

Cloning and Expression of the *Erwinia carotovora* subsp. *carotovora* Gene Encoding the Low-Molecular-Weight Bacteriocin Carocin S1[∇]

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The purpose of this study was to clone the carocin S1 gene and express it in a non-carocin-producing strain of *Erwinia carotovora*. A mutant, TH22-10, which produced a high-molecular-weight bacteriocin but not a low-molecular-weight bacteriocin, was obtained by Tn5 insertional mutagenesis using H-rif-8-2 (a spontaneous rifampin-resistant mutant of *Erwinia carotovora* subsp. *carotovora* 89-H-4). Using thermal asymmetric interlaced PCR, the DNA sequence from the Tn5 insertion site and the DNA sequence of the contiguous 2,280-bp region were determined. Two complete open reading frames (ORF), designated ORF2 and ORF3, were identified within the sequence fragment. ORF2 and ORF3 were identified with the carocin S1 genes, *caroS1K* (ORF2) and *caroS1I* (ORF3), which, respectively, encode a killing protein (CaroS1K) and an immunity protein (CaroS1I). These genes were homologous to the pyocin S3 gene and the pyocin AP41 gene. Carocin S1 was expressed in *E. carotovora* subsp. *carotovora* Ea1068 and replicated in TH22-10 but could not be expressed in *Escherichia coli* (JM101) because a consensus sequence resembling an SOS box was absent. A putative sequence similar to the consensus sequence for the *E. coli* cyclic AMP receptor protein binding site (−312 bp) was found upstream of the start codon. Production of this bacteriocin was also induced by glucose and lactose. The homology search results indicated that the carocin S1 gene (between bp 1078 and bp 1704) was homologous to the pyocin S3 and pyocin AP41 genes in *Pseudomonas aeruginosa*. These genes encode proteins with nuclease activity (domain 4). This study found that carocin S1 also has nuclease activity.

Erwinia carotovora subsp. *carotovora* (Jones) Bergey et al. is a phytopathogenic enterobacterium responsible for the soft rot, blackleg, or stem rot of a number of economically important crops (20). The disease is characterized by extensive maceration of the affected tissue caused by a variety of plant cell wall-degrading enzymes secreted by the pathogen. The major pathogenicity determinants are an arsenal of extracellular pectinases, including several pectate lyase isozymes, pectin lyase, pectin methylesterase, and pectin polygalacturonase. In addition, a range of other degradative enzymes, such as cellulase and protease, are secreted, but their roles in virulence are equivocal.

Various aspects of the epidemiology of the disease caused by this phytopathogen are understood, but there is no efficient method, either chemical or otherwise, to control the global disease. Biotechnology is progressing rapidly, and science is opening avenues to solve this difficult problem. Agrochemicals are generally used for the control of the disease, but in a quest for more environmentally friendly control methods, biological control using avirulent bacteriocin-producing mutants of *E. carotovora* subsp. *carotovora* is under investigation.

Some bacteria living in a competitive environment secrete proteinaceous toxins, known as bacteriocins, that kill closely related bacteria but not the producer strain itself. According to Klaenhammer, 99% of all bacteria may make at least one bacteriocin (13). All major groups of bacteria produce these

inhibitors (22). Their mode of killing can be either membrane pore formation, nonspecific degradation of cellular DNA, cleavage of 16S rRNA and tRNA, or inhibition of peptidoglycan synthesis, resulting in cell lysis (23). Other strains release bacteriocins, such as pyocin S (produced by a *Pseudomonas aeruginosa* strain) or colicin (produced by an *Escherichia coli* strain), that are soluble and sensitive to proteases. S-type pyocin, or colicin, is composed of two proteins of different sizes, one responsible for antibiotic activity (the killing protein) and the other conferring immunity (the immunity protein). A conserved consensus sequence (a P box for pyocin and an SOS box for colicin) in the 5' upstream region of each operon may act as a regulatory element for bacteriocin production (5, 26). Among the colicins, there are two main evolutionary lineages, which also distinguish the two primary modes of killing: pore formation and nuclease activity (23).

According to Kikumoto et al. and Nakatani and Tsuyama, the antibacterial activities of two types of bacteriocin produced by avirulent bacteriocin-producing biocontrol agents may contribute to suppression of soft rot disease (11, 17). There is also strong evidence that avirulent mutant strains of *Erwinia carotovora* subsp. *carotovora* effectively control the soft rot disease of Chinese cabbage (12, 28). A biological-control agent with the trade name “Biokeeper” has also been developed for the control of this disease in Japan (Central Glass Co., Japan). In view of these reports, identification and cloning of the gene(s) controlling bacteriocin production may facilitate its use in the development of resistant cultivars of Chinese cabbage and tobacco plants, using technology to introduce the genes into plants. Among *Erwinia* species, high-molecular-weight bacteriocins (or large bacteriocins) have structures similar to those of bacteriophages (10). Electron microscopy showed that

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TABLE 1. Bacteria and plasmids used in this study

