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Genus-Specific Distribution and Pathovar-Specific Variation of the Glycinecin R Gene Homologs in *Xanthomonas* genomes

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***Xanthomonas axonopodis* pv. *glycines* produces bacteriocins called glycinecin, and two glycinecin genes, *glyA* and *glyR*, were reported previously. In this paper, we describe genomic distribution and variation of the *glyR* gene revealed by extensive Southern hybridization analysis. In contrast to the *glyA* gene present only in *X. axonopodis* pv. *glycines,* the *glyR* gene was found to be distributed widely in all the pathovars of *Xanthomas* genus. It was also found that the *glyR* gene is a multigene family while the *glyA* is a single copy gene. Moreover, the copy number and the variation of the *glyR* multigene are unique to each pathovar of *Xanthomonas*. The uniqueness can be easily detected by the patterns resulted from Southern hybridization using the genomic digests. Thus, we suggest the *glyR* gene can serve as a useful genus-specific and pathovar-specific DNA marker for *Xanthomonas*. One of the *glyR* homologs was further isolated from *X. axonopodis* pv. *glycines*, and analyzed to be functional with strong inhibitory activity against several members of *Xanthomonas*.**

***Keywords*:** glycinecin, glyR, multigene, *Xanthomonas*, genus-specific, pathovar-specific

Xanthomonas axonopodis pv. *glycines* is known to produce bacteriocins named glycinecin (Fett *et al.*, 1987). So far two distinct genomic regions responsible for glycinecin production were isolated, and the gene structures for glycinecin A (*glyA*) and glycinecin R (*glyR*) were reported, respectively (Ahn and Cho, 1996). The active form of glycinecin A is a heterodimer consisting of 39- and 14-kDa subunits, products of the two adjacent *gly* genes, *glyA* and *glyB* (Ahn and Cho, 1996; Jung *et al*., 1998; Wu *et al*., 1998; Heu *et al.*, 2001). Southern hybridization and PCR amplification indicated that one copy of the *glyA* gene is present in all the pathovars of *X. axonopodis* pv. *glycines* but not in other bacterial strains tested (Oh *et al.*, 1999). Studies by Pham *et al.* (2004) suggested the bacteriocidal mechanism of glycinecin A is correlated with the permeability of membranes to hydroxyl and potassium ions.

The second glycinecin gene, *glyR*, was isolated and studied by our group (GenBank accession no. AY520840). The *glyR* gene was estimated to be over 4 kb long, extremely large as a bacterial gene. It encodes a high molecular weight bacteriocin, glycinecin R, and part of the protein shows homology to the *Rhs* element of unknown function in *Escherichia coli* and toxin complex (tc) gene of *Photorhabdus* at the protein level (Hill *et al*., 1995; Browen *et al*., 1998; ffrench-Constant *et al*., 2007). The gene structure and the biochemical nature of the bacteriocin activity is being characterized in detail by analyzing a cosmid clone carrying a *glyR* gene.

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In this paper, existence of *glyR* homologs in the genomes of *Xanthomonas* and also in other bacterial genomes of different genus were examined. Genomic distributions and variations were revealed by extensive Southern hybridization analysis, and one of the *glyR* homologs was tested for its functionality as a bacteriocin.

Materials and Methods

Bacterial strains

Strains of *Xanthomonas* and other bacterial species used in this study were isolated from various host plants at locations in Korea. 104 isolates of *X. axonopodis* pv. *glycines* were also collected from various parts of 20 susceptible soybean cultivars for three years. Strains were identified by Biolog GN microplate system (Biolog Inc., USA). Peptone sucrose agar (PSA) and yeast-dextrose-$CaCO_3$ agar (YDC) were used.

