gly Gene Cloning and Expression and Purification of Glycinecin A, a Bacteriocin Produced by Xanthomonas campestris pv. glycines 8ra

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Glycinecin A, a bacteriocin produced by Xanthomonas campestris pv. glycines, inhibits the growth of X. campestris pv. vesicatoria. We have cloned and expressed the genes encoding glycinecin A in Escherichia coli. Recombinant glycinecin A was purified from cell extracts by ammonium sulfate precipitation followed by chromatography on Q-Sepharose, Mono Q (ion exchange), and size exclusion columns. Purified glycinecin A is composed of two polypeptides, is active over a wide pH range (6 to 9), and is stable at temperatures up to 60°C. Glycinecin A is a heterodimer consisting of 39- and 14-kDa subunits, as revealed through size exclusion chromatography and cross-linking analysis. Two genes, glyA and glyB, encoding the 39- and 14-kDa subunits, respectively, were identified based on the N-terminal sequences of the subunits. From the nucleotide sequences of glyA and glyB, we conclude that both genes are translated as bacteriocin precursors that include N-terminal leader sequences. When expressed in E. coli, recombinant glycinecin A was found primarily in cell extracts. In contrast, most glycinecin A from Xanthomonas was found in the culture media. E. coli transformed with either glyA or glyB separately did not show the bacteriocin activity.

Bacteriocins are bactericidal compounds, usually proteinaceous, whose activities are often restricted to bacterial strains that are closely related to the producing bacterium (6, 23). Bacteriocins are produced in all major groups of Eubacteria and Archaebacteria (22). Some bacteriocins, the halocins from Halobacteria, have no protein sequence homology to any known bacteriocins (20), whereas others, such as the S-type pyocins of Pseudomonas aeruginosa, some of the colicins of Escherichia coli, and a cloacin of Enterobacter cloacae, reveal protein sequence homologies (16, 17, 19).

Despite their diversity, the bacteriocins share several characteristics (6, 23). They are generally high-molecular-weight proteins that gain entry into target cells by binding to cell surface receptors. Their bacterialicid mechanisms vary and include pore formation, degradation of cellular RNA, disruption of specific cleavage of 16S rRNA, and inhibition of peptidoglycan synthesis.

Many phytopathogenic bacteria, including members of the corynebacteria, erwinias, pseudomonads, and xanthomonads, produce proteinaceous bacteriocins (1, 2, 4, 5, 21, 24, 25). Since these bacteriocins are highly specific, can be produced at low cost, and are likely to be safe for both users and the environment, they appear to be excellent candidates for agricultural use in controlling plant pathogens. However, little is known about the chemical compositions, structures, modes of action, and genetics of most bacteriocins. Only a few isolated and/or purified bacteriocins have been reported from Agrobacterium radiobacter (18), Corynebacterium ulcerans (1), Erwinia carotovora subsp. carotovora (4), and Pseudomonas syringae pv. syringae (21).

Thus far, bacteriocin production by xanthomonads has received little attention. Bacteriocin production by Xanthomonas campestris pv. glycines was first reported by Fett et al. (5), who chose the term glycinecin to differentiate the X. campestris bacteriocin from the glycin produced by P. syringae pv. glycinea (21). Previously, X. campestris pv. glycines 8ra was tested for its antimicrobial activity, and it was found to be effective against most tested phytopathogenic Xanthomonas species, such as X. axonopodi, X. campestris pv. campestris, X. campestris pv. citri, X. campestris pv. pruni, X. campestris pv. vesicatoria, and X. oryzae pv. oryzae (24). To identify genes involved in glycinecin production, we screened an X. campestris pv. glycines 8ra cosm id library for bacteriocin production. We found five cosm id clones showing bacteriocin activity against X. campestris pv. vesicatoria Ds94-9. We subcloned a 6.0-kb DNA fragment with antimicrobial activity, which hybridized with only two of the five cosm id clones, suggesting that there was more than one gene encoding the bacteriocin in X. campestris pv. glycines 8ra (2). To distinguish this bacteriocin from the glycinecin discovered by Fett et al. (5), we named it glycinecin A. Testing of 24 X. campestris pv. glycines strains isolated from various geographic regions and soybean cultivars showed that all carried homologous DNA in the region corresponding to glycinecin A production (15).