Bacterium or plasmid	Relevant characteristics	Source
<i>E. coli</i> 1830	<i>pro met</i> Km ^r Nm ^r ; containing transposon Tn5 on the suicide plasmid pJB4JI	Gantotti et al. (7)
JM101 DH5	<i>supE thi Δ(lac-proAB) F' [traD36 proAB⁺ lacI^q lacZ ΔM15] supE44 hsdR17 recA1 endA1 gyrA1 thi-1 relA1</i>	Messing et al. (15) Hanahan and Reusch et al. (8, 21)
<i>E. carotovora</i> subsp. <i>carotovora</i>		
89-H-4	Putative biocontrol agent	Laboratory stock
H-rif-8-2	89-H-4; Rif ^r	This work
Ea1068	Wild type	Laboratory stock
T-29	Wild type	Laboratory stock
E108	Wild type	Laboratory stock
A-100	Wild type	Laboratory stock
86-H-2	Wild type	Laboratory stock
TH22-10	H-rif-8-2; <i>caroS1K</i> :: Tn5 Rif ^r Kan ^r	This work
Plasmids		
pACYC177	Amp ^r Kan ^r ; low copy number	Change et al. (2)
pBR322	Amp ^r Kan ^r	Bolivar et al. (1)
pAYL4	Amp ^r <i>caroS1K caroS1H</i>	This work

carotovoricin Er has an antenna-like structure, a base plate, several tail fibers (18, 19), a contractile sheath, and a flexible rod-like structure (10). Sequence comparison showed high homology between carotovoricin and phage proteins (19). To date, no genes encoding the low-molecular-weight (LMW) bacteriocin (LMWB) (or small bacteriocin) of *E. carotovora* have been isolated or characterized.

Here, we report the cloning and sequencing of DNA encoding one LMWB designated “carocin S1” in *E. carotovora* subsp. *carotovora* 89-H-4 and characterize its expression in a non-bacteriocin-producing strain of *E. carotovora* subsp. *carotovora*, Ea1068. A carocin S1 induction mechanism involving regulation by glucose and lactose is proposed.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The strains and plasmids used are shown in Table 1. The putative biocontrol agent produces two types of bacteriocins: low- and high-molecular-weight bacteriocins. *E. carotovora* subsp. *carotovora* strains were propagated at 28°C on 1.4% nutrient agar (NA) or with shaking in Luria-Bertani (LB) medium with 5 g rather than 10 g of NaCl per liter. *E. coli* strains were propagated at 37°C in LB medium with shaking. Rifampin, kanamycin, and ampicillin (all at 50 mg per liter) were added to NA and LB agars where necessary.

Bacterial mating. Bacterial mating was done on NA by the membrane filter mating method (7), using 0.22-μm-pore-size membrane filters (Millipore, Inc., Bedford, MA). The filters were placed on NA and incubated overnight at 28°C. Appropriate dilutions of the suspension of the progeny of the mating were spread on modified Drigalski’s agar plates (27) containing 50 μg/ml rifampin and kanamycin and incubated at 28°C for 24 to 48 h before the colonies were isolated.

Bacteriocin assays. Bacteriocin production was examined by the double-layer method of Fredericq (6), but hard and soft IFO-802 media (1% polypeptin, 0.2% yeast extract, 0.1% MgSO₄ · 7H₂O [pH 7.0]) containing 1.4% and 0.65% agar, respectively, were used. Growth inhibition zones around the colonies were considered an indication of bacteriocin production.

Instead of UV induction, 1 M glucose or lactose was added to induce bacteriocin synthesis.

Genetic-engineering technique. Plasmids of *E. carotovora* subsp. *carotovora* and *E. coli* plasmids were isolated by the method of Sambrook et al. (24). Total DNA was isolated as previously described (16).

Oligonucleotide DNA primers were synthesized by MD Bio Inc. (Taipei, Taiwan). General PCR has been described by Sambrook et al. (24). Thermal

asymmetric interlaced PCRs (TAIL-PCRs) were performed according to the method of Liu and Whittier (14), but the annealing temperature was decreased from 63°C to 60°C for specific primers. For TAIL-PCR, specific primers that are complementary to the respective sequences of Tn5 (PR-1, PR-2, PR-3, PF-1, PF-2, and PF-3) or known sequences after the first TAIL-PCR analysis (TH22-10F1 to TH22-10F4 and TH22-10R1 to TH22-10R4) were synthesized (Table 2).

