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Cloning of the Bacteriocin Gene from *Xanthomonas campestris* pv. *glycines* 8ra

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콩 불마름병균 *Xanthomonas campestris* pv. *glycines* 8ra의 박테리오신 유전자 Cloning

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ABSTRACT : Glycinecin is a bacteriocin produced by *Xanthomonas campestris* pv. *glycines*, which inhibits the growth of *X. c.* pv. *vesicatoria* that is the causal agent of the bacterial spot on pepper. In order to identify the genes involved in glycinecin production, the cosmid library of *X. c.* pv. *glycines* 8ra was tested directly for bacteriocin production using *X. c.* pv. *vesicatoria* Ds94-9 as an indicator strain. Five of two thousands cosmids, pG011, pG013, pG08, pG33, and pG35, that composed the genomic library of *X. c.* pv. *glycines* conferred glycinecin production to *Escherichia coli* HB101, as indicated by the formation of a large clear zone on nutrient agar covered with indicator strain *X. c.* pv. *vesicatoria* Ds94-9. One clone pG08, was arbitrarily chosen and further analyzed to narrow down the region responsible for the production of glycinecin. Several subclones from pG08 were made with restriction enzyme *Eco*RI. One of the subclones, pBL5, carrying 6.0 kb DNA from pG08 showed the bacteriocin activity. Based on restriction enzyme map and bacteriocin-producing activity of all the subclones, about 3.0 kb DNA region was responsible for the bacteriocin production. Among 5 isolated clones which were able to produce bacteriocin, only two clones were hybridized with 1.6 kb *Hin*dIII fragment from pBL5. Therefore, *X. c.* pv. *glycines* 8ra seems to carry more than one bacteriocin-producing gene.

***Key words* : *Xanthomonas campestris* pv. *glycines*, *X. campestris* pv. *vesicatoria*, bacteriocin, glycinecin.**

The ability of various bacteria to inhibit the growth of other bacteria has been well documented (9, 10, 27). In many cases it was demonstrated that the antagonistic activity was attributable to molecules of a proteinaceous nature, termed bacteriocin (9). The first bacteriocin to be discovered was the colicins produced by *E. coli*, and extensive knowledge is now available concerning their genetics (19). Many plant pathogenic bacteria, including members of the corynebacteria, erwinia, and pseudomonads, are known to produce proteinaceous bacteriocins (10, 27). Agrocin 84, produced by *Agrobacterium radiobacter* strain 84 that inhibits *A. tumefaciens*, the causal agent of the crown gall, was an unusual bacteriocin which is a structural analog of an adenine nucleotide (20). However, few studies have been carried out to elucidate the bacteriocins produced by xanthomonads (25). Bacteriocin-like substances were detected in *Xanthomonas oryzae* pv. *oryzae* (17) and glycinecins, bacteriocins produced by *Xanthomonas campestris* pv. *glycines*, were reported (5). However, their genetics is not elucidated and it has been suggested that glycinecin genes may be located on genomic DNA.

Here, we describe the isolation of the DNA region containing the gene coding for glycinecin production from *X. c.* pv. *glycines* 8ra.

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MATERIALS AND METHODS

Bacterial strains, plasmids and media. Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* was cultured on Luria-Bertani (LB) medium at 37°C and *Xanthomonas* was grown on nutrient agar medium (NA) routinely. Genomic library of *X. c.* pv. *glycines* 8ra constructed in cosmid pLAFR3 was a gift of E. J. Braun (University of Illinois, Urbana-Champaign).

Bacteriocin detection and assay. Standard methods were used for screening for bacteriocin production (4). Briefly, bacterial suspensions were prepared in sterile water with cells grown on peptone sucrose agar (PSA) overnight at 30°C. The suspensions were adjusted to OD_{600}=0.01 (approximately 2×10^8 CFU/ml), and 5 μl droplets of inoculum were spotted onto the surface of NA. Dishes were incubated at 30°C for 24 hr, and then the producer cells were killed with 10% chloroform vapors. Sterile water suspensions of *X. c.* pv. *vesicatoria* Ds94-9 used as an indicator strain were prepared from overnight cultures grown on NA at 30°C. Suspensions were adjusted to OD_{600}=0.1, and 0.2 ml of that suspensions were added to 9.8 ml of sterile 0.7% (wt/vol) water agar (pH 7.0) maintained at 50°C in a water bath. The inoculated water agar was poured over the surface of the producer plate, and dishes were incubated at 30°C for 48 hr.

