한국식물병리학회지 14(5) : 376-381(1998)
Korean J. Plant Pathol. 14(5) : 376-381(1998)

# Influence of Growth Conditions for the Production of Bacteriocin, Glycinecin, Produced by *Xanthomonas campestris* pv. *glycines* 8ra

**Woo Jung, Sunggi Heu and Yong Sup Cho***
*Department of Agricultural Biology, College of Agriculture and Life Sciences, Seoul National University, Suwon 441-744, Korea*

# 콩 불마름병균의 생장 조건이 박테리오신인 glycinecin의 생성에 미치는 영향

**우 정 · 허성기 · 조용섭***
서울대학교 농업생명과학대학 농생물학과

**ABSTRACT :** ***Xanthomonas campestris*** **pv.** ***glycines*** **8ra causes bacterial pustule disease on susceptible soybean leaves and produces a bacteriocin, named glycinecin, against related bacteria such as** ***Xanthomonas campestris*** **pv.** ***vesicatoria.*** **The antimicrobial activity of the glycinecin was effective to most tested** ***Xanthomonas*** **species.** ***X. c.*** **pv.** ***glycines*** **8ra was able to produce the glycinecin in liquid media as well as solid media. Maximal productivity of glycinecin was obtained at 30°C in the early stationary phase of growth of the** ***X. c.*** **pv.** ***glycines*** **8ra. The production of glycinecin was not dependent on the initial inoculum level but on cell density. Glycinecin was very sensitive to proteolytic enzymes such as trypsin and proteinase K but resistant to DNase and RNase. The culture supernatant of** ***X. c.*** **pv.** ***glycines*** **8ra retained some of its antimicrobial activity after 15 min at 60°C. It is stable at wide range of pH. The glycinecin showed the bactericidal activity after the adsorption of the glycinecin to the sensitive bacterial cell.**

*Key words* **: biological control,** ***Xanthomonas campestris*** **pv.** ***vesicatoria.***

Bacteriocin is antibiotic-like compounds with specificity primarily restricted to bacterial strains related to producer strains (11, 22). Species in over 30 bacterial genera, including many plant pathogens such as *Corynebacteria, Erwinias* and *Pseudomonas,* are known to produce proteinaceous bacteriocins (3, 5-8, 19, 22, 25, 26). The bacteriocin production from *Xanthomonas albilineans, Xanthomonas betiicola* and *Xanthomonas campestris* pathovars *juglandis, phaseoli* and *vesicatoria*, had been reported (9), but no data in support of their contention was presented. Bacteriocin production was first reported for *Xanthomonas campestris* pv. *glycines* by Fett *et al.* (6) and named as a glycinecin comparing to the glycin produced by *Pseudomonas syringae* pv. *glycinea* (18, 19). This glycinecin was inhibitory towards *X. c.* pv. *phaseoli* and *X. c.* pv. *vesicatoria* (7). However, no bacteriocin have been further characterized since all strains of *X. c.* pv. *glycines* were found to be capable of bacteriocin production only on agar media but not in liquid media.

*Corresponding author.

It is widely assumed that bacteriocin plays an important role in bacterial population dynamics with the resistance to and production of a bacteriocin being advantageous traits (7, 18, 22, 24). Bacteriocins are used in epidemiological investigations to type or differentiate strains that are otherwise indistinguishiable. Bacteriocin are also useful in identifying bacteria, because production of particular groups of bacteriocins tends to be restricted to certain taxonomic groups (7). Several biological control studies that use nonpathogenic mutants as a biological control agent have aimed at utilizing the production of bacteriocin as a mechanism for enhancing the biological control (22). In order to exploit the many advantages of using bacteriocin as a biological control agent, several problems associated including the stability in the field and the inducibility of the production of the bacteriocin should be solved.

In this work, we present the detection of the bacteriocin production of *X. c.* pv. *glycines* 8ra in liquid media and the influence of certain growth factors on the production of the bacteriocin by *X. c.* pv. *glycines* 8ra.

## MATERIALS AND METHODS

**Bacterial cultures and media.** The bacterial strain used for glycinecin production was a *X. c.* pv. *glycines* 8ra and a *X. c.* pv. *vesicatoria* YK93-4 was used as an indicator strain. The bacterial cultures were grown in Nutrient broth (NB, Difco) at 30°C.