TABLE 2. Primers used in this study^a

Name	Sequence (5'→3')
PR-1.....	5'-GCCGAAGAGAACACAGATTTAGCCCA
PR-2.....	5'-CCGCACGATGAAGAGCAGAAGTT
PR-3.....	5'-CAGATCTCTGGAAAACGGGAAAGG
PF-1.....	5'-AGAGAACACAGATTTAGCCCAGTCGG
PF-2.....	5'-CCGCACGATGAAGAGCAGAAGTTAT
PF-3.....	5'-GATCCTGGAAAACGGGAAAGGTTTC
TH22-10F1.....	5'-GAGCATGGTGTAAACAGAAGAACAG
TH22-10F2.....	5'-CTTCGTCCATGTAGTGGTGATTC
TH22-10F3.....	5'-GGGTTGTACTAATGTCTGTAAACG
TH22-10F4.....	5'-CTTCGTCCATGTAGTGGTGATTC
TH22-10R1.....	5'-GAATCACCACATACATGGACGAAG
TH22-10R2.....	5'-CTGTTCTTCTGTTACACCATGCTC
TH22-10R3.....	5'-AATCACCACATACATGGACGAAG
TH22-10R4.....	5'-CGTTTACAGACATTAGTACAACCC
TH22-10R5.....	5'-GTGATCGGCCTGATAACTTCC
TH22-10R6.....	5'-TTGTCGTGAGCGTCCAATG
TH22-10R7.....	5'-CATCAAGCAAGTTAACAGGCAAC
TH22-10R8.....	5'-TCTCCGGAAACACAGAATG
TH22-10R9.....	5'-CTCTCCGCCATTGGTATTC
TH22-10R10.....	5'-TTCATCTGATGTGTAGTGC
TH22-10R11.....	5'-TGATTGGCCTGGACTACAGC
TH22-10R12.....	5'-TGCCGTACCTGCCATTACC
N-1.....	5'-NGTCGA(G/C)(A/T)GANA(A/T)GAA
N-2.....	5'-GTNCGA(C/G)(A/T)CANA(A/T)GTT
N-3.....	5'-(A/T)GTGNAG(A/T)ANCANAGA
P-3.....	5'-CTCGACTTGTCACTGAAGCGGGAAG
P-4.....	5'-AAAGCAGGGAAGCGGTCAGCCCAT
PCAR-R2.....	5'-TCAATCTGGCATCGATAACAGGG
PCAR-F2.....	5'-TGCTGGATCCAGCTTACGTGG
PCAR-F3.....	5'-GGTTGTACTAATGTCTGTAAACG
PCAR-R3.....	5'-CGTTTACAGACATTAGTACAACC

^a All primers were purchased from MD Bio Inc., Taipei, Taiwan.

In addition, three arbitrary degenerate primers (N-1, N-2, and N-3) were used (Table 2).

For sequencing of TAIL-PCR products, the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit was used. Cycle sequencing was carried out on a GeneAmp System 9600 thermocycler. Sequencing using an automated DNA sequencer 373S (ABI) was carried out according to the manufacturer's protocol.

Southern and colony hybridizations and probe labeling and detection were performed using the digoxigenin (DIG) DNA Labeling and Detection Kit (Boehringer Mannheim GmbH, Mannheim, Germany) as described by the manufacturer. Hybridization was performed overnight, and the membrane was washed according to the manufacturer's instructions.

The DNA electrophoresis, restriction digestion, ligation, and transformation procedures for *E. coli* were done as described by Sambrook et al. (24). Plasmid DNA transformation for *E. carotovora* subsp. *carotovora* was performed using the methods of Hinton et al. (9) and Hanahan (8). *E. carotovora* subsp. *carotovora* cells were incubated at 35°C until the optical density of the cell suspension at 550 nm was 0.40 to 0.70 before transformation.

RNA preparation and Northern hybridization experiments. BSM (bacteriocin screening medium; 0.5% sucrose, 0.1% NH₄Cl, 0.2% KH₂PO₄, 0.02% MgSO₄ · 7H₂O, pH 7.5) was used for carocin S1 production. Total RNA was isolated from *E. carotovora* subsp. *carotovora* constructs grown in BSM without drugs at 28°C. To determine the stability of the H-rif-8-6, TH22-10, TH22-10/carocin S1, Ea1068, and Ea1068/carocin S1 strains, the bacteria were grown to a Klett value of ca. 150, at which point rifampin (0.2 mg/ml) was added to block further initiation. Culture samples (8 ml each) were then withdrawn at various time points into tubes containing 5 ml of ice-cold water, and total RNA was extracted.

Northern blot hybridization was done using 10 µg of total RNA isolated using Trizol (Invitrogen, San Diego, CA) according to the manufacturer's protocol. RNA samples were denatured at 65°C for 10 min in RNA sample buffer (250 µl of formamide, 83 µl of 37% [wt/vol] formaldehyde, 83 µl of 6× loading dye [Promega, Madison, WI], 50 µl of 10× MOPS [morpholinepropanesulfonic acid] buffer [10× MOPS buffer is 20 mM MOPS, 5 mM sodium acetate, and 1 mM EDTA, pH 7.0]) and 34 µl of distilled water. RNA samples were separated through 1% agarose gels in MOPS buffer with 2% (vol/vol) formaldehyde. DNA probes were synthesized by PCR using specific oligonucleotides, PCAR-R2 (for *caroSII*) and PCAR-R3 (for *caroS1K*), derived from the *E. carotovora* subsp. *carotovora* sequence as a template (Table 2). Template DNAs, *caroS1K* and *caroSII*, were obtained by PCR amplification. The probes were nonradioactively labeled by random priming using the DIG High Prime kit (Roche, Mannheim, Germany). To add the correct amount of probe for hybridization, serial dilutions of each probe (0.05 to 10 µg) were spotted on a nylon membrane, and the labeling sensitivity (the amount of labeled DNA per spot) was determined. RNA was transferred overnight to a positively charged nylon membrane (Amersham Life Science, Arlington Heights, IL) by capillary transfer using 20× SSC (20× SSC is 0.3 M NaCl plus 0.03 M sodium citrate, pH 7). Hybridization was performed for 16 h at 50°C in DIG Eazy Hyb buffer solution (Roche). The membrane was washed, and specific transcripts on the blots were detected using the DIG luminescence detection kit (Roche) according to the manufacturer's protocol.

