Genetic Analysis of the Chitinase System of Serratia marcescens 2170

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To carry out a genetic analysis of the degradation and utilization of chitin by Serratia marcescens 2170, various Tn5 insertion mutants with characteristic defects in chitinase production were isolated and partially characterized. Prior to the isolation of the mutants, proteins secreted into culture medium in the presence of chitin were analyzed. Four chitinases, A, B, C1, and C2, among other proteins, were detected in the culture supernatant of S. marcescens 2170. All four chitinases and a 21-kDa protein (CRP21) lacking chitinase activity showed chitin binding activity. Cloning and sequencing analysis of the genes encoding chitinases A and B of strain 2170 revealed extensive similarities to those of other strains of S. marcescens described previously. Tn5 insertion mutagenesis of strain 2170 was carried out, and mutants which formed altered clearing zones of colloidal chitin were selected. The obtained mutants were divided into five classes as follows: mutants with (i) no clearing zones, (ii) fuzzy clearing zones, (iii) large clearing zones, (iv) delayed clearing zones, and (v) small clearing zones. Preliminary characterization suggested that some of these mutants have defects in chitinase excretion, a negatively regulating mechanism of chitinase gene expression, an essential factor for chitinase gene expression, and a structural gene for a particular chitinase. These mutants could allow researchers to identify the genes involved in the degradation and utilization of chitin by S. marcescens 2170.

The number of studies dealing with bacterial chitinases—their biochemical properties, the structure of the genes encoding them, the catalytic mechanism involved, and their tertiary structures—has been increasing rapidly. The hydrolysis of chitin by chitinases is the most critical step in the degradation and utilization of chitin by bacteria. However, the study of chitinases is not sufficient to elucidate the process by which chitin is degraded and utilized by bacteria. The process involves a number of steps, including the recognition of chitin outside of the cell, the induction of chitinases, the maintenance of proper levels of chitinase production, and the incorporation and catabolism of degradation products. In this study our intent was to identify the genes involved in the degradation and utilization of chitin. Our long-term goal is to answer the following questions. How do bacteria recognize chitin? How is chitinase production induced and regulated? Why do chitinolytic bacteria produce multiple chitinases? How are degradation products processed? Our ultimate goal in these studies is a general understanding of how bacterial cells degrade and utilize chitin.

We have studied the chitinase system of Bacillus circulans WL-12 and have provided comprehensive findings on biochemical properties, structure-function relationships, the identification of essential amino acid residues for catalytic activity, and the mechanisms by which multiple chitinases are generated (1, 2, 4, 23, 31–35). However, this bacterium is not a suitable model for the genetic analysis of the degradation and utilization of chitin. Therefore, we chose to study Serratia marcescens 2170, an active producer of chitinase (9, 25, 27) and an excellent model for studying the degradation and utilization of chitin. S. marcescens is an efficient degrader of chitin, and the chaA and chaB genes encoding chitinases A and B of three S. marcescens strains, QMB1466, BJL200, and 27117, have been cloned and sequenced (5, 6, 10–12, 16, 17). Recently, the three-dimensional structure of chitinase A from strain QMB1466 was described by Perrakis et al. (26).

In the present study, we analyzed proteins excreted into culture medium in the presence of chitin to elucidate how S. marcescens 2170 degrades chitin. In addition, we isolated various Tn5 mutants with characteristic defects in chitinase production.

MATERIALS AND METHODS

Bacterial strains, plasmids, phage, and culture conditions. S. marcescens 2170 (25) was used for chitinase production and Tn5 insertion mutagenesis and as a source of chromosomal DNA for chitinase gene cloning. Chitinases produced by S. marcescens 2170 were compared with those of S. marcescens QMB1466 (24) obtained from the American Type Culture Collection. For chitinase production, S. marcescens strains were grown at 30°C in yeast extract-supplemented minimal (YEM) medium (24) containing various carbon sources, with shaking.