**Assay for bacteriocin activity.** Bacteriocin production by *X. c.* pv. *glycines* 8ra was detected by the direct colony method (4). An overnight culture of strain *X. c.* pv. *glycines* 8ra was spotted onto nutrient agar (NA, Difco) media and incubated for 24~48 hr to allow colonies to develop and then the producer cells were killed with chloroform vapors. The indicator strain grown overnight in NB at 30°C was resuspend in a streile $H_2O$ at $OD_{600nm}$=0.1 and 0.2 ml of the suspension was added to a 9.8 ml of sterile agar solution (0.7%) and incubated at 50°C. The producer plates were overlaid with the mixture and incubated at 30°C for 24 hr. In order to screen the bacteria sensitive to glycinecin, 0.5 ml of the overnight culture filtrate of *X. c.* pv. *glycines* 8ra was spotted on bacteria streaked on NA plate and incubated at 30°C for 24~48 hr.

Bacteriocin activity of the supernatant was assayed by the agar diffusion method (16), with some modifications. About 10 μl of chloroform was added to 500 μl of *X. c.* pv. *glycines* 8ra culture and vortex vigorously for 1 min to kill bacteria. After centrifugation for 1 min at 11,000 g, 5 μl of serial dilutions of the cell-free and chloroform-free supernatant were spotted on the NA plate. Indicator strains were overlaid as explained above and incubated for 24 hr at 30°C. The bacteriocin activity was defined as the reciprocal of the highest dilution forming a visible halo was expressed as activity units per ml (AU/ml).

**Bacteriocin production by initial inoculation levels.** Washed overnight culture of *X. c.* pv. *glycines* 8ra was resuspended in NB and a portion of the resuspension was transferred into a new tube containing 5 ml of NB to final concentraion of $10^8$, $10^7$, $10^6$, $10^5$, $10^4$, $10^3$, $10^2$ and $10^1$ cfu/ml, respectively and incubated for 2 days at 30°C with 150 rpm. Bacteriocin activity was tested every 6 hour as described above.

**Influence of temperature and pH on bacteriocin production.** The inhibitory activity of the bacteriocin was tested at different temperatures and pH values. In order to study the effect of pH, the initial pH of NB was adjusted with HCl or NaOH from 4.0 to 9.0. Changes in cell density (optical density at 600 nm), pH and bacteriocin activity were monitored every 6 hour as mentioned above. The effect of temperature was studied using a NB that is adjusted to pH 7.0.

**Effect of temperature and pH on bacteriocin activity.** Cell-free culture supernatants were obtained by centrifugation (16,000 g for 5 min) after chloroform-treated. The bacteriocin was incubated at different pH (from 3.0 to 11.0) at 30°C for a day and the activity was assayed as described above.

**Sensitivity of bacteriocin to enzymes and heat.** Inactivation of glycinecin by various enzymes or heat was conducted with crude cell-free supernatant of *X. c.* pv. *glycines* 8ra. Trypsin (bovine pancreas, Type III, Sigma Chemical Co., St. Louis, Missori) and proteinase K (Type XIV, Sigma Chemical Co.) were prepared in 0.05 M Tris-HCl buffer, pH 7.3, with 0.1 M $CaCl_2$. Ribonuclease A (RNase A) (93.7 Kunitz units/mg, bovine pancreas, protease free, Calbiochem-Behring, San Diego, California) was prepared in 0.05 M Tris-HCl buffer, pH 8.1. Deoxyribonuclease I (DNase) (bovine pancreas, Type III, Sigma Chemical Co.) was prepared in 0.05 M Tris-Hcl buffer, pH 8.1 with 10 mM $MgSO_4 \cdot 7H_2O$. All enzyme solutions were prepared at 10 mg/ml. ProteinaseK was also prepared at 5 mg/ml. Cell-free supernatants were incubated with various enzymes each at final concentration of 500 μg/ml for 60 min at 37°C. To determine the thermal stability of glycinecin, the cell-free supernatant (0.5 ml in different tubes) was treated separately at 40, 50, 60, 70, 80 and 90°C for 15 min and kept in a boiling water bath for 10 and 15 min. The tubes were cooled in ice water and assayed for bacteriocn activity as mentioned above.

