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Molecular Cloning of Chitinase Genes Family from *Serratia marcescens*

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Sau3AI으로 부분절단한 Serratia marcescens genomic DNA(5Kb 이상)을 pUC19의 BamHI site에 삽입하여 total genomic library를 준비하였다. Swollen colloidal chitin media에서 halo를 형성하는 2개의 *E.coli* 형질전환주를 선별하였다. 이들 colony가 chitinase 유전자를 갖음을 재확인하기 위하여 4-methylumbelliferyl N-acetyl-β-D-glucosaminide(4-MuFGlcNAc)를 이용하였다. 4-MuFGlcNAc는 chitinase에 대한 기질특이성을 나타내며 형광을 나타내는 기질로서 positive clone들은 360nm의 자외선을 조사하였을 경우 밝은 형광을 나타낸다. pUC19으로 부터 유래된 2 종류의 다른 chitinase clone, pCH1(11.0Kb) 및 pCH2(7.5Kb)를 genomic DNA library로 부터 분리하였으며, 이들의 제한효소지도를 작성한 결과 서로 다른 제한효소지도를 나타내었다. pCH1EA 및 pCH2로 부터 각각의 EcoRI-XbaI fragment를 subcloning함으로써 두개의 다른 chitinase 유전자의 위치를 결정하였다. pCH1EA 및 pCH2를 cross hybridization 한 결과 hybridization signal을 나타내지 않아 서로 유사성이 없는 것으로 사료된다.

Key Words : Serratia marcescens, Chitinase gene, Cloning, DIG labeling, Hybridization

Chitin, an insoluble linear β-1, 4-linked polymer of N-acetylglucosamine(NAG), is a major structural component of fungal cell wall except oomycestes and of arthropod exoskeleton like marine organisms including shrimp and crab. Chitinase(EC 3.2.1.14) is an enzyme that is widely distributed in nature and plays an important part in the degradation of chitin. Various reports have revealed that the modified chitin as well asits deacetylated compound, chitosan, act as an antibacterial agent and have been used in wastewater treatment(Austin et al., 1981). N-carboxybutyl chitosan shows antimicrobial properties(Muzzarelli et al., 1990) and 6-O-carboxymethyl-chitin(CM-chitin) has been used as a drug carrier(Watanabe et al., 1990). Sulfated chitin derivatives inhibit tumor-induced angiogenesis (Murata et al., 1991). However, the production of chitin, chitosan and its various derivatives mainly depend on chemical method required for large amount of the concentrated hydrogen chloride and sodium hydroxide during the acidification and neutralization process, which causes serious environmental pollution. Because the tremendous amount of natural chitin has been disposed in nature, it is great problem to reduce a growing waste-disposal of chitin in the shellfish food industry. To recycle the waste and reuse as a carbon source and biomedical products, we have to isolate chitinase gene in *E.coli*. *E. coli* should serve as a convenient host for cloning and expression of chitinase gene

since it completely lacks of endogenous chitinase activity.

Various bacterial and fungal chitinase have been purified and several chitinase genes have been cloned in *Serratia marcescens*(Jones et al., 1986 ; Fuchs et al., 1986), *Bacillus ciurculans*(Watanabe et al., 1990), *Vibrio vulnificus*(Wortman et al., 1986), *Streptomyces*(Miyashita et al., 1991 ; Robbins et al., 1992), *Mucor rouxii*(Pedraza-Reyes and Lopez-Romero., 1991), *Aphanocladium album*(Blaiseau and Lafay., 1992) and *Aspergillus carneus*(Sherief et al., 1991). As Serratia marcescens produce at least five different chitinase enzymes, it is interesting to understand the regulation mechanisms.

Jones et al.(Jones et al., 1986) have been already cloned two different chitinase genes and revealed the nucleotide sequences for one of chitinase genes. We hoped to clone all of the chitinase gene from *S. marcescens* to study the regulation system among several chitinase genes and characteristics of each chitinase enzyme. In this report, we describe the cloning of other two different chitinase genes from *Serratia marcescens* and its characteristics.

MATERIALS AND METHODS

Bacterial strains and plasmid : *Serratia marcescens*, which is clinically isolated and characterized, was kindly supplied from Pusan Baik Hospitals. pUC19 was used for cloning in *E. coli* JM109

Restriction enzymes, DNA modifying enzymes and chemicals : All restriction enzymes were purchased from KOSCO. DNA modifying enzymes like T4 DNA ligase and Alkaline Phosphatase(CIAP) purchased from Boehringer Mannheim. X-Gal and IPTG was provide from Jersey Lab & Glove Supply,Inc.(New Jersey, USA). SeaPlaque Low Melting Agarose was supplied from FMC, Co.(USA). Crude chitin powder were purchased from Sigma,Co.