Escherichia coli DH5α was used as a host organism, and pUC119 was used as a vector for chitinase gene cloning from S. marcescens 2170. E. coli DH5α carrying pUC119 or its derivatives was grown in Luria-Bertani (LB) medium containing 100 μg of ampicillin per ml. Plasmid pTROY11 (30) is a derivative of pBR322 containing the lamB gene of E. coli encoding the receptor for λ phage. A 467-bp Tn5 (8) was the donor phage for Tn5 mutagenesis and was propagated in E. coli LE392 (supE44 supF58 hsdR514 galK2 galT22 metB1 trpR55 lacY1). E. coli LE392 was grown in Trypticase broth supplemented with 0.2% maltose.

For Tn5 insertion mutagenesis, S. marcescens 2170 carrying pTROY11 was grown in Trypticase broth medium containing 0.2% maltose and 2 mg of ampicillin per ml. Mutants were selected on LB agar plates containing 50 μg of kanamycin per ml and 2 mg of ampicillin per ml.

Transposon mutagenesis. Tn5 transposon mutagenesis of S. marcescens 2170 harboring the plasmid pTROY11 was carried out as described previously (25). Kanamycin-resistant colonies were transferred onto agar plates of YEM medium containing 0.2% colloidal chitin, 50 μg of kanamycin per ml, and 2 mg of
amplification per ml and were incubated at 30°C. Clearing zones that formed around mutant colonies were visually inspected, and mutants with altered clearing zones were selected.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 12.5% slabs was conducted as described by Ames (3) with the buffer system of Laemmli (19). After electrophoresis, renaturation of the enzymes in the gel and detection of chitinase activity were performed as described previously (33).

Enzyme and protein assay. Chitinase activity was measured by a modification of Schales' procedure (14), with colloidal chitin as an assay substrate. One unit of chitinase activity was defined as the amount of enzyme which produces 1 μmol of reducing sugar per min.

Protein concentration was measured according to the method of Lowry et al. (21), with bovine serum albumin as the standard.

Secretion of nuclease by Tn5 mutants was examined by visual inspection of color-changed zones formed around the mutant colonies on an indicator plate containing 0.2% (wt/vol) salmon sperm DNA after staining with 0.1% toluidine blue. Secretion of lipase and protease was detected with an indicator plate containing 0.2% tributyrine (18) and 0.5% (wt/vol) milk casein, respectively. The clearing zone of milk casein was visualized by treatment with 5% trichloroacetic acid.

Chitin affinity column chromatography. Crude enzyme preparation was prepared from the supernatant of a 72-h culture of S. marcescens 2170 cultivated in medium containing 0.5% colloidal chitin, applied to a chitin column (3.5 by 13 cm) previously equilibrated with 20 mM sodium phosphate buffer (pH 6.0) and eluted stepwise with (i) 4 column volumes of 20 mM sodium phosphate buffer (pH 6.0), (ii) 4 column volumes of 20 mM sodium acetate buffer (pH 5.5), (iii) 4 column volumes of continuous gradient from 20 mM sodium acetate to 20 mM acetic acid (pH 5.5) to 20 mM acetic acid, and (iv) 2 column volumes of 20 mM acetic acid at a flow rate of 1 ml/min.

N-terminal amino acid sequence analysis. Proteins which have affinity for chitin were isolated from the culture supernatant of S. marcescens 2170 by chitin affinity chromatography and were separated by SDS-PAGE. The proteins on the polyacrylamide gel were electroblotted onto a polyvinylidene difluoride membrane, as described by Matsudaira (22). The membrane was briefly stained with Coomassie brilliant blue R-250 for protein band visualization, and excised chitinase bands were sequenced on an Applied Biosystems (Foster City, Calif.) 473 gas phase sequencer.