Screening of cosmid library

Genomic cosmid library of *X. axonopodis* pv. *glycines* in pLAFR was a gift of E. J. Braun (University of Illinois, Urbana-Champaign). The individual clones of the library were grown in 96-well plates. Cosmid clones were transferred onto the solid media in rectangular plates of the same size as a 96-well plate using a replica stamp with a 96-pin head. After clones were grown overnight, soft agar overlay method (described in the following section) was applied to screen clones containing the genomic region responsible for producing bacteriocin using *X. campestris* pv. *vesicatoria* 833 (race 3) as an indicator.

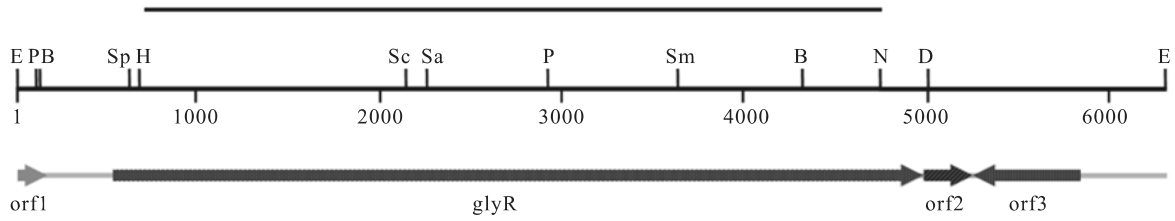


Fig. 1. Location of the probe used in Southern hybridization analysis. The restriction map of 6.3 kb *Eco*RI fragment was shown on the top, and the corresponding gene map was shown on the bottom. The probe was *Hin*dIII-*Nsi*I fragment (4,061 bp) indicated by a bar above the restriction map. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; N, *Nsi*I; Sa, *Sal*I; Sc, *Sca*I; Sp, *Sph*II

DNA preparation from cosmid and plasmid clones

For sequencing and Southern hybridization, modified PEG DNA minipreparation method was used (Moon *et al*., 1991), and two-step minipreparation method was used for brief analysis of clones and subclons (Moon *et al*., 1991).

Genomic DNA preparation from various bacteria

Single colony was inoculated and grown in Luria-Bertani broth at 30°C for 16 h. After collected by centrifugation at 4,000×g for 15 min, the cells were incubated with lysozyme for 60 min at room temperature. Then 10% SDS was added, and the mixture was incubated at 37°C for 30 min. It was further incubated for 1 h at 37°C with RNaseA (10 mg/ml). After treated with proteinase K (20 mg/ml), the mixture was extracted with phenol/chloroform, and then precipitated with 1/10 vol of 3 M sodium acetate (pH 7.0) and 2.5 vol of ethanol. DNA pellet was washed in 70% ethanol and dried in the air, and then resuspended in TE buffer by heating in 50~60°C water bath for 10 min.

Southern hybridization

Sourthern hybridization was performed as described in a previous paper (Moon *et al.*, 1991). The *Hin*dIII-*Nsi*I fragment (4,061 bp) of the *glyR* gene contained in the cosmid clone was purified as the probe from a low-melting point agarose gel, and the probe DNAs were labeled using random hexamer as described in the paper. Total genomic DNA digested with *Eco*RI was electrophoresed in either 0.8% or 1% agarose gel. Transfer of the DNA onto a Nytran membrane and hybridization was carried out as described in the paper.

Bacteriocin activity assay

Soft agar overlay (overlay halo assay) method was used for the detection of bacteriocin activity. Indicator strain, *X. campestris* pv. *vesicatoria* 833 (Race1) was grown in 5 ml LB broth at 28~30°C for 16~20 h. Producer strains or clones were dotted (0.3 cm in diameter) and grown on a LB plate at 37°C for 20 h. Then 500 µl of chloroform was dropped on inside of the cover, and then the bottom part of the plate was placed upside down on the cover. After the producer cells on the surface of agar were lysed by exposure to chloroform for 5 min, chloroform was evaporated completely for 10 min. One hundreds microliter of indicator cells prepared as mentioned above was mixed with 7 ml of soft agar (0.7% agar in LB, 50°C), and then overlaid on producers grown on the agar plate. Plates were then incubated overnight at 28 or 37°C and inspected for a clear zone (halo) surrounding producer cells.