Preparation of swollen colloidal chitin media : Swollen colloidal chitin was prepared as following the Monreal and Reese s method(Monreal and Reese., 1969). After stirring 100 g of crude chitin in 2 liter of the concentrated HCl for 12 hr, the precipitates were neutralized with 5N NaOH and filtered with D.W. White colloidal chitin was obtained after washing the filtrate with D.W. to remove NaCl which was formed during neutralization steps.

Preparation of total genomic library : Above 5 kb in length of Sau3A1 digested pool were eluted from 0.7% of SeaPlaque GTG agarose gel and purified, ligated to the BamH1 digesting pUC19 which dephosphorylated by CIAP. We could obtain about 20,000 recombinants by electroporation of JM109 with ligation pool.

Transformation of *E.coli* : *E. coli* gene pusler(Bio-Rad, USA) was used to obtain high transformation efficiency of *E. coli* in order to prepare total genomic library. Preparation of competent cell and electro-transformation were performed according to manufacture's protocols. In other subcloning procedure, fresh or frozen competent cell were prepared according to Hanahan's method.

Recombinant techniques : General recombinant techniques were followed by the basic protocols of Sambrook et al. High quality plasmid DNA was purified by the differential ammonium acetate precipitation (Lee and Rasheed., 1990) from *E. coli* cell grown in

Terrific Broth including the appropriate antibiotics and DNA fragments from SeaPlaque GTG agarose gel were recovered by LiCl method(Favre., 1992).

Screening of Chitinase clones : 4-MUF.GlcNAc stock substrate solution are prepared as the procedure of O'Brien and Colwell(O'Brien and Colwell., 1987) by dissolving 50 mol of 4-Methylumbelliferryl-N-Acetyl-β-D-glucosaminide in 2.0 mol of dimethylformamide. Mix well 0.6 ml of stock substrate solution with 9.4 ml of phosphate buffer(0.1 M, pH 7.4). Spread colonies on Whitman No.1 filer paper and drop 20 μl of 4-MU.GlcNac buffer substrate solution. After incubation at 37℃ for 10 min., a few drops of saturated sodium bicarbonate added and then expose under U. V. light(366 nm).

Hybridization and probe preparation : DNA probe (1~2 ug) were prepared with digoxigenin-11-dUTP by 2 units of Klenow fragment including hexanucleotide mixture. DNA fragments separated on agarose gel electrophoresis were capillarily transferred to the charged nylon membrane.

Prehybridization and hybridization was performed using Enprotech hybridization bottles at 68℃ in Integrated Separation System hybridizationoven. Detection of probe-target DNA hybrids and color developing was done in vinyl bag within a few minutes.

RESULTS AND DISCUSSION

Screening of chitinase genes from genomic library : To screen the chitinase clones from genomic library, we used the conventional plate method for chitinase activity by using colloidal chitin as the substrate. About 10,000 recombinant, we identified two colonies producing very small clear halo around colony. Although Jones et al.(Jones et al., 1986) have demonstrated very large halo around chitinase clone after 10 days incubation, we could not observe the halo was growing with the longer incubation. To prove the two colonies fished out from library harbor the true chitinase gene, we used the filter paper spot test with 4-methylumbelliferryl-N-β-D-glucosaminide. It has the advantages of being rapid, simple to perform and very sensitive to determine the chitinase activity.

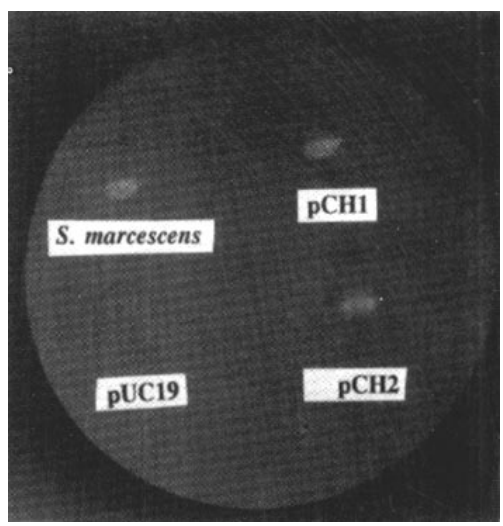

Fig. 1. Screening of chitinase clones by exposure under long wave U.V. light(366nm) directly on colonies that soaked with 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide. Two colonies show the light-blue fluorescence, 4-methylumbelliferone, formed by the action of chitinase.

