chitinase activity was defined as the amount of enzyme that produces 1 μmol of reducing sugar per min. Chitobiase activity was measured using 4-methylumbelliferyl N-acetyl-β-D-glucosaminide (4-MU-GlcNac) (Sigma, St. Louis, MO) as substrate. (19)

**SDS–PAGE.** SDS–PAGE was carried out as described by Laemmli using 12.5% polyacrylamide gels under reducing conditions. (20) Proteins separated by SDS–PAGE were stained with Coomassie Brilliant Blue R-250.

**Chemicals.** N-Acetylglucosamine was purchased from Seikagaku Kogyo (Tokyo). Water soluble chitin (WS chitin) and N,N′-diacetylchitobiose were from Yaizu Suisan Chemical (Tokyo). The degree of deacetylation of WS chitin is 38.8%, and its approximate molecular weight is 200,000 to 300,000. Because WS chitin is soluble in water due to partial deacetylation and the limited size of the chitin chain, it is possible to measure cell growth in a medium containing WS chitin by optical density.

**Sequence analysis.** Putative promoters were identified using the Neural Network Promoter Prediction Program with a cutoff score of 0.90.

### Results and Discussion

#### Isolation of chitobiase-deficient mutants and identification of transposon-inserted genes

To identify the novel regulators of chitobiase gene expression in *S. marcescens* 2170, we screened for transposon insertions that alter chitobiase activity. Plasmid pUTmini-Tn5 Sm/SP-lacZ was used for transposon mutagenesis. Transposon mutagenesis was conducted as described previously. (14) Mutants with altered chitobiase activity when grown on a colloidal chitin plate were detected by means of 4-MU-GlcNac hydrolysis and isolated. Four of approximately 6,000 transposon mutants, TT116, TT120, TT401, and TT327, were selected based on their chitobiase-deficient phenotype. These mutants showed little chitobiase activity when grown in liquid medium containing colloidal chitin as carbon source (data not shown). Mutations that caused defects in the production of chitobiase were found by inverse PCR or arbitrarily primed PCR and sequencing of the PCR products to lie within the coding regions of yabfM, yabfN, and chitobiase (ctb) or at position −59 bp relative to the translation start codon of yabfM (Fig. 1). The genomic organization of the yabfM-chitobiase gene region strongly suggests that yabfMN and ctb constitute an operon (accession no. AB728624; Fig. 1). *S. marcescens* 2170 yabfMN was highly homologous to the *E. coli* yabfMN operon. In *S. marcescens* 2170, yabfN was located 46 bp downstream of yabfM, and there was no potential promoter of yabfN or terminator of yabfM. The amino acid sequences of YbfM and YbfN of strain 2170 exhibited 74 and 64% identity to those of *E. coli* MG1655 respectively. However, unlike *E. coli*, which lacks a ctb gene, the ctb gene without any promoter candidates was located 13 bp downstream of yabfN, and the sequence corresponding to the terminator of yabfN was not present in *S. marcescens*. In addition, deficiencies in the chitobiase activity of the mutants, which have a transposon insertion in yabfM (TT116) or yabfN (TT120), also confirm this presumption. To identify the transcriptional initiation site of the yabfM, 5′RACE was performed using 5′-RACE adapter and primers, yabfM-3′RT, 5′-adapter, and yabfM-3′PCR. The transcription start site was located 136 bp upstream of the yabfM translation start codon (Fig. 1B). The promoter of yabfM was predicted to be located at a position from −177 to −138 bp relative to the translation start codon of yabfM by the Neural Network Promoter Prediction program (http://www.fruitfly.org/seq_tools/promoter.html). The −35 and −10 elements of the promoter are shown in Fig. 1B. The transposon insertion site in TT327 was downstream of the predicted yabfM promoter region. Accordingly, the chitobiase deficiency was expected to be due to mutations within the coding regions or the 5′-untranslated leader region (5′-UTR) of the predicted yabfMN-ctb operon.