Screening genomic library for glycinecin gene(s). The screening for bacteriocin-producing gene(s) from *X. c.* pv. *glycines* 8ra genomic library was directly by observation of inhibition zone surrounding *E. coli* carrying DNA fragment from *X. c.* pv. *glycines* 8ra. Randomly chosen two thousand bacterial colonies from *X. c.* pv. *glycines* 8ra genomic library were transferred on NA with toothpicks. Bacteria were incubated at 30°C for 24 hr and then killed with chloroform vapors for 30 min. After drying the plates, bacterial colonies were overlaid with water agar suspensions of the indicator strain *X. c.* pv. *vesicatoria* Ds94-9 and further incubated at 30°C for 36 hr.

DNA isolation, analysis and modification. Plasmid DNA from *E. coli* was isolated and purified by alkaline lysis method (21). Restriction endonucleases were used in accordance with the supplier's specifications. Transformation of *E. coli* with plasmid DNA was conducted according to the method of Gerhardt *et al.* (21). DNA fragments were isolated from agarose gels by using dialysis tubes (21). Deletions of DNA inserts were obtained by the use of available restriction sites. In some cases, the sticky ends of DNA fragments were made blunt with a fill in reaction using deoxynucleotide triphosphates (dNTPs) and the Klenow fragment before ligation.

Table 1. Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant characteristics[a]	Sources
Bacterial strains		
Xanthomonas campestris		
pv. *glycines* 8ra	Wild type, soybean pathogen, Rif^r	E. J. Braun
pv. *vesicatoria* Ds94-9	Wild type, pepper pathogen	Y.-J. Ko
E. coli HB101	F^- *hsdS20*(r_B^- m_B^-) *recA13 leuB6 ara-14 proA2 lacY1 galK2 rpsL20*(*Str*^{r}) *xyl-5 mtl-1 supE55*	(1)
Plasmids		
pLAFR3	*Inc*P *cos*$^+$ *rlx*$^+$ *Tra*$^-$ *Mob*$^+$ Tc^r	(24)
pBluescript II SK(+)	Amp^r	Stratagene, La Jolla, CA.
pG011	Tc^r, pLAFR3 carrying 42 kb DNA from *X. c.* pv. *glycines* 8ra	This study
pG013	Tc^r, pLAFR3 carrying 44 kb DNA from *X. c.* pv. *glycines* 8ra	〃
pG08	Tc^r, pLAFR3 carrying 43 kb DNA from *X. c.* pv. *glycines* 8ra	〃
pG33	Tc^r, pLAFR3 carrying 47 kb DNA from *X. c.* pv. *glycines* 8ra	〃
pG35	Tc^r, pLAFR3 carrying 42 kb DNA from *X. c.* pv. *glycines* 8ra	〃
pG0808	Tc^r, 31 kb derivative of pG08	〃
pG0812	Tc^r, 37 kb derivative of pG08	〃
pG0813	Tc^r, 31 kb derivative of pG08	〃
pBL5	Amp^r, 6.0 kb *Bam*HI fragment from pG08 inserted into pBluescript II SK(+)	〃

[a] Rif^r=rifampicin resistance; Amp^r=ampicillin resistance; Tc^r=tetracycline resistance.

Southern analysis. In southern blotting experiments DNA was transferred to nylon membranes (Sigma Co.) by the capillary blot procedure. The transferred DNA was cross-linked on the membrane with UV for 3 min and baked for 30 min at 120°C. The dried membrane was prehybridized with hybridization solution (5×SSC, N-lauroylsarcosine, 0.1% (W/V); SDS, 0.02% (W/V); Blocking reagent, 1% (W/V)) at 68°C for 1 hr. Hybridization was performed with fresh 10 ml hybridization solution containing denatured labelled DNA probe to the final concentration of 10 μl (10 ng/ml) for 100 cm^2 of membrane at 68°C for 16 hr.

DNA fragments used as probes were labelled with digoxigenins as described by the manufacturer (Boehringer Mannheim). The template DNA (3 μg) was denatured for 10 min at 68°C and quickly chilled on ice/NaCl. The denatured DNA solution was mixed with 2 μl hexanucleotide mixture as a random primer, 2 μl dNTP mixture, and 1 μl Klenow fragment. The solution was incubated for 20 hr at 37°C. To stop the reaction, 2 μl of 0.2 M EDTA was added, and the labelled DNA was precipitated by adding 2.5 μl of 4 M LiCl and 75 μl prechilled (−20°C) ethanol. The mixture was mixed well, incubated for 10 hr at −20°C, and centrifuged for 20 min at 14,000 rpm in a microcentrifuge. DNA pellet was washed with 50 μl of cold 70% ethanol, dried, and dissolved in 200 μl of TE-SDS (pH 8.0, 0.1% SDS) buffer.