Here, we report the cloning and characterization of the two structural genes, glyA and glyB, encoding glycinecin A from X. campestris pv. glycines. We also show that glycinecin A exists as a heterodimer and that its stability is improved by chemical cross-linking of the two subunits.

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MATERIALS AND METHODS

Bacterial strains. X. campestris pv. glycines 8ra was obtained from E. J. Braun of the University of Illinois at Urbana-Champaign. E. coli and Xanthomonas strains were grown in Luria-Bertani (LB) broth or M9 minimal broth supplemented with 0.4% (wt/vol) of appropriate carbon sources. Liquid cultures of E. coli and Xanthomonas were grown in a shaking incubator at 37 and 30°C, respectively.

Bacteriocin activity assay. Bacteriocin activity was determined by examining inhibition zones created on the indicator strain X. campestris pv. vesicatoria Ds94-9 (10). Cell-free bacterial culture supernatants or cell extracts were serially diluted (twofold) in sterile water. A 10-μl aliquot of each sample was then spotted on LB agar plates and allowed to dry for 10 min. The plate was overlaid with 7 ml of soft agar (0.7%, wt/vol) containing 0.1 ml of the indicator strain (optical density at 600 nm, 0.1) and incubated overnight at 30°C. Bacteriocin titer was defined as the reciprocal of the highest dilution factor that showed inhibition of the indicator strain. The activity was calculated as titer = \( \frac{1}{2^{100}} \) and indicated in arbitrary units per milliliter. When formation of a turbid zone followed formation of a clear inhibition zone, the critical dilution was taken to be the average of the final two dilutions.

Expression and purification of glycinecin A. For separate expression of each gene product of glyA and glyB, E. coli strain HB101 was transformed with plasmids pKEYH1 and pSGEV1, respectively. Cells were incubated at 37°C overnight and harvested through centrifugation, and cell lysates and culture supernatants were assayed for bacteriocin activity as described above.

Recombinant glycinecin A was purified from E. coli transformed with pSGEB1 (Fig. 1). E. coli carrying pSGEB1 was grown at 37°C in LB containing ampicillin (50 μg/ml). Bacterial cells were harvested through centrifugation and suspended in lysis buffer (0.1 M phosphate buffer containing 1 mM phenylmethylsulfonyl fluoride). The cells were sonicated on ice in 1-min bursts at 250 W at 1-min intervals, and the cell extracts were precipitated with ammonium sulfate (30 to 60%). The final precipitate was resuspended in 50 mM Tris-HCl (pH 8.0) and dialyzed against 20 mM Tris-HCl (pH 8.0) overnight. The dialyzed solution was applied to a 3.0- by 15-cm Q-Sepharose (Pharmacia, Uppsala, Sweden) column. The column was washed with 20 mM Tris-HCl (pH 8.0) until A280 returned to