**Influence of culture media on bacteriocin production.** Various media including NB (Nutrient broth, Difco), PSB (peptone-sucrose broth : Bacto-peptone, 10 g; Sucrose, 10 g; Sodium glutamate, 1 g per liter), LB (Luria-Bertani broth : Bacto-peptone, 10 g; Yeast extract, 5 g; NaCl, 10 g per liter), and M9 minimal media (Sambrook *et al.*, 17), were tested for their ability to support the growth of *X. c.* pv. *glycines* 8ra and the production of the bacteriocin.

**The bacteriocin adsorption test.** The tested bacteria were grown overnight in NB and spun down the cell by centrifugation at 6,000 rpm for 10 min. The active *X. c.* pv. *glycines* 8ra supernatant was added to the bacterial pellet and series of diluted bacteria with the same supernatant were incubated at 30°C. After 1 hr incubation, the bacterial culture was centrifuged for 1 min at 11,000 g and the supernatant was taken to measure the bacteriocin activity as explained above.

## RESULTS

**Bacteriocin production.** *X. c.* pv. *glycines* 8ra showed antibacterial activity against indicator strain *X. c.* pv. *vesicatoria* YK93-4 on Nutrient agar at 30°C overnight. The antibacterial action appeared to be highly specific to other *Xanthomonas* strains, whereas it was not active against various gram-positive and gram-negative species tested (Table 1). Bacteriophage was ruled out as the inhibitory agents by two methods. In the first method, the active agent was tested for transfer from the inhibition zone to a freshly prepared lawn of the indicator strain by use of a sterile wooden toothpick. No inhibitory agent was transferred in this manner. Secondly, agar plug from the inhibition zones were removed, crushed in a small volume of Difco nutrient broth (NB) and spotted onto a freshly prepared lawn of the indicator strain. Again no tranfer of an inhibitor agent occured.

*X. c.* pv. *glycines* 8ra was shown to produce antibacterial substance in liquid culture too. Overnight culture supernatants of *X. c.* pv. *glycines* 8ra were sterilized by filtration and tested for the presence of antibacterial activity by clear inhibition zone assay on agar plates. *X. c.* pv. *glycines* 8ra was found to produce and secrete a protein factor in liquid culture, called glycinecin hereafter, which strongly reduced the growth of the *X. c.* pv. *vesicatoria* YK93-4 indicator strain utilized. The glycinecin production and/or secretion was not affected by the growth medium for the producer strain *X. c.* pv. *glycines* 8ra. Antibacterial activity was observed for growth in various minimal media or rich media (Data not shown).

In order to find out the relationship between the glycinecin production and the growth phase of the *X. c.* pv. *glycines* 8ra, culture supernatant of *X. c.* pv. *glycines* 8ra grown in NB at 30°C were collected at various times during exponential and stationary growth phase. Samples were filter-sterilized and tested for bacteriocin activity by clear inhibition zone assay on agar plates. As shown in Fig. 1, bacteriocin activity was increased exponentially entering the stationary phase. To test whether the detection of the bacteriocin activity from the early exponential growth phase was dependent on the growth phase of the bacteria or the concentration of the bacteria, several 5 ml nutrient broth were inoculated with various initial inoculum sizes of *X. c.* pv. *glycines* 8ra. After incubation at 30℃, bacteriocin production by *X. c.* pv. *glycines* 8ra always occurred in liquid medium after certain time with any initial inoculum level ($10^8$-$10^1$ cfu/ml) (Table 2). It suggests that the production of glycinecin by *X. c.* pv. *glycines* 8ra was not dependent on initial inoculum level for bacteriocin production, but the certain numbers of bacteria in the medium. Whenever the concentration of bacteria in the medium was over $10^7$ cfu/ml, the bacteriocin activity was detected. Therefore, the higher inoculum, the sooner bacteriocin production was detected (Table 2). However, the bacteriocin production was not