As shown in Fig. 1., our two chitinase clones, pCH1 and pCH2, exhibit the light fluorescence in filter paper spot test with 4-methylumbelliferryl-N-β-D-glucosaminide as compared with *S. marcescens* and JM109 containing pUC19 plasmid. Like cellulase and other

polysaccharide degradation, the major product of extracellular hydrolysis of chitin is disaccharide by the action of endochitinase. The consecutive steps in chitin utilization is the transport of disaccharide into the cell by permease, hydrolysis of the disaccharide to aceylglucosamine by chitobiase and phosphorylation, deacetylation and deamination of aceylglucoamine. It has been revealed that bacterial chitinase consist of endochitinase, chitiobiase and permease(Jones et al., 1986). The exochitinase is not included in bacterial chitinase, which is found only in fungal chitinase(Butler et al., 1991). Because pCH1 and pCH2 did not produce large halo on the swollen chitin media and produced the light fluorescence in filter paper spot test with 4-methylumbelliferryl-N-β-D-glucosaminide, it is considered that the chitobiase gene is encoded in pCH1 and pCH2.

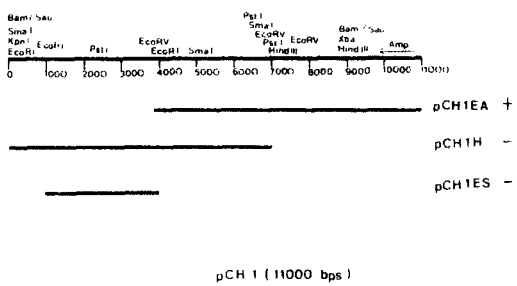

Fig. 2. The restriction map of pCHI. The 8.4kb of *Serratia marcescens* DNA fragment in pCHI do not have the restriction sites of BamHI, ClaI, NdeI, PvuII, SacII and XhoI. The derivatives (pCH1EA) from pCHI, which obtained from self-ligating of EcoR1 large fragment, show chitinase activity.

Subcloning of pCH1 and pCH2 : As shown in Fig. 3, pCH1 contain three EcoR1 sites and two HindIII sites. The HindIII large fragment was self-ligated, which named as pCH1H. pCH1H did not show chitinase activities. The EcoR1 large fragment of 7.1kb in length was self-ligated and small EcoR1 fragment of 3.0kb long was subcloned into pUC19, which were named as pCH1EA and pCH1ES. Only pCH1EA showed chitinase activity. Therefore, we could conclude that chitinase gene was localized with EcoR1 large fragment. The fine restriction map is represented in Fig. 3. As shown in Fig 4., the pCH2 contained two EcoRV sites and two KpnI sites. The large EcoRV fragment was self-ligated, which named as pCH2RVA. It did not show chitinase activity. The KpnI large fragment including pUC19 was self-ligated(pCH2KA) and the small fragment was subcloned into the KpnI site of pUC19(pCH2KB). These two recombinants also did not show chitinase activity. Because only one PvuII site was identified within foreign DNA of pCH2 about 4.8kb in length. The PvuII-HindIII fragment about 3.8kb long was subcloned into the HindIII

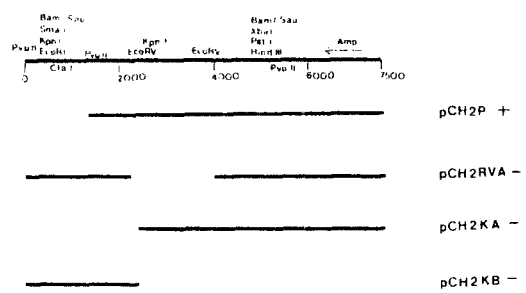

Fig. 3. The restriction map of pCH2. The 4.8kb of *Serratia marcescens* DNA fragment in pCH2 do not have the restricition site of BamHI, BglII, NdeI, SacII and XhoI. The derivatives(pCH2P +) from pCH2, chich obtained by subcloning of PvuII-HindIII fragment in pCH2 into the SmaI-HindIII site of pUC19, show the chitinase activity.

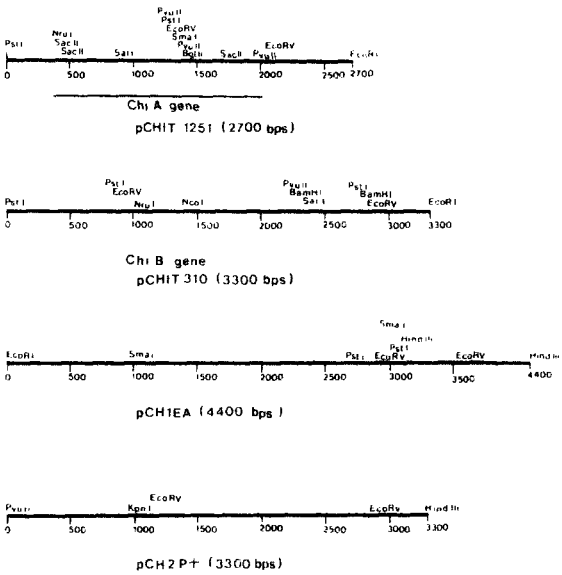

Fig. 4. The comparison of ChiA gene(pCHIT 1251) and ChiB(pCHIT 310) (Jone et al., 1986 ; Harpster and Cunsmur, 1989) with pCH1EA gene and pCH2 gene of *Serratia marcescens*.