#### Analysis of chitinase production in the chitobiase-deficient mutants

Our previously constructed ctb mutant produced chitinases and CBP21 normally. (15) Hence, we examined the effects of the mutations in the mutants produced in the present study on the production of chitinases. The mutations in TT116, TT120, and TT401, in which a transposon was inserted in the ORF of the yabfMN-ctb operon, did not affect the production of chitinases, and mutant TT327, in which a transposon was inserted at position −59 bp relative to the translation start codon of yabfM, did not produce chitinase on a plate containing colloidal chitin (Fig. 2). However, the chitinase defect in TT327 was restored by plasmid pTHybFM carrying only yabfM (Fig. 2B).

In *S. marcescens* 2170, (GlcNAC)₂ uptake into the cells is essential for the production of all chitinases and CBP21. The growth of the ctb-deficient mutant on (GlcNAC)₂ was retarded as compared with that of the wild-type strain, as described previously. (15) We have
proposed a model of the catabolic pathway of the degradation products of chitin, which includes a hypothetical pathway involving (GlcNAc)_2-specific PTS permease and phosphochitobiase. As for Salmonella enterica serovar Typhimurium, YbfM (ChiP) has been characterized as a porin required for the uptake of chitin oligosaccharides. The growth curve of TT327 was not significantly different from that of the ctb mutant or TT327 harboring plasmid pTHybFM (Fig. 3). This result suggests that the mutation in TT327 did not affect the ability to take up (GlcNAc)_2. Although ectopic expression of ybfM restored chitinase production in TT327, chitinase deficiency in the mutant appeared to be independent of YbfM function.

5'-UTR of the ybfM gene affected chitinase production
While the transposon insertion at position +827 bp relative to the translation start codon of ybfM in TT116 did not affect chitinase production, the transposon insertion in the 5'-UTR of ybfM in TT327 led to deficient chitinase production. These results suggest that the region between positions −59 and +827 bp relative to the translation start codon of ybfM affects the production of chitinases. With the aim of determining the minimum region of ybfM that complements chitinase production, the 5'-UTR of the ybfM gene was selected and used to express chitinase in TT327. The results showed that the 5'-UTR of ybfM affects chitinase production, although the exact mechanism by which the 5'-UTR affects chitinase production is not clear. Further studies are needed to elucidate the mechanism by which the 5'-UTR affects chitinase production.

Regulation of Chitinase Production by the 5'-UTR of ybfM
The results of the experiments showed that the 5'-UTR of ybfM affects chitinase production, but the exact mechanism by which the 5'-UTR affects chitinase production is not clear. Further studies are needed to elucidate the mechanism by which the 5'-UTR affects chitinase production.

Fig. 2. Chitinase Production of Chitobiase-Deficient Mutants on a Colloidal Chitin Plate.
A, Wild-type and chitobiase-deficient mutants were grown on YEM medium containing 0.2% colloidal chitin as carbon source at 33°C for 2 d. B, Wild-type and chitobiase-deficient mutant TT327 with pTHybFM or pTH18kr were grown on YEM medium containing 0.2% colloidal chitin as carbon source at 33°C for 2 d.

Fig. 3. Growth of S. marcescens 2170 and Mutants of It in YEM Medium Containing 0.1% (GlcNAc)_2.
Growth was monitored by obtaining OD_660 values. Wild-type (S. marcescens 2170, hollow circle), TT327 harboring pTH18kr (triangle), TT327 harboring pTHybFM (square), ctb mutant (diamond), no carbon source control (S. marcescens 2170, solid circle). Each point represents the mean ± SD for two or three independent experiments.

Fig. 4. Construction of Plasmids Containing Various Upstream Regions of ybfM.
Numbers indicate positions from the translation start site. Arrows indicate orientations of genes. lac P, lac promoter; ybfM P, ybfM promoter.