Alternatively, liquid culture was also used for bacteriocin activity assay. Cells were grown in 5 ml of LB with appropriate antibiotics at 37°C overnight or 16 h. Then, 100 µl of the culture was added to 10 ml (1/100 dilution) of new media and grown at 37°C for 12 h. The cells were collected by centrifugation at 4,000×g for 20 min, and then resuspended in 1 ml of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8). After the cell suspension was frozen at -20°C, it was melted again on ice. The cells were sonicated ten times at 13 watts for 5 sec burst and 5 sec rest interval. After centrifuged at 10,000×g for 30 min, the supernatant was trans-

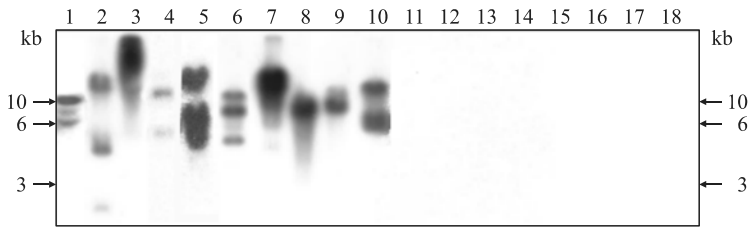


Fig. 2. Southern hybridization analysis using total genomic DNA from 18 bacterial species Genomic DNAs were digested with *Eco*RI, fractionated on a 0.8% agarose gel, and then transferred to a Nytan membrane. The membrane was hybridized with ^{32}P-labelled *glyR* gene (*Hin*dIII-*Nci*I fragment) as shown in Fig. 1. Bacterial strains corresponding to the lanes 1~18: 1, *X. axonopodis* pv. *glycines* 8ra; 2, *X. campestris* pv. *campestris* 1963; 3, *X. campestris* pv. *qorarium*; 4, *X. axonopodis* pv. *citri* 875; 5, *X. campestris* pv. *carotae* ATCC 10547; 6, *X. campestris* pv. *vesicatoria* sdl833; 7, *X. axonopodis* pv. *dieffenbachiae*; 8, *X. campestris* pv. *sesame* 914; 9, *X. arboricola* pv. *pruni* sdl2065; 10, *X. oryzae* pv. *oryzae* KXO169; 11, *A. tumefaciens*; 12, *B. subtilis*; 13, *E. coli*; 14, *P. syringae* pv. *aptata*; 15, *P. syringae* pv. *syringe* 61; 16, *P. syringae* pv. *tabaci* 14340; 17, *P. syringae* pv. *tabaci* 33618; 18, *P. syringae* pv. *tomato*