**Table 1.** Antimicrobial spectrum of glycinecin produced by *Xanthomonas campestris* pv. *glycines* 8ra.

| Tested organism | No. of strains inhibited/no. tested |
|---|---|
| *Agrobacterium radiobacter* | 0/1 |
| *Agrobacterium tumefaciens* | 0/1 |
| *Bacillus subtilis* | 0/2 |
| *Enterobacter faciens* | 0/2 |
| *Erwinia carotovora subsp. carotovora* | 0/2 |
| *Erwinia stewartii* | 0/1 |
| *Pseudomonas aeruginosa* | 0/1 |
| *Pseudomonas fluorescens* | 0/1 |
| *Pseudomonas putida* | 0/1 |
| *Pseudomonas syringae* pv. *glycines* | 0/3 |
| *Pseudomonas syringae* pv. *syringae* | 0/2 |
| *Pseudomonas syringae* pv. *tabaci* | 0/1 |
| *Pseudomonas syringae* pv. *tomato* | 0/1 |
| *Pseudomonas tolassii* | 0/2 |
| *Ralstonia solanacearum* | 1/1 |
| *Xanthomonas axonopodis* pv. *aurantifolii* | 1/1 |
| *Xanthomonas axonopodis* pv. *diefenbachiae* | 1/1 |
| *Xanthomonas campestris* pv. *campestris* | 3/3 |
| *Xanthomonas campestris* pv. *citri* | 5/5 |
| *Xanthomonas campestris* pv. *glycines* | 0/4 |
| *Xanthomonas campestris* pv. *pruni* | 1/1 |
| *Xanthomonas campestris* pv. *vesicatoria* | 30/30 |
| *Xanthomonas oryzae* pv. *oryzae* | 3/3 |

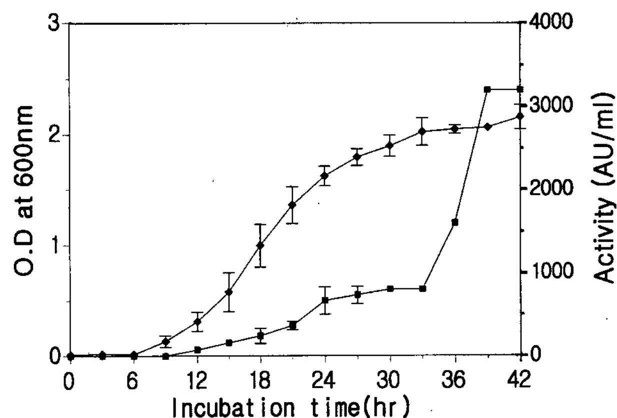


**Fig. 1.** Growth of *Xanthomonas campestris* pv. *glycines* 8ra (◆, OD at 600 nm) in Nutrient Broth at 30°C and the production of bacteriocin (■, AU/ml).

**Table 2.** Effect of initial inoculum size on glycinecin production by *Xanthomonas campestris* pv. *glycines* 8ra.

| Inoculum size (cfu/ml) | Bacteriocin activity | |
|---|---|---|
| | First detected (h/AU) | Max. (h/AU) |
| $10^9$ | 0/100 | 8/3200 |
| $10^8$ | 4/100 | 12/3200 |
| $10^7$ | 8/200 | 16/3200 |
| $10^6$ | 8/200 | 20/3200 |
| $10^5$ | 16/200 | 24/3200 |
| $10^4$ | 20/200 | 32/3200 |

induced by the concentration of the producer bacteria since the 100 times concentrated supernatant of the $10^5$ cfu/ml of *X. c.* pv. *glycines* 8ra with lyophylizer have shown the same bacteriocin activity with that of $10^7$ cfu/ml.

**Effects of enzymes, organic solvents, heat, and pH on glycinecin activity.** The chloroform-treated supernatnat of *X. c.* pv. *glycines* 8ra was tested for sensitivity to various enzymes, organic solvents, heat, and pH values. As shown in Table 3, the antimicrobial activity was not affected by treatment with lysozyme, ribonuclease A, DNase, or any of the organic solvents used, while it was completely lost after treatments with trypsin, chymotrypsin, and proteinase K, thus suggesting the proteinaceous nature of the bacteriocin. Glycinecin was also relatively resistant to heat; the full activity was maintained up to 50°C and some activity remained even after 15 min incubation at 100°C (Table 3). Glycinecin was also active over a wide pH range, with only partial sensitivity to extremely acidic or extremely basic conditions (Table 4).