Bacteriocin expression and purification. Bacteria in BSM were incubated in a sterilized stainless steel box with a stainless steel cover at 28°C for 24 h without any light. After centrifugation, the medium without cells was removed. Ammonium sulfate was added to 80% saturation to precipitate the protein, and the precipitate was collected on a 0.45-µm cellulose filter. One milligram of precipitated protein was dissolved in 100 µl of bacteriocin buffer (0.1 M Tris [pH 7.5], 0.01 M dithiothreitol, and 0.5 M MgCl₂).

Bacteriocin assay for nucleotidase activity. To determine the bacteriocin antibiotic activity, 100 µg/10 µl of the CaroS1K protein solution was added to an indicator plate containing the Ea1068 or Ea1068/pAYL4 strain growing on soft IFO-802 medium containing 0.65% agar. Growth inhibition zones at the point of addition were considered an indication of carocin S1 activity.

To confirm nucleotidase activity, 500 ng/1 µl genome DNA solution from strain Ea1068 was added to 100 µg/10 µl of the CaroS1K protein solution and incubated at 28°C for 3 h. After incubation, the samples were treated and analyzed by 1.0% agarose gel electrophoresis in Tris-acetate-EDTA buffer.

Computer analysis of sequence data. The nucleotide sequence and the deduced amino acid sequence of carocin H1 were compared using the BLAST and FASTA programs of the National Center for Biotechnology Information server. Sequence data were compiled by DNASIS-Mac software (Hitachi, Tokyo, Japan).

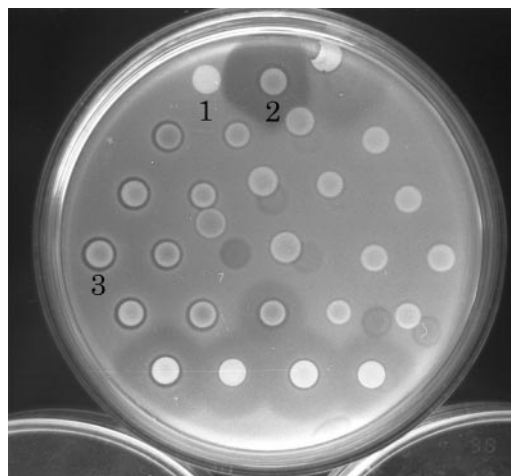


FIG. 1. Bacteriocin activities of Tn5 insertion mutants of *E. carotovora* subsp. *carotovora* strains: 1, *E. coli* 1830/pBJ4II (containing Tn5); 2, H-rif-8-6 (parent); and 3, TH22-10 (insertion mutant). The unlabeled strains are all Tn5 insertion mutants of the H-rif-8-6 parental strain. The indicator was Ea1068.

RESULTS

Isolation of transposon insertion mutants. The mating of strain H-rif-8-6 with *E. coli* 1830 resulted in 4,500 colonies that could grow on selective plates containing rifampin and kanamycin (50 µg/ml each). To ascertain their antibiotic resistances, colony growth on selective medium was rechecked and found to be a stable property.

Bacteriocin activities of the putative Tn5 insertional mutants. The bacteriocin activities of the test isolates were examined. The parental strain produced an LMW bacteriocin that diffused further from the colony than did the high-molecular-weight bacteriocin. The zones of inhibition around the putative isolates (insertion mutants) were restricted compared to those of the parent strain (Fig. 1). This suggested the possibility that transposon Tn5 had been successfully inserted into the genes of the LMW bacteriocin.

Detection of the Tn5 gene in the mutants. To ascertain whether Tn5 was actually inserted into the putative isolates, "nested PCR" was used to amplify the *nptII* gene (29), using two oligonucleotide DNA primers, P-3 and P-4 (Table 2). Almost all the test isolates except H-rif-8-6 produced an ~500-bp DNA fragment, indicating that H-rif-8-6 did not harbor the Tn5 gene. Southern blot hybridization also confirmed the above-mentioned results (data not shown).