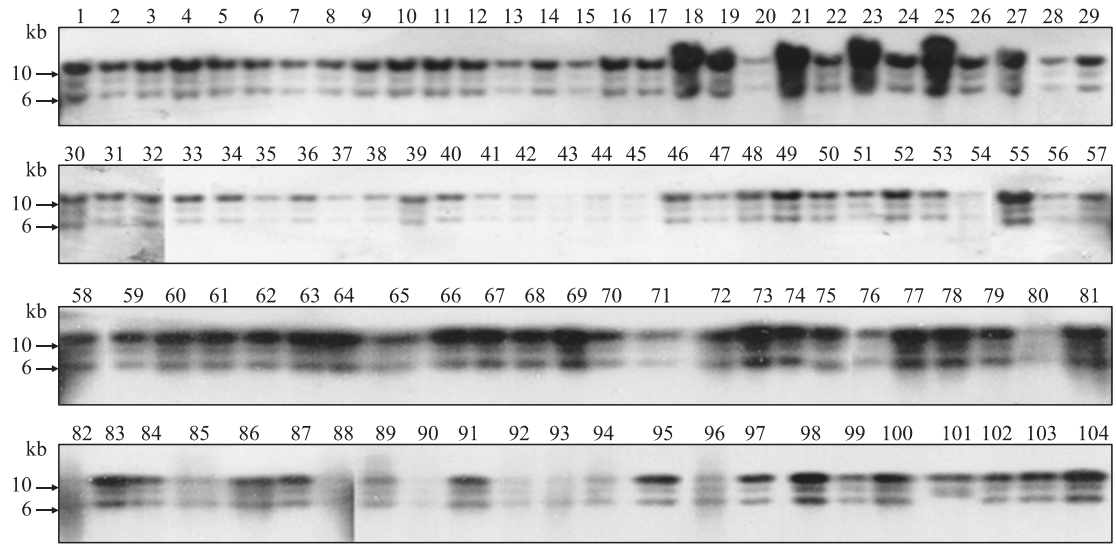


Fig. 3. Southern hybridization analysis using total genomic DNA from 104 isolates of *X. axonopodis* pv. *glycines*. Hybridization was carried out as in Fig. 2. Numbers 1~104 indicate 104 isolates of *X. axonopodis* pv. *glycines*, and isolates #1 is *X. axonopodis* pv. *glycines* 8ra from which the *glyR* gene was previously isolated and studied in detail.

ferred into a new tube. For each bacteriocin activity assay, 20 μl of the supernatant was applied onto filter discs on a agar plate or directly onto solid agar. Indicator cells were overalyed and clear zones were examined as described above for assay using colonies.

Results and Discussion

Genus-specific distribution of *glyR* homologs in *Xanthomonas genomes*

Previously, a cosmid clone was isolated from a genomic library of *X. axonopodis* pv. *glycines* 8ra, and the *glyR* gene responsible for the production of a bacteriocin, Glycinecin R was identified. In this study, the presence of the *glyR* gene in various bacterial species was examined. To systematically detect any *glyR* homologs in the genomes of wide variety of bacterial species, a series of Southern hybridization analysis using the *glyR* gene as the probe (Fig. 1) were performed. As shown in Fig. 2, all the *Xanthomonas* species contain at least one copy of the *glyR* homologs, while none of other bacterial species in different genus contains the homolog. Thus, it is reasonable to conclude the *glyR* gene is specific to the *Xanthomonas* genus, and we suggest that the *glyR* sequence may be useful as the DNA marker for the preliminary identification of *Xanthomonas* species.

Unique copy number and variation of the *glyR* homolog in the genomes of *X. axonopodis* pv. *glycines* isolates

Distribution and variation of the *glyR* homologs in the genomes of isolates of *X. axonopodis* pv. *glycines* were examined in detail by Southern hybridization analysis. As shown in Fig. 3, the hybridization results revealed all the 104 isolates have three copies of the *glyR* homologs. Furthermore, all but one isolate showed the same hybridization pattern of 6.3 kb, 8 kb, and 13 kb when the genomic DNAs were digested with *Eco*RI enzyme. In the case of the isolate #101, it is very likely that the lowest 6.3 kb band was shifted up by a point mutation which destroyed the *Eco*RI site. It is reasonable to conclude that the ladder-pattern of 6.3 kb, 8 kb, and 13 kb is typical for *X. axonopodis* pv. *glycines*, and thus we suggest that the specific ladder-pattern of the *glyR* homolog is useful as a DNA maker for the identification of the pathovar *glycines* among *X. axonopodis* species. In addition to the previously isolated cosmid clone containing the *glyR* gene within 6.3 kb *Eco*RI fragment, the second cosmid clone containing a *glyR* homolog within 13 kb *Eco*RI fragment was isolated in this study, and was discussed further in the following sections.