**Mode of action.** To determine whether glycinecin had a bactericidal or a bacteriostatic effect, the indicator *X. c.* pv. *vesicatoria* YK93-4 was grown in the cell free or/and heat-treated supernatant of the *X. c.* pv. *glycines* 8ra. The number of surviving indicator bacterial cells was determined by plate counting at various times after the addition of the supernatant. As shown in Fig. 2, the viable cell count of indicator strain *X. c.* pv. *vesicatoria* YK93-4 was decreased dramatically within 3 hours of incubation with the active supernatant and further reduction of cell number was observed with increasing time of incubation. In contrast to that, the cell number of the indicator grown in heat-treated and inactive supernatnat was increased with increasing time of incubation. As with the decreasing number of the bacterial cell, the absorbance of the supernatant was decreased dramatically.

**Table 3.** Effects of enzymes, organic solvents, and heat on glycinecin activity

| Treatment | Activity (Au/ml) |
|---|---|
| None | 3200 |
| trypsin | 0 |
| Chymotrypsin | 0 |
| Proteinase K | 0 |
| DNase | 3200 |
| RNase | 3200 |
| Lysozyme | 3200 |
| Acetone | 3200 |
| Acetonitrile | 3200 |
| Butanol | 3200 |
| Chloroform | 3200 |
| Ethyl alcohol | 3200 |
| Ether | 3200 |
| Ethyl acetate | 3200 |
| Hexane | 3200 |
| Methyl alcohol | 3200 |
| Toluene | 3200 |
| Incubation (15 min) at: | |
| 30°C | 3200 |
| 40°C | 3200 |
| 50°C | 3200 |
| 60°C | 800 |
| 70°C | 100 |
| 80°C | 100 |
| 90°C | 100 |

**Table 4.** Effects of pH on glycinecin activity

| Incubation 1 day at 30°C | Activity (Au/ml) |
|---|---|
| pH 2.0 | 100 |
| pH 3.0 | 200 |
| pH 4.0 | 800 |
| pH 5.0 | 1600 |
| pH 6.0 | 3200 |
| pH 7.0 | 3200 |
| pH 8.0 | 3200 |
| pH 9.0 | 1600 |
| pH 10.0 | 800 |
| pH 11.0 | 200 |

**Adsorption of Glycinecin to bacterial cells.** Glycinecin was adsorbed to sensitive bacterial cells such as *X. c.* pv. *vesicatoria* YK93-4. The viable cell count was decreased dramatically in the presence of active bacteriocin fraction (Table 5). The rate of the decreasing bacteriocin activity was dependent on the number of the susceptible bacteria. About 75% of the glycinecin was adsobed to the susceptible cell with $5\times10^9$ cfu/ml within an hour and about 50% of the glycinecin was adsorbed with $5\times10^8$ cfu/ml. More than 4 hour incubation with $5\times10^9$ cfu/ml made more than 90% adsorption of the bacteriocin to the susceptible cell.

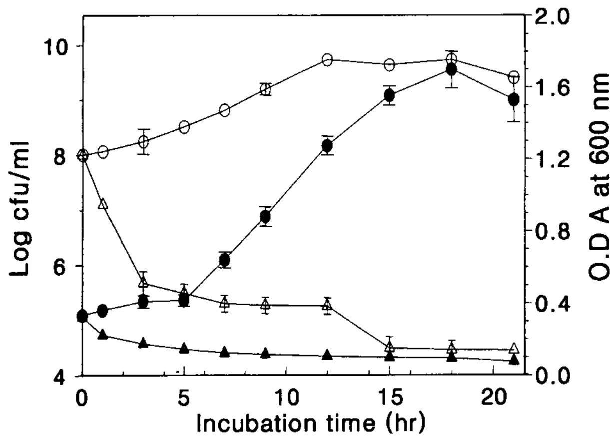


**Fig. 2.** Growth of *Xanthomonas campestris* pv. *vesicatoria* YK93-4 in *Xanthomonas campestris* pv. *glycines* 8ra cell-free supernatant; crude (△, ▲) and heat-treated (○, ●).