Cloning of the genes encoding chitinases A and B. Chromosomal DNA was prepared from S. marcescens 2170 as described by Silhavy et al. (29). The chromosomal DNA was partially digested with Sau3AI, and 2- to 10-kb DNA fragments were collected after they were separated by agarose gel electrophoresis. The DNA fragments were ligated to dephosphorylated BamHI-digested pUC119. E. coli DH5α cells were transformed with the ligated DNA, and transformants were selected on LB agar plates containing isopropyl-β-D-thiogalactopyranoside (IPTG; 40 μg/ml), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 40 μg/ml), and ampicillin (100 μg/ml). The transformants were transferred onto LB agar plates containing 0.2% (wt/vol) colloidal chitin and 100 μg of ampicillin per ml. The plates were incubated at 33°C for 24 to 72 h, and the transformants carrying chitinase genes were detected by the formation of a clearing zone around the colony.

Nucleotide sequence determination. Overlapping deletions were introduced into the inserted DNA fragments of the plasmids carrying chitinase genes and into a plasmid with the fragment in the reverse orientation with a deletion kit purchased from Takara Shuzo Co., Ltd. (Osaka, Japan). Regions of appropriate size of the deletion derivatives were sequenced with an automated laser fluorescence sequencer (Pharmacia). Sequencing reactions were done with the AutoRead sequencing kit (Pharmacia) according to the supplier's instructions, with double-stranded template.

Nucleotide sequence data were analyzed with the SDC Genetyx system (Software Kaihatsu Co., Tokyo, Japan). The amino acid sequence was compared with those available in the National Biomedical Research Foundation protein data bank with the Lipman-Pearson algorithm (20).

Chemicals. Glycol chitin and colloidal chitin were prepared from powdered chitin purchased from Funakoshi Chemical Co. (Tokyo, Japan) by the methods described by Yamada and Imoto (36), and Jeuniaux (15), respectively. The chitin EX (powdered chitin) (made by the Katokichi Co., Tokyo, Japan) used in chitin affinity chromatography was purchased from the Funakoshi Chemical Co. Chitobiose [GlcNAc2] and chitooligosaccharide mixture [[GlcNAc]2 + (GlcNAc)3] were obtained from the Yaizu Suisan Chemical Co. (Shizuoka, Japan) and the Pias Co. (Osaka, Japan), respectively.

RESULTS

Chitinase production by S. marcescens 2170. The production of chitinases by S. marcescens 2170 was examined in YEM medium containing various carbon sources and was compared with that of S. marcescens QMB1466. S. marcescens QMB1466 was initially isolated as an organism with high chitinase-producing activity (24), and chitinases of this bacterium, especially chitinase A, have been studied extensively at the molecular level (10, 12, 16, 26). Unexpectedly, strain 2170 produced a higher level of chitinase activity both in the medium containing colloidal chitin and in powdered chitin than QMB1466 did, as shown in Fig. 1A. When colloidal chitin was used as an inducer...
substrate, the chitinase activity of strain 2170 maximized at day 2 of cultivation and remained constant until day 7. On the other hand, when powdered chitin was used as a substrate, chitinase activity gradually increased, maximizing at day 5 of cultivation. The maximum activity observed with chitin was more than two times higher than that observed in the medium containing colloidal chitin. The addition of N-acetyl-D-glucosamine (GlcNAc) into the medium containing powdered chitin repressed chitinase production almost completely. Induction of chitinase production by soluble low-M_r substrates, such as glucose, GlcNAc, and chitobiose [(GlcNAc)_2], was also examined. As shown in Fig. 1B, (GlcNAc)_2 induced a level of production of chitinase similar to that induced by medium containing colloidal chitin. The most abundant protein (95 kDa) observed in the culture supernatant was not adsorbed. Three major (57, 52, and 48 kDa) proteins and one minor (36 kDa) protein eluted with acetic acid exhibited chitinase activity on the agar replica of polyacrylamide gel. The 21-kDa protein (designated CBP21) eluted with sodium acetate buffer did not show any chitinase activity (Fig. 3).