Hybridized membrane was washed twice with washing solution I (2×SSC, 0.1% SDS) at room temperature and followed by washing solution II (0.1× SSC, 0.1% SDS) twice at 68°C for 15 min. For the detection of hybridization, the membrane was rinsed with washing buffer (0.1 M maleic acid, pH 7.5; 0.15 M NaCl; 0.3% Tween 20 (v/v)) for 5 min and incubated in 100 ml of buffer 2 (0.1 M maleic acid, pH 7.5; 0.15 M NaCl; 10% Blocking reagent (W/V)) for 30 min at room temperature. The membrane was incubated in 20 ml of newly prepared buffer 2 added with anti-digoxigenin-AP conjugate to the final concentration of 75 mU/ml, for 30 min at room temperature. The membrane was washed with 100 ml of washing buffer with gentle agitation for 15 min twice at room temperature, and the equilibration of the membrane was performed in 20 ml of buffer 3 (0.1 M Tris-Cl; 0.1 M NaCl; 50 mM $MgCl_2$, pH 9.5) for 5 min at room temperature. The substances, 45 μl NBT and 35 μl X-phosphate, were added to 10 ml of newly prepared buffer 3 and the membrane was immerged in the solution and incubated during the color development at room temperature in the dark. When desired spot or band intensities were achieved, the reaction is stopped by washing the membrane for 5 min with 50 ml of TE (pH 8.0) buffer.

RESULTS

Cloning of genetic determinant for glycinecin gene(s). To identify glycinecin biosynthesis genes of *X. c.* pv. *glycines* 8ra, we screened individual members of a genomic library of *X. c.* pv. *glycines* 8ra for bacteriocin production. Five of 2,000 cosmids pG011, pG013, pG08, pG33, and pG35, that composed the genomic library of *X. c.* pv. *glycines* 8ra conferred glycinecin production to *E. coli* HB101, as indicated by the production of large clear zone on NA covered with indicator strain *X. c.* pv. *vesicatoria* Ds94-9 (Fig. 1). All five clones were able to produce bacteriocin on *X. c.* pv. *vesicatoria* Ds94-9 equally well. One of those five clones, pG08 was chosen arbitrarily for further detail characterization.

A restriction map of pG08 was constructed using *Bam*HI, *Hin*dIII, and *Eco*RI. The insert DNA has five *Eco*RI, four *Hin*dIII, two *Bam*HI sites (Fig. 2). A summation of the restriction fragments indicated that the size of the insert was about 21 kb.

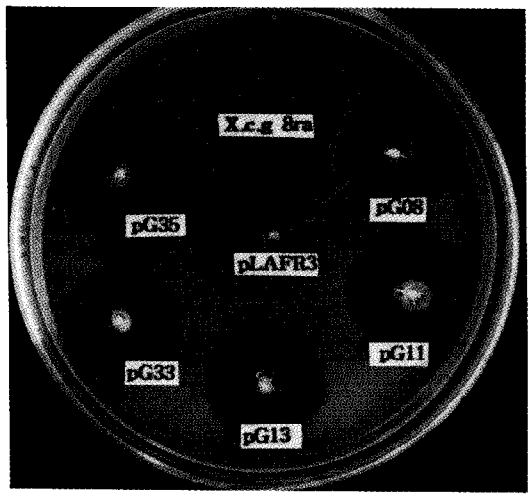

Fig. 1. Inhibition of *Xanthomonas campestris* pv. *vesicatoria* on nutrient agar medium by *X. c.* pv. *glycines* 8ra and *E. coli* HB101 carrying each subclone. *E. coli* HB101 carrying plasmid vector pLAFR3 was used as a negative control.