Pathovar-specific variation of the *glyR* homologs in the genome of *Xanthomonas*

In addition to *X. axonopodis* pv. *glycines*, distribution patterns were analyzed in detail for other pathovars of *Xanthomonas* chosen from Fig. 2. The genomic Southern hybridization analysis was also carried out for three pathovars, *X. axonopodis* pv. *citri*, *X. axonopodis* pv. *dieffenbachiae*, and *X. campestris* pv. *vesicatoria.* All the isolates of pathovar *citri* showed the multiple pattern of two bands (Fig. 4), while the pattern of three bands is typical in pathovars *dieffenbachiae* and *vesicatoria* as seen in *pathovar glycines* (Fig. 4). However, the three-band patterns of *glycines*, *dieffenbachiae*, and *campestris* are not identical, but unique to each pathovar.

Since bacteriocins are so widespread and diverse, it could provide clues to genomic variations in even closely related bacteria. Frey *et al*. (1996) carried out bacteriocin typing for *B. salaccearum*, and reported its correlation with genomic variation of the pathogen. Strains of the sweet potato soil rot pathogen *Streptomyces ipomoeae* had been divided into three groups based on their ability to inhibit one another

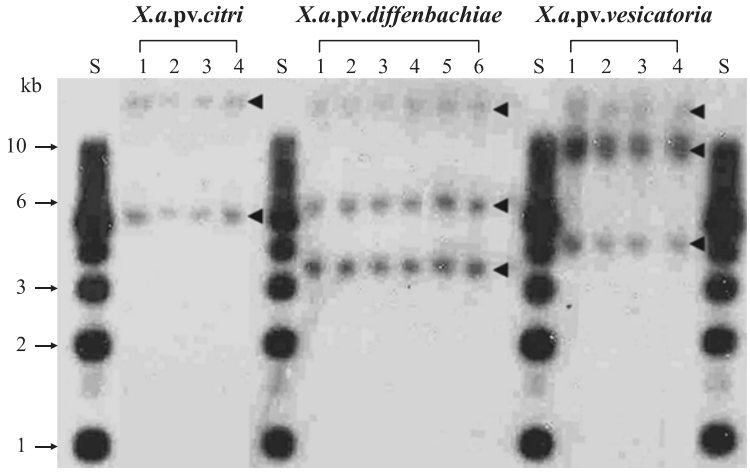


Fig. 4. Southern hybridization analysis using total genomic DNA from isolates of *X. axonopodis* pv. *citri, X. axonopodis* pv. *dieffenbachiae*, and *X. campestris* pv. *vesicatoria*. Hybridization was carried out as in Fig. 3. Numbers indicate isolates of each pathovars, respectively. S; 1 kb size maker

by the production of various bacteriocins including ipomicin during pairwise cocultivation (Zhang *et al*., 2003). Our findings also support the idea that bacteriocin typing could be powerful to detect genomic variation.

In summary, the multiple copies of the *glyR* homolog exist in pathovar-specific pattern in the pathovars *glycines*, *dieffenbachiae*, *citri*, and *campestris.* Not only these four pathovars studied in detail in this section, but all the seven different pathovars *Xanthomonas* showed a unique multiplicity pattern as shown in Fig. 2. Therefore, we suggest the unique pathovar-specific variation of the *glyR* multigene family is useful as a DNA marker, and thus can serve as the critical criteria in identifying various pathovars of *Xanthomonas* species.