FIG. 1. Restriction enzyme map and associated bacteriocin activity of plasmid pBL5 and related subclones. (A) Plasmid pBL5 was digested with several restriction enzymes, and each fragment was ligated into pBluescript or pRK415. E. coli was transformed with each construct, and the bacteriocin activities of the cell lysates were determined. Locations of glyA and glyB are indicated. (B) Plasmids containing glyA or glyB were cotransformed into E. coli. The plasmid pRK415 containing an RK2 replicon was used for the construction of pKYEH1, and the plasmid pBluescript was used for pSGEV1 and pKYS26B. Resultant cell lysates were tested for bacteriocin activity. Enzyme abbreviations: E, EcoRI; B, BamHI; H, HindIII; PstI, PstI; He, HincII; S1, SalI; Sm, SmaI; RV, EcoRV; Xb, XbaI.
baseline, and the bound proteins were eluted with a linear gradient of NaCl from 0 to 1.0 M in the same buffer at a flow rate of 2 ml/min. The active glycinecin A peak fractions were pooled, concentrated, and desalted with Centricon 10 concentrators (Millipore, Bedford, Mass.). The concentrate was applied to a Mono Q HR 5/5 (Pharmacia) column preequilibrated with 20 mM Tris-HCl (pH 8.0). The column was washed with the same buffer and eluted with a linear gradient of NaCl from 0 to 1.0 M at a flow rate of 1 ml/min. The fractions were analyzed for bacteriocin activity as described above.

**N-terminal amino acid sequencing.** The subunits of purified glycinecin A were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinyl dithiole membrane. The transferred polypeptides were visualized with Coomassie brilliant blue, and the glycinecin A bands were cut for N-terminal amino acid sequencing, which was performed using Edman degradation on an Applied Biosystems 477A automatic sequencer.

**Molecular weight determination.** The subunit composition of glycinecin A was examined by size exclusion chromatography and by SDS-PAGE analysis of chemically cross-linked glycinecin A. Purified glycinecin A was analyzed via size exclusion using a Superdex-75 HR 10/30 fast-performance liquid chromatography column (Pharmacia). The column was equilibrated with 20 mM Tris-HCl (pH 8.0) containing 150 mM NaCl, and the separation was carried out isocratically at a flow rate of 0.4 ml/min. Both A and glycinecin A activities were determined for all fractions. For cross-linking of glycinecin A, disuccinimidyl suberate (DSS) was used according to the manufacturer’s instructions. Cross-linked glycinecin A was analyzed by SDS-PAGE (8) and visualized by silver staining.

**Localization of recombinant glycinecin A.** *E. coli* cells expressing glycinecin A were incubated in a 15-ml test tube containing 5 ml of LB medium at 29°C for 21 h with aeration. Cells were harvested by centrifugation at 4,000 × g for 15 min, and the supernatant was analyzed for secreted bacteriocin activity. The cell pellet was resuspended in lysis buffer (phosphate-buffered saline containing 1% Triton X-100), sonicated (5 cycles of 20-s bursts at 300 W at 1-min intervals), and then centrifuged at 10,000 × g for 20 min. The resulting supernatant was used as the cytoplasmic fraction (14).

**Subcloning of the genes for glycinecin A.** Plasmid pBL5, which contains a 6.0-kb DNA fragment that confers bacteriocin activity, was further digested with various restriction endonucleases for restriction enzyme mapping. Fragments of different sizes were subcloned into pBluescript using various restriction enzymes. Each construct was transformed into *E. coli* HB101 and cultured in an LB medium containing ampicillin (50 µg/ml) overnight at 37°C.

**Nucleotide sequence accession number.** The nucleotide sequence reported here has been submitted to the GenBank database under accession no. AF281069.

## RESULTS

**Cloning and nucleotide sequencing of the genes encoding glycinecin A.** In a previous work (2), it was shown that plasmid pBL5 carrying a 6.0-kb DNA fragment isolated from *X. campestris* pv. glycines 8ra conferred bacteriocin activity against *X. campestris* pv. vesicatoria Ds94-9. We termed this activity glycinecin A. Here, we subcloned different parts of the 6-kb DNA insert from pBL5 into pBluescript. Each subclone was expressed in *E. coli* HB101, and its ability to produce bacteriocin was tested on nutrient agar media using *E. coli* HB101 and cultured in an LB medium containing ampicillin (50 µg/ml) overnight at 37°C.