The proteins adsorbed on the chitin column were separated in SDS-PAGE and transferred onto polyvinylidene difluoride membrane. Their N-terminal amino acid sequences were then determined. As shown in Table 1, the N-terminal amino acid sequences of the 57- and 52-kDa proteins were identical to those deduced from nucleotide sequences of the chiA gene encoding chitinase A and the chiB gene encoding chitinase B of S. marcescens QMB1466, respectively. Accordingly, the 57- and 52-kDa proteins of S. marcescens 2170 were termed chitinase A and chitinase B, respectively. The protein bands of 48 and 36 kDa with chitinase activity both showed double peaks in each cycle of N-terminal amino acid sequence determination, and amino acid residues detected in each cycle were identical for the two proteins. These results suggest that the protein bands of 36 and 48 kDa contain two polypeptides and that the polypeptides in the 36-kDa band...
are derived from the polypeptides in the 48-kDa band, presumably by proteolytic modification of the C-terminal portion. These 48- and 36-kDa protein bands were named chitinase C1 and chitinase C2, respectively.

**Cloning of the genes encoding chitinases A and B of S. marcescens** 2170 and their deduced amino acid sequences. To examine the similarity of the chitinases of 2170 and other S. marcescens strains, chiA and chiB genes encoding chitinases A and B were cloned and sequenced. Sau3AI-digested fragments of chromosomal DNA of S. marcescens 2170 were ligated with BamHI-digested pUC119 and transformed into E. coli DH5α. Transformants carrying chitinase genes were selected by the formation of clearing zones around the colonies on agar plates containing colloidal chitin. Approximately 4,000 transformant colonies were examined, and five chitinase-positive transformants were obtained. Restriction enzyme analysis of the inserted DNA in the plasmids isolated from these transformants identified two groups, each carrying the gene corresponding to either the chiA or the chiB gene of S. marcescens QMB1466. Nucleotide sequences of the genes were determined, and deduced amino acid sequences are shown in Fig. 4. Calculated sizes of the deduced polypeptides of the chiA and chiB genes were 61,109 and 55,390, respectively. The amino acid sequence of chitinase B detected in the culture supernatant of S. marcescens 2170 matched the deduced sequence from Ser-2, indicating that chitinase B is excreted from the cell without signal sequence cleavage.

**Transposon mutagenesis.** S. marcescens 2170 was first transformed with the plasmid pTROY11 carrying the lamB gene from E. coli to make this strain susceptible to λ phage. Then, the strain was infected with λ 467::Tn5, and Tn5 insertion mutants were selected on LB agar plates containing 50 μg of kanamycin per ml. Approximately 3,000 mutants were transferred onto agar plates of YEM medium containing colloidal chitin and then incubated at 30°C for 1 to 3 days. Clearing zones of colloidal chitin forming around mutant colonies were visually inspected, and mutants with altered clearing zones were selected. The obtained mutants were divided into five classes as follows: (i) 6 mutants with no clearing zones, (ii) 3 mutants with fuzzy clearing zones, (iii) 1 mutant with a large clearing zone, (iv) 1 mutant with a delayed clearing zone, and (v) 10 mutants with small clearing zones.

**ChiB gene of strain 2170.** Amino acid sequences matching the N-terminal amino acid sequences are shown in Fig. 5. Mutants with fuzzy (F1 to F3), large (L1), and small (S1 to S6) clearing zones or no clearing zones (N1 to N6) are shown. Six of ten mutants with small clearing zones are shown.

### TABLE 1. N-terminal amino acid sequences of the proteins obtained by chitin affinity column chromatography

<table>
<thead>
<tr>
<th>Protein (kDa)</th>
<th>N-terminal amino acid sequence</th>
</tr>
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<tbody>
<tr>
<td>57</td>
<td>A P G R E L I A W S P R S P T I P T I A W (ChiA)*</td>
</tr>
<tr>
<td>52</td>
<td>S T R K A V I G Y Y F I P T N Q N I N Y (ChiB)*</td>
</tr>
<tr>
<td>48</td>
<td>A/V V/D A/D A/D I/A/M I/P I/M H/P A/P A/N/A/K</td>
</tr>
<tr>
<td>36</td>
<td>A/V V/D A/D A/D I/A/M I/P I/M</td>
</tr>
<tr>
<td>21</td>
<td>H G Y V E S P A S R A Y Q K L Q L N T</td>
</tr>
</tbody>
</table>

* Lowercase letters indicate deduced amino acid sequences of the chiA and chiB genes of S. marcescens QMB1466.