Isolation of the *glyR* homolog from *X. axonopodis* pv. *glycines*

As shown in Fig. 2 and 3, we detected three *glyR* homologs

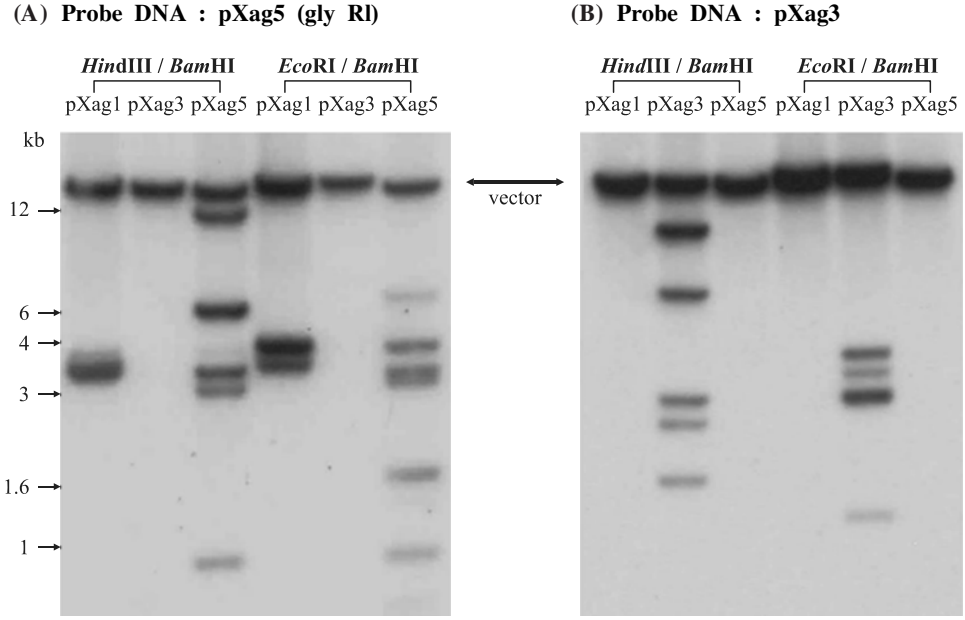
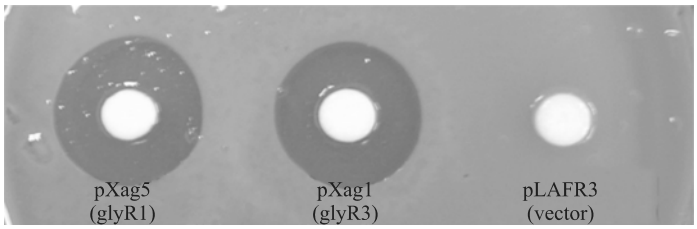


Fig. 5. Cross hybridization of cosmid clones containing genes for glycinecin production. Recombinant cosmid DNAs isolated from each clone were digested doubly with *Hin*dIII/*Bam*HI or *Eco*RI/*Bam*HI, respectively. Digested DNAs were fractionated on a 1% agarose gel, and then transferred to a Nytan membrane. DNA from pXag5 and DNA from pXag3 were used as the probes for panel A and panel B, respectively. The whole cosmid DNA from the clones was digested triply with *Hin*dIII, *Bam*HI, and *Eco*RI, and then labelled using random hexamer method. Hybridization was carried out as described in Fig. 2.

(A) Target strain : *Xanthomonas campestris* pv. *vesicatoria*



(B) Target strain : *Xanthomonas oryzae* pv. *oryzae*

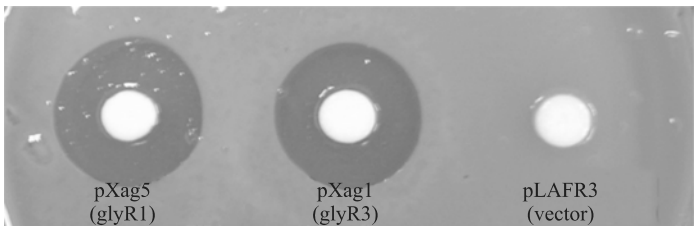


Fig. 6. Inhibition activity of *glyR* gene homologs. Cosmid clones pXag5 containing *glyR1* gene and pXag1 containing *glyR3* gene were used as the producers and cosmid vector clone pLAFR3 was used as the negative control. Extracts obtained from the producers and the control were applied to the filter discs, and then placed on agar medium. Target strains were overlayed on the agar medium as described in 'Materials and Methods'.

in the genome of *X. axonopodis* pv. *glycines.* Each homolog is contained in 6.3 kb, 8 kb, and 13 kb *Eco*RI restriction fragments, and referred to as *glyR1*, *glyR2*, and *glyR3*, respectively. The *glyR1* contained in 6.3 kb fragment is identical to the *glyR* which is the first glycinecin R gene previously reported to GenBank as mentioned earlier.