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**Biological Sequence Analysis (12), the first methionine appeared to be more suitable for the start codon. ORF2, which partially overlapped with ORF1, had the start codon located 22 bp inside ORF1, indicating that a different reading frame was used. A comparison (using BLAST) of the sequences of ORF1 and ORF2 to known sequences in the NCBI database revealed no similarities to those reported thus far.

**Purification of glycinecin A.** Production of glycinecin A in pSGEB1-transformed *E. coli* was maximal at pH 7, and a minimum of 10^7 CFU/ml was required for the detection of bacteriocin activity on the indicator strain. The recombinant glycinecin A was found predominantly in the *E. coli* cell extracts rather than in the culture medium, whereas glycinecin A from *X. campestris* pv. glycines 8ra cultures was found predominantly in the culture media (Table 1).

**Recombinant glycinecin A was purified via a four-step procedure, and after each step, bacteriocidal activity was assayed by determining the maximal dilution that would result in the appearance of a clear zone in the indicator strain. The initial purification step, ammonium sulfate precipitation of cell lysates, resulted in an 8.1-fold increase in specific activity. After ion-exchange chromatography on Q-Sepharose and Mono Q resins, bacteriocin activity was found in a peak fraction of the elution profile of the Mono Q column. The final step, size exclusion column chromatography on Superdex-75, resulted in purification of glycinecin A to near homogeneity (Fig. 2A). Overall, this purification procedure typically resulted in an 18-fold increase in specific activity.**

**The purified glycinecin A was active at pH 6 to 9 and retained its full activity after incubation at 60°C for 2 h (data not shown). SDS-PAGE analysis of purified glycinecin A verified that it was composed of two subunits, with molecular masses of 39 and 14 kDa (Fig. 2B). The N-terminal amino acid sequences derived from the purified SDS-PAGE bands were found to be the same as those deduced from the ORF1 and ORF2 sequences, with the exception of the leader sequence encoded by ORF1.**

**Functional analysis of glycinecin A.** Sequence of the pSGEB1 insert revealed two ORFs, and SDS-PAGE of purified glycinecin A showed two bands. In order to determine which ORF was responsible for the bacteriocin activity, several subclones covering various regions of the insert were tested for bacteriocin production (Fig. 1A). Only subclones that carried both ORFs were shown to produce antimicrobial activity against *X. campestris* pv. vesicatoria Ds94-9. No bacteriocin activity was detected when either ORF1 or ORF2 was expressed separately (Fig. 1A), but activity was detected when cells were cotransformed with two compatible plasmids, each...
carrying one of the two genes. Hence, bacteriocin activity did not require cis-acting \textit{glyA} and \textit{glyB} genes (Fig. 1B). However, a mixture of cell extracts from cells expressing \textit{glyA} and \textit{glyB} individually did not show activity.

To verify that the naturally occurring glycinecin protein is composed of 39- and 14-kDa subunits, we analyzed crude extracts of \textit{X. campestris} pv. glycines 8ra. After nondenaturing gel electrophoresis to locate the protein species showing bacteriocin activities against \textit{X. campestris} pv. vesicatoria Ds94-9, the active band was eluted and analyzed by SDS-PAGE. This analysis resulted in two bands corresponding to polypeptides of 39 and 14 kDa (data not shown). These results suggest that glycinecin A activity requires both \textit{glyA} and \textit{glyB} gene products.

The quaternary structure of glycinecin A was investigated through gel filtration column chromatography and by covalent cross-linking. Purified glycinecin A was loaded onto a Superdex-75 HR 10/30 analytical column, and the fractions were assayed for bacteriocin activity. Based on the elution profile, the molecular mass of glycinecin A was estimated to be 50 kDa, which closely approximates the sum of the above two bands of 14 and 39 kDa. However, the activity peak was relatively broad, indicating a possibility that the \textit{glyA} and \textit{glyB} gene products interact weakly, allowing dissociation to occur during elution.