Amplification of Tn5 insertion junction DNA and sequencing. After the first TAIL-PCR experiment, two or more different-size bands were obtained for each sample. All of the fragment products were isolated by electrophoresis and purified, and the sequences of the recovered products were analyzed. Analysis of the respective bands showed the same sequence. On the basis of the sequence obtained from the first TAIL-PCR experiment, specific primers (left side, TH22-10F1 to TH22-10F4; right side, TH22-10R1 to TH22-10R12) were synthesized for subsequent TAIL-PCR experiments.

Sequence analysis. A DNA fragment of 2,279 base pairs was sequenced. Analysis of the Tn5 insertions showed that two

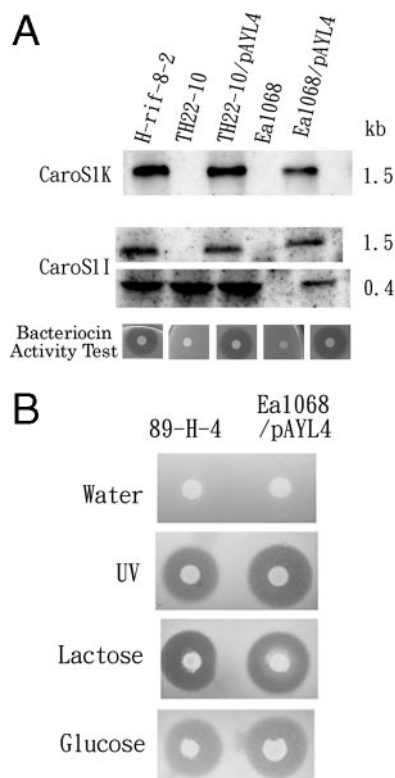


FIG. 2. Transcription analysis of the carocin S1 gene. (A) Northern hybridization analysis of *caroSIK* and *caroSII*. Total RNAs (20 μ g) from H-rif-8-2, TH22-10, TH22-10/pAYL4, Ea1068, and Ea1068/pAYL4 cells incubated in BSM at 28°C for 24 h were subjected to Northern blot analysis. *E. carotovora* subsp. *carotovora* strain Ea1068B was used for the bacteriocin activity test. (B) Carocin S1 expression after water, UV, glucose, and lactose stimulation. The producer strains were 89-H-4 and Ea1068/pAYL4, and the indicator strain was Ea1068.

complete open reading frames (ORF2 and ORF3) were present, and Tn5 was located in ORF2 between bp 1581 and bp 1582. The 3' end of another open reading frame, ORF1, was located upstream of ORF2. A noncoding region and a putative promoter were located between ORF1 and ORF2. Downstream from ORF3, the 5' end of another ORF (ORF4) was found.

Homology with other genes and proteins. The predicted amino acid sequences of ORF2 and ORF3 were compared with amino acid sequences deposited in the Swiss-Prot protein sequence database. Significant similarity was found between the sequences of ORF2 and ORF3 in *E. carotovora* subsp. *carotovora* and those of *pyoS3A* and *pyoS3I* of *P. aeruginosa*, respectively. Designation of ORF2 as *caroSIK* and ORF3 as *caroSII* was therefore proposed. The two genes were called the carocin S1 genes.

Subcloning and expression of the carocin S1 gene from H-rif-8-6. The DNA fragment of the carocin S1 gene from H-rif-8-6 was amplified by PCR. After PCR amplification of two oligonucleotide primers, CAR-F2 and CAR-R2, the carocin S1 gene was purified, digested by restriction enzymes (ClaI and BamHI), and subcloned into plasmid pACYC177 by T4 ligase. The new plasmid was designated pAYL4. One hundred transformed colonies were isolated using selective LB agar