**FIG. 5.** Clearing zones of colloidal chitin formed by the mutants. Mutants with fuzzy (F1 to F3), large (L1), and small (S1 to S6) clearing zones or no clearing zones (N1 to N6) are shown. Six of ten mutants with small clearing zones are shown.
medium containing colloidal chitin for 3 days, and proteins in the culture supernatant were analyzed by SDS-PAGE. As shown in Fig. 6, all of the mutants lack a protein band corresponding to chitinase A.

(iii) The mutant with a large clearing zone. The mutant L1 formed a large clearing zone on agar containing colloidal chitin compared with wild-type 2170. As shown in Fig. 7A, L1 produced a higher level of chitinase activity than the wild-type strain did at the early stage of cultivation in YEM medium containing powdered chitin. Chitinase production in the medium containing powdered chitin was repressed by GlcNAc almost completely in wild-type 2170, as shown in Fig. 1. Repression by GlcNAc was not as severe with this mutant (data not shown). In addition, this mutant, unlike wild-type 2170, produced significant chitinase activity in LB medium (data not shown).

(iv) Mutants with delayed clearing zones. When wild-type 2170 was inoculated on YEM agar containing colloidal chitin and incubated at 30°C, a clearing zone became visible the next day. In contrast, the clearing zone of mutant D1 was not visible until the 3rd day of incubation. Once a clearing zone formed, it seemed to grow faster, attaining the same size as that of the wild-type strain.

When the mutant was grown in YEM liquid medium containing chitooligosaccharide mixture ([GlcNAc]3 and [GlcNAc]4), chitinase activity was mainly recovered in the cell-associated fraction, as shown in Fig. 7B, suggesting that chitinases were produced but not properly excreted into culture medium in this mutant.

(v) Mutants with small clearing zones. At the initial stage of screening, approximately 22 mutants were judged to have small clearing zones. These mutants were tested for growth on YEM agar containing glucose instead of colloidal chitin and were excluded if they formed small colonies. The chitinase production of the remaining mutants was examined in liquid medium, and 10 mutants clearly defective in chitinase production were selected. The time course of chitinase production of mutants S1, S2, S3, and S4 in liquid culture is shown in Fig. 7C. Some mutants did not exhibit any detectable chitinase activity in liquid culture when grown in the medium containing colloidal chitin. Other mutants did not show any activity for the first 3 days and then suddenly produced detectable levels of chitinase activity. Another mutant produced chitinase activity at a level significantly lower than that of the wild-type strain from the beginning to the end of cultivation.

**DISCUSSION**

*S. marcescens* 2170 produced a higher level of chitinase activity than the more extensively studied strain QMB1466. Another reason we chose to study strain 2170 is that it is more amenable to genetic analysis than QMB1466. Chitinases A and B of strain 2170 are very closely related to those of previously reported *S. marcescens* strains in terms of amino acid sequences. Brurberg et al. demonstrated periplasmic localization of chitinase B in *S. marcescens* BJL200 cells harboring recombinant plasmid pMAY2-10 encoding chitinase B (6). These researchers postulated that the role of chitinase B is to digest the shorter GlcNAc oligomers capable of entering the periplasm (7). In the case of strain 2170, a significant amount of chitinase B was observed in the culture supernatant, as shown in Fig. 3B and 6. Much less chitinase B than chitinase A was detected in the culture supernatant, but the ratio of these two chitinases in the culture supernatant did not change significantly during cultivation. Proteins detected in the culture supernatant were restricted to chitinases and a few other types, especially in the medium containing colloidal chitin. From these observations, at least in strain 2170, it seems that chitinase B is specifically excreted into culture medium together with other chitinases, although chitinase B does not possess a signal sequence at its N terminus and the excretion path of this chitinase probably differs from that of other chitinases.