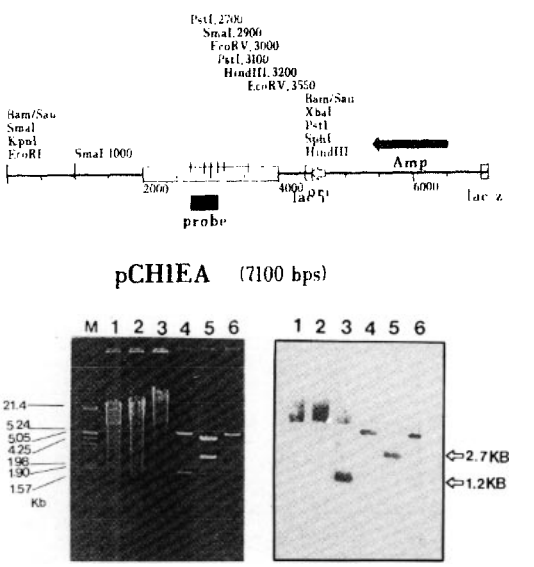

Fig. 5. Genomic southern hybridization of *Sarratia marcescens* with DIG-labeled 400bp of PstI fragment from pCH1EA as DNA probe(lane 1, 2 and 3). Cross hybridization of pCH2 with DIG-labeled 400 bp of PstI fragment from pCH1EA(lane 4, 5 and 6). Lambda-EcoRI/HindIII digested fragments were used as a size marker. The *Serratia marcescens* DNA fragment digested with BamHI, EcoRI and HindIII were run on lane 1, 2 and 3, respectively. The pCH2 DNA fragments of EcoRV, EcoRI/HindIII and PstI digestion were run on lane 4, 5 and 6.

-SmaI site of pUC19. The resulting recombinant, pCH 2P, showed chitinase activity. The fine restriction map of pCH2 are represented in Fig 4.

Comparison of the restriction map of several chitinase gene : As shown in Fig. 5, the restriction map of ChiA gene and ChiB gene of *Serratia marcescens* was different to pCH1 and pCH2 described in this report. Therefore, it is considered that pCH1 and pCH2 harbor other chitinase gene of *Serratia marcescens*.

Southern hybridization : Genomic southern hybridization and cross hybridization of pCH2 with EcoRV, EcoRV/HindIII and PvuII digestion are shown in Fig. 6. BamHI and EcoRI digestion pattern of *S. marcescens* DNA did not showed any distinct band, but HindIII digestion pattern showed one distinct band about 1.2kb long(lane 3) which hybridized with 400bp of PstI fragment from pCH1EA. Although it showed strong hybridization signals with 400bp probe, it did not represent the fragment of chitinase gene in pCH1EA. It may suggest of being any other homologous region in *S. marcescens* genome with PstI fragment of pCH1EA. In cross hybridization experiment, the PstI fragment of pCH1EA did not show any homologous region in pCH2.

Although EcoR1-HindIII fragment of 2.7kb long was hybridized to PstI probe in lane 5, it contained only pUC19 DNA. Other two signals shown in lane

4 and 5 were also resulting from pUC19 DNA. Therefore, we could conclude that 400bp of PstI fragment in pCH1EA did not represent any homology with S. marcescens DNA in pCH2. The EcoRV fragment about 1.9kb long within pCH2 were labeled with DIG and hybridized to the EcoRV(lane 3), EcoRI/HindIII (lane 4) and PstI(lane 5) fragments of pCH1EA. Hybridization signals were only detected in DNA fragment which contained pUC19 DNA fragment. Therefore, We could conclude that the *S. marcescens* genomic DNA within pCH1 and pCH2 did not show any homology.

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Total genomic DNA library of Serratia marcescens was prepared by inserting Sau3A1 partial digesting fragments(above 5 kb) into the dephosphorylated BamHI site of pUC19.

In primary screening, two colonies were selected by observing the halo around E. coli transformants grown on the swollen colloidal chitin media. Secondary screening was performed by soaking two colonies with a few drops of 4-methylumbelleliferryl N-acetyl-β-D-glucosaminide(4-MuNGlcNAc). As 4-MuNGlcNAc is a specific, fluorogenic substrate for chitinase, the positive clones produce light fluorescence by the exposure under the long wave U.V. light(360 nm). From genomic DNA library derived from pUC19, we have isolated two different chitinase clones, pCH1(11.0Kb) and pCH2(7.5Kb), which show completely different restriction map to each other.

The cross-hybridization of pCH1EA and pCH2 have not revealed any hybridization signals to each other.