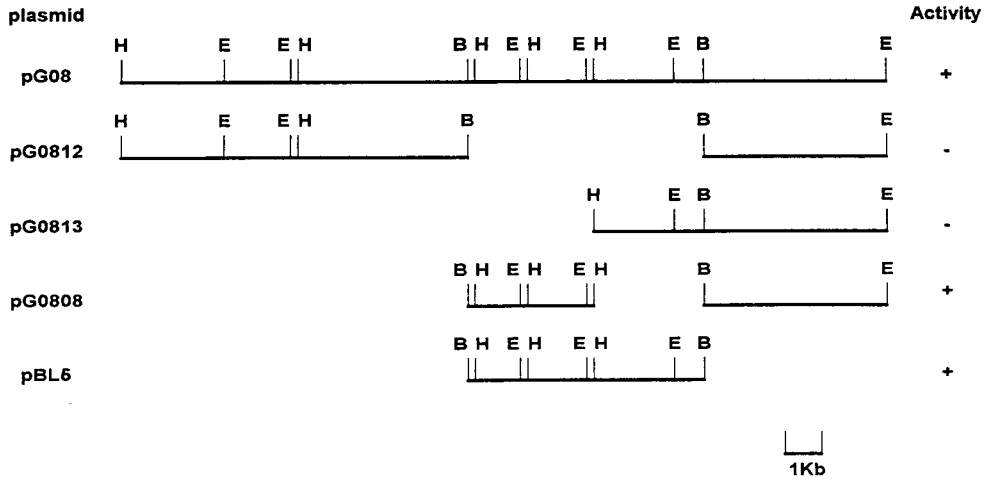

Fig. 2. Restriction maps of inserts of pG08 and recombinant plasmids. + : bacteriocin-producing; − : bacteriocin-nonproducing; E : *Eco*RI; H : *Hin*dIII; B : *Bam*HI.

In order to define regions responsible for the bacteriocin production, deletions were made in the 21 kb insert of pG08. According to the restriction map of pG-08, *Hin*dIII and *Bam*HI digestion and religation would delete various fragments of pG08. Manipulated three plasmids, pG0808, pG0812, and pG0813, were transformed into *E. coli* HB101. Three subclones were able to cover all the region of the insert DNA of pG08. Among three subclones only one clone, pG0808 was able to produce bacteriocin on NA media covered with *X. c.* pv. *vesicatoria* Ds94-9. Subclone pG0808 had two portions of DNA from pG08, 3.0 kb center and 6.7 kb left portions. Since other subclones which were not able to produce bacteriocin also included the 6.7 kb left portion, it is assumed that this 6.7 kb is not responsible for the bacteriocin production, but the 3.0 kb center portion seems responsible for the bacteriocin production. To confirm this assumption, 6.0 kb *Bam*HI DNA fragment from pG08 covering the 3.0 kb center portion of pG0808 was cloned into the plasmid vector pBluescript II SK (+) (Fig. 2), and bacteriocin production was tested. Plasmid pBL5 was able to produce bacteriocin (Fig. 3). Therefore, the 3.0 kb *Bam*HI-*Hin*dIII fragment from pG08 was responsible for the bacteriocin production.

Southern analysis for the DNA region for bacteriocin production. All five clones were screened for bacteriocin producing activity. To clarify that those five clones are carrying the same DNA portion with plasmid pG08 for bacteriocin production, southern analysis was done. Each plasmid DNA from the five clones and chromosomal DNA from *X. c.* pv. *glycines* 8ra were digested with restriction enzyme *Eco*RI and hybridized with labelled 1.6 kb *Hin*dIII fragment from pG08 as a probe. Among the five clones, two clones, pG33 and pG08 showed similarity and the other three clones were not hybridized with that fragment (Fig. 4). Chromosomal DNA also showed one strong band. Therefore, clone pG33 had the same DNA region with pG08 for the bacteriocin production.

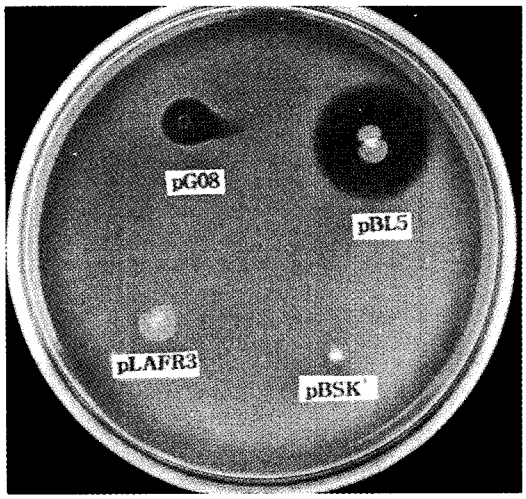

Fig. 3. Inhibition of *Xanthomonas campestris* pv. *vesicatoria* on NA by *E. coli* HB101 carrying pG08 or pBL5. *E. coli* HB101 carrying pLAFR3 was used as a negative control for pG08, and pBSK$^+$ (pBluescript II SK(+)) was used as a negative control for pBL5.