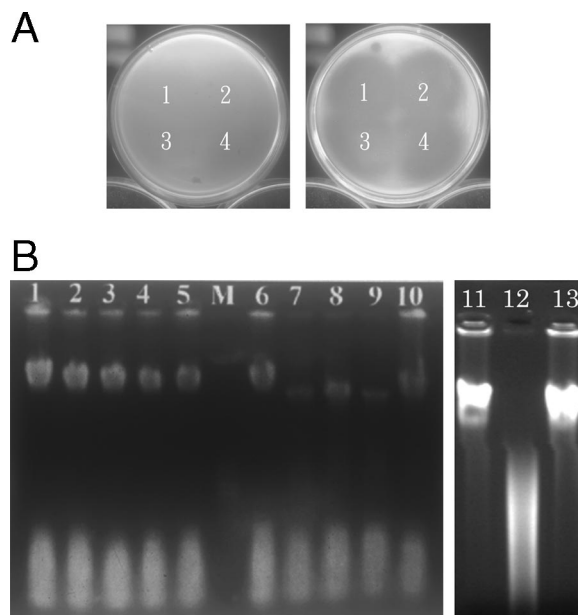


FIG. 3. DNase activity of carocin S1 against genomic DNA from *E. carotovora* subsp. *carotovora* Ea1068. (A) Analysis of the killing activity of purified carocin S1. Carocin S1 was purified from 89-H-4 (1), H-rif-8-6 (2), TH22-10/pAYL4 (3), and Ea1068/pAYL4 (4) strains and then added to the indicator plates to test its killing activity. The indicator strains were Ea1068/pAYL4 in the left plate and Ea1068 in the right plate. (B) The reaction mixture (50 μ l) containing 1 μ g of DNA and 20 μ l of sample solution in 10 mM Tris, pH 7.5, 4 mM MnCl₂ was incubated at 28°C for 2 h. Samples 1 to 5 were collected from Ea1068, and samples 6 to 10 were collected from Ea1068/pAYL4. Lanes 1 and 6 were from the 11th fraction tubes, lanes 2 and 7 from the 12th fraction tubes, lanes 3 and 8 from the 13th fraction tubes, lanes 4 and 9 from the 14th fraction tubes, and lanes 5 and 10 from the 15th fraction tubes. Lane 11 was genomic DNA isolated from Ea1068. Lane 12 was the positive control (DNA mixed with EcoRI and buffer), and lane 13 was the negative control (DNA and buffer). Samples were treated and analyzed by agarose gel electrophoresis.

medium containing 100 μ g/ml ampicillin after the transfer of pAYL4 into *E. coli* DH05 and JM101. The presence of the carocin S1 gene was detected by using electrophoresis after digestion with ClaI and BamHI. The *caroS1* band size was 1.9 kb (data not shown). Carocin S1 activity was not detected after a bacteriocin assay using the indicator strain Ea1068 of *E. carotovora* subsp. *carotovora*. The pAYL4 plasmid DNA was isolated from DH05/pAYL4 and transferred into the insertion mutant TH20-10 and the wild-type strain of *E. carotovora* subsp. *carotovora* Ea1068 (a non-bacteriocin-producing bacterium sensitive to carocin S1). *E. carotovora* subsp. *carotovora* strains Ea1068 and E108 were used as indicators to detect bacteriocin-producing colonies. Thirty-two colonies were isolated by selection on LB medium containing kanamycin, rifampin, and ampicillin (50 μ g/ml each), and the carocin S1 gene was detected as previously described. The colonies exhibited zones of growth inhibition when the host was Ea1068, and also, many colonies exhibited zones when the host was TH22-10, a mutant derived from H-rif-8-6 (Fig. 2A).

Transcription analysis of the carocin S1 genes. The plasmid pAYL4, which contains the carocin S1 gene, is expressed in *E. carotovora* subsp. *carotovora* Ea1068 and TH22-10 strains from