To investigate the quaternary structure of glycinecin A in solution, we treated purified glycinecin A with the cross-linking reagent DSS. Cross-linked products were analyzed by SDS-PAGE followed by silver staining. The molecular mass of cross-linked glycinecin A was estimated to be 55 kDa, which confirmed that glycinecin A exists as a heterodimer (Fig. 3). SDS-PAGE of glycinecin A under nonreducing conditions also resulted in 39- and 14-kDa bands (data not shown), indicating that heterodimer formation does not depend on the formation of disulfide bonds. Interestingly, DSS-treated glycinecin A was more stable at high temperature than was untreated glycinecin A. Cross-linked glycinecin A remained active even when heated to 90°C for 15 min, whereas native glycinecin A lost its activity at 75°C (Fig. 4). However, the cross-linking treatment reduced glycinecin A activity by approximately one-third.

**DISCUSSION**

The genes responsible for the production of glycinecin A in \textit{X. campestris} pv. glycines 8ra were identified and cloned. Nucleotide sequence analysis revealed two structural genes, \textit{glyA} and \textit{glyB}, whose deduced amino acid sequences correspond to the N-terminal amino acid sequences of the two components of glycinecin A. Recombinant glycinecin A produced in \textit{E. coli} was found predominantly in cell extracts, whereas native glycinecin A produced in \textit{X. campestris} pv. glycines was found predominantly in the culture media.

Colonies of \textit{E. coli} transformed with pBL5 containing \textit{glyA} and \textit{glyB} on a 6-kb DNA insert show a wide inhibitory zone,
whereas those transformed with pSGEB1 show a very small inhibitory zone, if any (data not shown). These results suggest that the secretion of glycinecin A may require a specific secretory apparatus that is not present in E. coli, and genes for its secretion may also be located on the 6-kb insert of pBL5. Genes related to bacteriocin production are usually organized in an operon comprised of a structural gene, a dedicated immunity gene, and a gene encoding an accessory protein essential for externalization. The above results indicate that the presence of glycine residues in positions 2 and 1 relative to the processing site. However, neither glyA nor glyB has a double-glycine residue in its leader sequence, suggesting that glycinecin A requires a specific transporter distinct from those found in lactic acid bacteria.

Our results also indicate that glycinecin A exists as a heterodimer (Fig. 2A and 3), and coexpression of both subunits in the same host is essential for bacteriocin activity (Fig. 1B). Some bacteriocins require the complementary action of two different peptides to achieve biological activity; this two-peptide group includes lactacin F (3), lactococcin G (13), and cytolyisin L (7). The complementary action of bacteriocins is a rather common occurrence in gram-positive bacteria, but bacteriocins from gram-negative bacteria are rarely multimeric. Glycinecin A is unique in this respect, since its natural producer, X. campestris pv. glycines, is gram negative and its subunits are relatively large (39 and 14 kDa). Since BLAST searches did not match glycinecin A to any known protein or gene sequence, it is unclear whether both subunits are responsible for bactericidal activity or whether one subunit acts as a receptor and the other acts as the bacteriocin. Although each of the two subunits contains a separate leader peptide, mixtures of extracts from cells transformed with either glyA or glyB did not show bacteriocin activity. This finding suggests that both subunits are required for proper folding in the periplasm after translocation of each subunit through the plasma membrane.

Using the promoter analysis Neural Network Promoter Prediction program (M. G. Reese, Neural Network Promoter Prediction program, 1998 version [http://www.fruitfly.org/seq_tools/promoter.html]), we found typical –35- and –10-type promoter sequences upstream of glyB with high probability (96%), as well as a ribosome-binding site upstream of glyA. This putative promoter gave a low probability score (45%) and had high homology to the –10 but not to the –35 sequence. Several genes related to bacteriocin are commonly organized as operons under the control of a single promoter (4, 9). Since we cloned only the structural genes, the strong promoter that controls the expression of the operon may be located outside the cloned sequence. On the other hand, the high probability score for the putative promoter of glyB is consistent with independent control of each gene.

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