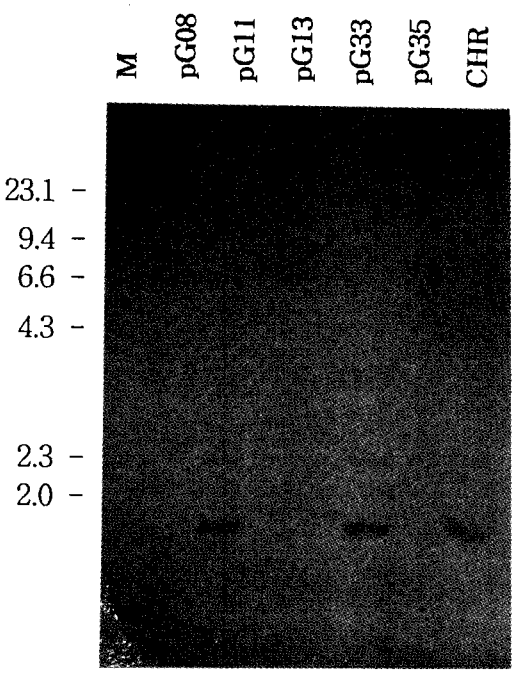

Fig. 4. Southern hybridization of plasmids isolated from five bacteriocin-producing clones and chromosomal DNA (CHR). DNA was digested with restriction enzyme *Eco*RI and hybridized with dig-labelled 1.6 kb *Hin*dIII fragment from pG08.

DISCUSSION

X. c. pv. *glycines* 8ra produced glycinecin, a bacteriocin, which is bactericidal to *X. c.* pv. *vesicatoria* Ds94-9. In a previous study by Fett *et al.* (5), the plasmid profiles of global *X. c.* pv. *glycines* strains were very similar to each other and plasmids did not carry glycinecin gene(s). Based on their study, the genomic library of *X. c.* pv *glycines* 8ra was tested directly for bacteriocin production. In general, bacteriocins are active only against closely related bacteria. Since glycinecin is not active against *E. coli*, a direct assay for glycinecin producing gene(s) in *E. coli* was possible.

Five clones with bacteriocin activity were isolated from two thousand cosmids that composed the genomic library of *X. c.* pv. *glycines* 8ra. This is the first report of the cloning genetic determinant for glycinecin production.

Among the five clones, two clones showed the similarity, however, the other clones did not. This suggests that *X. c.* pv. *glycines* 8ra contains at least two genetically different genes for glycinecin production. Several bacteria produce more than one bacteriocin. In the case of *E. coli*, about 17 genes are involved in colicin production and secretion. Almost all genes involved in colicin production were genetically well characterized and most of their functions are known. In the case of phytopathogenic bacteria, the genetic study for bacteriocin production has rarely done. Recently several studies about bacteriocin production of *Erwinia herbicola* and *Pseudomonas syringae* pv. *syringae* were reported (11, 23, 29). Both of them may have more than one bacteriocin. However, no genetic study supports this suggestion.

Bacteriocin has all the best characteristics which ideal biological control agent can have such as, 1) bacteriocin is very specific to target bacteria and it is not harmful against saprophytic beneficial bacteria; 2) it is easy to produce and requires low cost for production; and 3) it is very safe for environment and human being. However, bacteriocin also has several problems to be a practical and successful biological control agent in the field such as, low survivals, low stability, and lacks of carriers, etc. If we apply advanced molecular biology techniques to solve these problems, bacteriocin will be a ideal biological control agent in the near future.

요 약

콩 불마름병균 *Xanthomonas campestris* pv. *glycines* 8ra는 *X. c.* pv. *vesicatoria*에 길항력이 있는 bacteriocin인 glycinecin을 생성 분비한다. Bacteriocin 생성 분비 능력이 있는 콩 불마름병균을 효과적인 생물학적 방제원으로 활용하기 위해서는 좀더 체계적인 연구가 필요하여, bacteriocin 생성에 관계되는 유전자의 분리를 시도하였다. 약 2,000개의 *Xanthomonas campestris* pv. *glycines* 8ra cosmid library에서 bacteriocin의 생성 분비 능력을 조사하여 다섯 개의 clone을, pG011, pG013, pG33과 pG35, 선발하였다. 그중 한 clone pG08을 임의로 선택하여 plasmid DNA를 분리하였다. Plasmid pG08은 vector plasmid pLAFR3에 약 21 kb의 *X. c.* pv. *glycines* 8ra의 DNA가 끼어 있었으며, pG08에서 약 6.0 kb의 DNA를 떼어내어 다른 plasmid vector에 넣은 subclone pBL5는 bacteriocin의 생성 분비 능력이 있었다. Plasmid pG08을 제한효소 처리후 다시 접합시켜 만든 몇 개의 subclone과 pBL5의 제한효소 지도를 비교 분석한 결과 약 3.0 kb의 *Bam*HI-*Hin*dIII 부분의 DNA가 bacteriocin의 생성에 관계함을 알았다.

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