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      10      20      30      40      50      60      70
carocin S1K 1 : -MRQCCRQPQVVMAGLAAQAGRIIAAGGAVAAQANQDPAKALTHQMKRLGPSLWGGQMA SFLLMGALQ
pyocin S3A 1 : -----MDAPPPLIVRMSRPGKPGVSIIV--SRD-R--ODIISNPFPGNPF-RGFTL
pyocin AP41 1 : MSDVFDLGSMTTATATAGQYSFYTPPPETPIPLTYIAREGINKFEL--PEGAKT-KDLIKRYQYIGSQIPAAIM
      80      90      100     110     120     130     140     150
carocin S1K 73 : HHLKGEKADLLPDKIL--EADVQAALDTEAA-KYPAL-PASFTPEWLERSTVVVVTGLNRRRPEIDNELDVVQ
pyocin S3A 49 : --AMEADFKRR-KESL---EADVQAALDTEAA-KYPAL-PASFTPEWLERSTVVVVTGLNRRRPEIDNELDVVQ
pyocin AP41 73 : IRGVQVEIKKSNLNTALANVGATVDGELAYLRSQKKEKNDPEATPLMASAEKAAAVELLSKQKELADARTIAN
      160     170     180     190     200     210     220
carocin S1K - : -----
pyocin S3A 116 : NSASLKLQASNTIILDQINSLOEFLNKLNAETDRRRAEAERQAEADAARKAEEAAR--EAAQVVTQQLGA
pyocin AP41 148 : AFFGYDPIITVNVVNV-MNE-LYGRRLDKDFSFDNWSKSYSA--AKIRLIEAETISVLNRSSSAIDGKVAE-LTR
      230     240     250     260     270     280     290     300
carocin S1K - : -----
pyocin S3A 189 : DFNAITTPTATKVQQRVKVDASISVSTVSGAVASATQAVQVKTQAQQQANSQISSSQNLISAFRNQIALA
pyocin AP41 217 : LQRLEDAQHAEAAARQTEAER--LAE--EQRAEAR-QQAEARROAEARQELRLAEAEAKRVAEAKKRQD
      310     320     330     340     350     360     370
carocin S1K - : -----
pyocin S3A 264 : A-QASGEAQTAFNTKVKQVNEATAFSTARQALAQFAANAFAEVETEENRLTQVKASPTKAASQAQATL-R
pyocin AP41 287 : EINARLQAIIVS-ESEAKRDEEYKRLLEEQDKISNPTVTTPPVVDAGSRDDALAHGTTRVYSGGETGTGGSGR
      380     390     400     410     420     430     440     450
carocin S1K - : -----
pyocin S3A 337 : QFTESYAKVNFALQTEAALQAKAKEVSAVAEAOARRPTNDQKADVVGGQIQAAANTLAMPATSTBLVSVAG
pyocin AP41 361 : DVDTGEGGGIIT-ARPDVGVSVIPRRRDKIPDQPPRRDLGSLVPIPFDFTFPSPFPGVGV--PAAAKPLIPAGG
      460     470     480     490     500     510     520
carocin S1K - : -----
pyocin S3A 412 : FESPAWETARLAASITNAVNRLIQIAGSGPGAYVATFALSLYSDDGKDSD-KVPAEVENFLALEASFLGLEG
pyocin AP41 433 : -EASV--SE---TLKTAVD-ILSVARKTPGAMLGQVAAVAT---MAVSSFWPRLNNGEQASFAIPVAELSEPP
      530     540     550     560     570     580     590     600
carocin S1K 92 : -----ANKOCTVPSRVRYQWMEDEETGRLLK--AVGYHTSMESGRDQ--VVRRLKYIFPNRRAAFWEEGA
pyocin S3A 485 : TADLQSVAKA--GGTVDMPVRLTSAAQESPSGKSLAAMLT---NGATVPKGVVPRRNTATATERVEVTPAK
pyocin AP41 498 : LAVDWQAIANAK-ETVDLRYKIKTLN---GSIQILAMPT---DPSGAA--VEVRAITLISASCTIKYI----
      610     620     630     640     650     660     670
carocin S1K 153 : -TGF--IL-WTPEN-EGMELTDTLAHGEQVIVSAVPG-FETP-EMDDV-SILATPMPPE-ERKDFRDYI--LV
pyocin S3A 554 : STVVENPPLILTWTPATPPGSONPSSSTTP-VVQ-PAPVYEGATHTPVQAEPEYPGV--PLDLDLIL-VI---
pyocin AP41 558 : TTGPGGGT-ILV-PPDTPPGQIDPSSSTP-AVERGELIMPECTLLLEKPKQIE-SPELDQRPE-FNNGYVY----
      680     690     700     710     720     730     740     750
carocin S1K 215 : EEPNSPFFI-YIYLSKLEVNLDVDLISNETHGSRQKYQADHMPESAAVKAYIKRLVFDLRKDKLEMAKAEVAA
pyocin S3A 620 : EAGSDVPPPIYLVFSKFPVKLEVDIYGNAGRPDGNHLDHMDGQALATSRAIYEDITEYGETRRLMKRGGG
pyocin AP41 624 : -PEDSGIFPPIYVYRDTRDE-GVAT-GN--GQVVTGNWLAG--ASQC---DGV---E-IE-SQADQLRQKEF
      760     770     780     790     800     810     820
carocin S1K 289 : IILPTEIHKISETFYGGRRNSDNIEL-SODLRGHWIVILHAIKPKLKEHGVTEQQLPLARDKM-HKLNIE-EOGL
pyocin S3A 694 : VALPAVHORESEFYGGRRNTKEKQKQAS-DLEAAVDSENFDAVKKELLEEFSEDDIIRAEAL-HMVNK-EOGW
pyocin AP41 683 : KSW--RD---RRQF---WMLVSK--PS-ALENLSPNRYVFSQGLAPYAWPEPHIGSKEKFEIHHVPLESGG
      830     840     850
carocin S1K 361 : Y-----
pyocin S3A 766 : Y-----
pyocin AP41 747 : ALYNIDNLVIVTPKRHSEIHKELKLRREK

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B

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      10      20      30      40      50      60      70
carocin S1I 1 : M-SVMVEALIDCLGKTYQELFD-----AGLIPYK--KKPTGFGSDPDIS-EDMIRESIYLSNVRDVKVNEITL
pyocin S3I 1 : MEKRLIVKLIIDSIGRSHEEIV-----GAGLVKSG--KLESPIYHSEHL-TLSPER-GLEINRAENKIL-EKIH
pyocin AP41 1 : MDIKKN--SDYTESEFLEIEEFFKNKSLKGSLELRMDKLVKHFEVISHRRSSEVI--RHPKPGF--ETPE
      80      90      100     110     120     130     140     150
carocin S1I 66 : RIPVRNKLKLGIFPNELPAPLRKNMIPWLGAENICFP-EKEPSPRKKLKERWLYQIILYDF--KRI-----
pyocin S3I 65 : ISLIDTVEGSAAYSGLPEPLVRKMSKEQVRSVMKKEFETRESR-EPVIGILGGWD-VFMHPQANLRIGFTY
pyocin AP41 70 : G--IVVEKWEWAANGLEPGFKAG-----
      160
carocin S1I - : -----
pyocin S3I 137 : TSDSNVCNLTFFALLGSK
pyocin AP41 - : -----