In addition to chitinases A and B, chitinase bands previously detected in the culture supernatant of *S. marcescens* QMB1466 (10) were identified in the culture supernatant of 2170; these were termed chitinases C1 and C2. Since these two chitinases gave two peaks at each cycle of amino acid sequence determination, protein bands of chitinases C1 and C2 both contain two distinct polypeptides. The amino acid residues that appeared at each cycle are the same for chitinases C1 and C2. Therefore, the N-terminal amino acid sequences of the two polypeptides in C1 are identical to those of C2, suggesting that the double peaks were not due to contaminants. Chitinase C2 is likely to be a proteolytic derivative of C1, and each protein band of chitinases C1 and C2 is probably composed of two similar proteins, one of them a few amino acids shorter at its N terminus. Recently, we succeeded in cloning the new chitinase gene *chic* for chitinases C1 and C2. Therefore, chitinases C1 and C2 are not proteolytic derivatives of either chitinase A or B (unpublished data).

The protein corresponding to chitin binding protein CBP21 found in this study was also detected by Fuchs et al. (10) as one of the chitinolytic proteins with a subunit molecular mass of 21 kDa. This protein adsorbed to chitin but did not have chitinase activity in our experiment. The property of chitin adsorption seemed to be different from that of the other chitinases, as demonstrated by the elution pattern from chitin column (Fig. 3), that is, CBP21 was eluted with sodium acetate buffer, and the chitinases were eluted with a mixture of sodium acetate buffer and acetic acid. The N-terminal amino acid sequence of this protein did not match any region of chitinases A and B deduced from the *chiA* and *chiB* genes, indicating that this protein is not the chitin binding domain of chitinase A or B. It matched the amino acid sequence of the deduced polypeptide from the open reading frame located downstream of the *chiB* gene of *S. marcescens* 27117 in the EMBL data bank (submitted by Gal et al.) (11). Chitin binding activity and the fact that only a few proteins including chitinases are produced by strain...
large clearing zones. Mutant L1 (7116 WATANABE ET AL. J. BACTERIOL. chitin, and chitinase activity in the culture supernatant was measured at wild-type strain 2170 were grown in YEM medium containing 0.5% colloidal strain 2170. (C) Mutants with small clearing zones. Mutants S1 through S5 andated fraction (sonication, and chitinase activity in the culture supernatant (and cell-associ-F) was measured. Solid lines, mutant D1; dashed lines, wild-type strain 2170. (C) Mutants with de-Cells and culture supernatant were separated by centrifugation. After being sus-pression caused by the addition of GlcNAc was less severe than that observed on the wild-type strain. Therefore, this mutant seems to have a defect in the gene involved in the negative regulation of chitinase gene expression. The partial effect of the mutant on the repression caused by GlcNAc suggests that more than two regulating systems (or regulation elements) participate in the negative regulation of chitinase production in this bacterium.

The mutant with a delayed clearing zone accumulated chitinase within the cells when it was grown in the medium containing chitooligosaccharides as an inducer substrate. Although the location of the chitinases within the cell and the effect of the mutation on other extracellular enzymes remain to be elucidated, it is likely that this mutant contains a Tn5 insertion in the region affecting chitinase excretion.

The most abundant mutants were those with small clearing zones. The phenotype of this class of mutants is not clear. Half of these mutants produced chitinase normally when they were grown in liquid medium. However, five did not show any activity, and three did not produce chitinases until the 3rd day of cultivation in liquid culture. These mutants are the most likely to have defects in the genes that are involved in the very early stage of chitinase production.

The ultimate goal of this series of studies is to elucidate the system that is utilized by bacteria to degrade chitin. The iso-
loration of the mutants in this study should help researchers identify the genes that are involved in the degradation of chitin by this bacterium.

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