Fig. 5. Chitinase Production of TT327 Harboring Various Plasmids.
A, Chitinase production on colloidal chitin plate. B, Chitinase activity of TT327 harboring various plasmids grown in liquid medium. Strains were grown at 33°C with aeration in YEM medium containing 0.2% glycerol until OD_660 = 1.0 was reached. Numbers represent plasmids, as follows: −137 + 117-F, pkTer − 137 + 117-R; −137 + 117-F, pkTer − 137 + 117-R; −75 + 117-F, pkTer − 75 + 117-R; −75 + 117-F, pkTer − 75 + 117-R; −137 + 30-F, pkTer − 137 + 30-F; −137 + 3-F, pkTer − 137 + 3-F.
production in TT327, plasmids containing various ybfM regions (pTHybfM, carrying the region from −328 relative to the translation start codon to 74 bp downstream of the stop codon of ybfM; pybfM +117, carrying the region from −328 to +117 bp relative to the translation start codon of ybfM; pybfM +30, from −328 to +30 bp; pybfM +3, from −328 to +3 bp; and pybfM +59, from −328 to −59 bp) were introduced into TT327 (Fig. 4). TT327 harboring plasmid pTHybM or pybfM +117 formed a clearing zone on the colloidal chitin plate, like the wild-type strain. A slightly smaller clearing zone was formed in the case of TT327 with pybfM +30, as compared with that of the wild-type strain. TT327 with pybfM +3, which contained only the first codon and 328 bp of the upstream region of ybfM, formed a significantly smaller clearing zone than the wild-type strain did (Fig. 5A). TT327, harboring plasmid pybfM −59 and containing the region from −328 to −59 bp relative to the translation start codon of ybfM, did not form a clearing zone on the colloidal chitin plate. These observations indicate that the region from −59 to +3 bp relative to the translation start codon of ybfM is the minimum requirement for the production of chitinases. These observations suggest that transcripts from the region from −59 to +3 bp relative to the translation start codon of ybfM, which is part of the 5′-UTR of ybfM, are required for the production of chitinases. To test this hypothesis, we designed plasmids containing the regions from −137 to +117 bp, −75 to +117, −137 to +30, and −137 to +3 bp relative to the translation start codon of ybfM under the control of the lac promoter in pTH118kr in both directions (Fig. 4). To avoid transcription from the opposite direction, the phage T4 terminator of mini-Tn5 Sm/Sp was added at the end opposite to the inserted regions regulated by the lac promoter. TT327, harboring the plasmids that carry part of ybfM in the forward orientation under the control of the lac promoter, showed chitinase activity when grown on a colloidal chitin plate (Fig. 5A) and in liquid medium containing 0.2% glycerol without chitin substrates (Fig. 5B). In contrast, no chitinase activity was detected in the culture supernatants of TT327, harboring plasmids that carry part of ybfM in the orientation opposite to the lac promoter when grown in the same medium (Fig. 5A, B). The minimum region required for activation of chitinase production was from −75 to +3 bp relative to the translation start codon of ybfM. These results suggest that chitinase production was induced by the transcription of ybfM 5′-UTR, and that induction occurred in the absence of chitin substrates.

The mutations in the coding regions of ybfM, ybfN, and ctb did not interfere with chitinase induction in S. marcescens. Although the reason for the activation of chitinase production by 5′-UTR in S. marcescens remains unclear, these results indicate that transcriptional activation of ybfM activates chitinase production in this bacterium. In Escherichia coli and Salmonella enterica serovar Typhimurium, a base-pairing small regulatory RNA, MicM, was recently reported to be involved in the regulation of ybfM expression.21,22 MicM binds to the ribosome binding site of ybfM mRNA and inhibits translation, leading to degradation of messages.23 An RNA trapping mechanism has been reported to be responsible for MicM regulation. MicM was trapped by the first intergenic region of the chb operon, which encodes a component of the chitobiase/triose phosphotransferase system along with the ChbR transcription factor.24 Trapping of MicM makes possible expression of the ybfM/N operon.25 YbfM in Salmonella is a chitoporin essential for growth on chitotriose and chitobiose as sole nitrogen and carbon source.26 Further study is required to clarify the involvement of MicM in the regulation of chitinase production by the 5′-UTR of ybfM in S. marcescens. We found that ChiR is an essential factor in the induction of all chitinases and CBP21. Hence, ChiR is expected to prove one of the key factors in ybfM 5′-UTR dependent chitinase regulation. We are conducting further research to elucidate the mechanism of chitinase induction by the 5′-UTR of ybfM.

References