**Table 5.** The decrease of the activity by the adsorption of the glycinecin to the indicator bacterial cell.

| Tested strain | Concentration (cfu/ml) | Activity after 1 hr (Au/ml) | Adsorption (%) |
|---|---|---|---|
| *X. c.* pv. *vesicatoria* YK93-4 | $5\times10^9$ | 800 | 75 |
| | $5\times10^8$ | 1600 | 50 |
| | $5\times10^7$ | 3200 | 0 |
| | $5\times10^6$ | 3200 | 0 |
| *Escherichia coli* | $5\times10^9$ | 3200 | 0 |
| | $5\times10^8$ | 3200 | 0 |

* % adsorption=(1-AU in cell-free supernatant fluid / AU control) ×100

However, the activity of the supernatant coincubated with resistant *E. coli* cells still remained the maximum activity. These suggest that the bacteriocin was adsorbed to the bacterial cell and coprecipitated with bacterial cell by centrifugation.

## DISCUSSION

Though the production of glycinecin by *X. c.* pv. *glycines* in a solid medium has been reported (Fett *et al*, 1985), this is the first report on the production of glycinecin by *X. c.* pv. *glycines* 8ra in a liquid medium. Filtered bacterial culture contained bactericidal activity against *X. c.* pv. *vesicatoria* YK93-4. Maximal productivity of glycinecin was obtained in a NB maintained at initial pH between 6.0 and 8.0, and incubated at 30 °C in the early exponential to the early stationary phase of growth of *X. c.* pv. *glycines* 8ra. The glycinecin is produced even in logarithmic growth but just the concentration of produced bacteriocin may not be enough to be detected. This indicated that glycinecin might not be secondary metabolites produced during the stationary phase. Saucier *et al.* (1995) reported that bacteriocin production by *Carnobacterium piscicola* LV17 was dependent on the size of inoculum because of the inducing effect of the residual bacteriocin in the inoculum size. However, in this study, the production of glycinecin by *X. c.* pv. *glycines* 8ra was not dependent on the initial inoculum. Regardless of the level of initial inoculum, the glycinecin activity was detected when the bacterial concentration reached more than $10^7$ cfu/ml. The most bacteriocin production was induced by the UV or mitomycinC but glycinecin produced by the *X. c.* pv. *glycines* 8ra was not induced by the UV or mitomycinC (data not shown). It suggested that the mechanism for bacteriocin production by *X. c.* pv. *glycines* 8ra may be different to the mechanism of other bacteriocin production.

The glycinecin is very stable protein. Glycinecin was sensitive proteases such as proteinase K, chymotrypsin, and trypsin, but resistant to other chemicals tested (Table 3). It is relatively stable between pHb4.0~10.0. The supernatant of *X. c.* pv. *glyciens* 8ra has kept bacteriocin activity even after 1 year incubation of supernatant at room temperature.

Though we don't know exact mechanism for this glycinecin, this glycinecin activity is the bacteriocidal after the bacteriocin adsorbed to the susceptible cells.

All these characteristics of glycinecin produced by *X. c.* pv. *glycines* 8ra may be a good nature as a biological control agent. It is known that the bactericoin can be good biological control agent. But only few bacteriocin is a active biological control agent since the restricted characteristics of the bacteriocin such as the induction of the production of the bacteriocin by the UV or mytomycinC. Those inducible characteristics made bacteriocin useless as a biological control agent in the field. Since the glycinecin production is not induced and glycinecin is very stable at wide range of temperature, both supernatant and the bacterial culture can be used as a biological control agent in the field.

## 요 약

콩 불마름병을 일으키는 병원균인 *Xanthomonas campestris* pv. *glycines* 8ra는 *Xanthomonas campestris* pv. *vesicatoria*와 같이 비슷한 균에 대해 glycinecin 이라는 bacteriocin을 분비한다. Glycinecin의 길항 작용은 test한 거의 모든 *Xanthomonas* 종에 대하여 효과가 있었다. *X.*