To characterize the newly identified *glyR* homologs in detail we carried out systematic approach to isolate the clones containing the homolog. A cosmid library of *X. axonopodis* pv. *glycines* was screened by assaying the bacteriocin activity against *X. campestris* pv. *vesicatoria* 833 as described in 'Materials and Methods'. Five cosmid clones with strong inhibitory activity were selected, but two of them were eliminated since they were hybridized to the *glyA* gene (data not shown). The three remaining clones, pXag1, pXag3, and pXag5 were subjected to cross-hybridization analysis. As shown in Fig. 5, when probed with the recombinant cosmid DNA of pXag5, both pXag1 and pXag5 were hybridized, while pXag3 was not. When the pXag3 DNA was used as the probe, only pXag3 itself was hybridized. The results revealed that the pXag1 and pXag5 cosmid clones contain the same glycinecin gene which is different from the glycinecin gene in pXag3. Based on the restriction digestion and the partial sequencing (data not shown), we found pXag5 is identical to the previously isolated cosmid clone containing the *glyR* gene in 6.3 kb *Eco*RI fragment (*glyR1* gene). Since pXag1 was hybridized to pXag5, but showed different restriction patterns, we concluded the cosmid clone pXag1 contains a *glyR* homolog different from the *glyR1* gene in the pXag5. Detailed restriction analysis revealed pXag1 clone contains the *glyR3* in 13 kb *Eco*RI fragment shown in Fig. 2 and 3 (data not shown).

Functionality of the *glyR* homologs in pathovars of *Xanthomonas*

As mentioned earlier, we isolated a cosmid clone containing the *glyR* gene in 6.3 kb *Eco*RI fragments from the genome of *X. axonopodis* pv. *glycines*, and this is the first member of the *glyR* family studied. The glycinecin R produced by this gene was shown to strongly inhibit several *Xanthomonas* species (Roh *et al*., manuscript in preparation). Since the second *glyR* homolog, *glyR3* is not identical to the *glyR1* gene studied previously, it is interesting to examine if there are any functional differences between the two *glyR* homologs. The functionality of the *glyR3* gene as a bacteriocin was analyzed using the cosmid clone pXag1 as the producer. As shown in Fig. 6, the *glyR3* gene is also functional with inhibitory activity against *X. campestris* pv. *vesicatoria* as strongly as *glyR1* (Fig. 6A). It also inhibits the growth of two other *Xanthomonas* species, *X. oryza* pv. *oryza* (Fig. 6B) and *X. albilineans* (data not shown). However, both of *glyR1* and *glyR3* did not show any significant inhibition to other *Xanthomonas* species. Thus, two *glyR* genes are similar in terms of target selectivity and strength of inhibition. So far we showed the two *glyR* genes, *glyR1*, and *glyR3*, are functional, and the third *glyR* homolog, *glyR2* in the 8 kb fragment remains to be isolated and analyzed.

In conclusion, our results revealed that the *glyR* is the multigene family distributed widely in the genomes of *Xanthomonas* species. Considering the fact that bacterial genomes rarely allow copies of a gene, it is very unusual that the *glyR* gene exists in multiple copies. It is even more perplexing in that the gene is exceptionally large, over 4 kb in size, as a bacterial gene. We assume the gene plays essential roles in controlling the size of population, in competition with closely related bacteria and thus in survival, and

our finding of its function as a bacteriocin strongly supports the assumption. The multiplicity of non-identical copies implies the possibility of minor differences in the function of each *glyR* gene homolog, and it still remains to be elucidated.

Acknowledgements

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