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their native promoters. Northern blots of total RNAs from H-rif-8-2, TH22-10, TH22-10/pAYL4, Ea1068, and Ea1068/pAYL4 cells incubated in BSM at 28°C for 24 h are shown in Fig. 2A. PCR products specific for *caroS1K* and *caroS1I* were used as probes in hybridizations. In both cases, the *caroS1K* gene was expressed and detected in H-rif-8-2, TH22-10/pAYL4, and Ea1068/pAYL4 cells. However, the *caroS1I* gene was also detected in all host cells except Ea1068 cells. Only a 0.4-kb RNA band was detected in the Tn5 insertional mutant strain, TH22-10. Sequence analysis found similarity to the consensus sequence of the *E. coli* cyclic AMP receptor protein binding site (−312 bp) upstream of the start codon. To prove this finding, the 89-H-4 and Ea1068/pAYL4 cells were treated with water, UV, or lactose. Carocin S1 gene expression was stimulated by either lactose or UV (Fig. 2B).

Carocin S1 purification and nucleotidase assay. Carocin S1 was collected and purified, and its activity was tested (Fig. 3B). This product, produced from 89-H-4, was highly toxic. Homology analysis of the protein showed similarities to pyocin S3, which has nucleotidase activity (Fig. 3A). Here, carocin S1 was shown to inhibit growth of the indicator strain Ea1068 and to have nucleotidase activity.

Nucleotide sequence accession number. The GenBank accession number of the sequence of the carocin S1 gene is AF205141.

DISCUSSION

The results described here show that *E. carotovora* subsp. *carotovora* 89-H-4 has a functional antibacterial gene. Expression of the gene in a non-bacteriocin-producing strain of *E. carotovora* subsp. *carotovora*, strain Ea1068, resulted in the production of a bacteriocin (named carocin S1) that was released into the growth medium. This study is the first to find, clone, and express an LMWB gene of an *Erwinia* species. The carocin S1 genetic determinant consists of two structural genes, *caroS1K* and *caroS1I*, which have homology to the pyocin S3 and pyocin AP41 genes that encode the killer protein and immunity protein of *P. aeruginosa* strains.

Analysis of the *caroS1K* gene showed a potential Shine-Dalgarno sequence (ATGGAA), which may be a ribosome binding site, 5' of the putative ATG start codon at position −44 bp. Several *E. coli* sigma 70-like promoter sequences were found 5' of this putative ribosome binding site. A possible CTGATA(17 bp)CAGTAT was found at positions −186 to −214 bp relative to the translational start codon of the *E. coli* sigma 70 binding consensus sequence. This may be the promoter for *caroS1K* and could be expressed only in *E. carotovora* subsp. *carotovora*, but not in *E. coli*.

Upstream of the translational start codon, no sequence resembling the consensus sequence for an SOS box (the site for binding of LexA, the repressor of the DNA damage-inducible genes of *E. coli*) (30) was found. This may explain why *caroS1* could not be expressed in *E. coli* HB101 but was successfully

expressed in *E. carotovora* subsp. *carotovora* strain Ea1068 (a non-bacteriocin-producing strain).

Analysis of the genomic sequence around the carocin S1 gene revealed a sequence similar to the consensus sequence of the *E. coli* cyclic AMP receptor protein binding site (−312 bp) upstream of the start codon, which is activated by lactose. From our studies, glucose, as well as SOS agents, can also induce the carocin S1 gene.

Similar to *caroS1K*, *caroS1I* had a putative Shine-Dalgarno sequence (AAGGAA) located at the 3' end of the *caroS1K* gene. This sequence was 13 nucleotides from the stop codon, TAA, of *caroS1K* and 15 nucleotides from the initiation codon of the *caroS1I* gene. Also, several *E. coli* sigma 70-like promoter sequences were found at the 5' end of this putative ribosome binding site. A putative TAGAAC(19 bp)TAAACT was found at positions −25 to −55 bp relative to the translational start codon of the *E. coli* sigma 70 binding consensus sequence. This may also be the promoter for the *caroS1I* gene. However, its sequence is very similar to that of the pyocin S3 gene of *P. aeruginosa* (25, 26). With this structure, ribosomes can remain in simultaneous contact with the termination codon of the first gene (*caroS1K*) and the initiation codon of the second (*caroS1I*). Thus, *caroS1K* and *caroS1I* can be transcribed as a two-gene operon, because the two genes are translationally coupled.

The homology search found that the carocin S1 gene was homologous to the pyocin S3 and AP41 genes in *P. aeruginosa* from positions 1078 to 1704; these genes encode nuclease (domain 4) (27) (Fig. 4). This suggests that the carocin S1 gene may also encode a similar nuclease. Although the remaining sequence (622 to 1078 bp) had low homology with domain 1 or 3, it may encode functions that are the same or similar to those of domain 1 (receptor binding domain) or domain 3 (translocation domain) or both (27).

The LMWB of *Erwinia* species was not induced by mitomycin C (data not shown), which is a DNA-damaging agent commonly used for colicin production in *E. coli*, but was induced by UV exposure and glucose. Recently, similarity between an LMWB receptor in *Erwinia carotovora* subsp. *carotovora* and receptors for lipopolysaccharide, sucrose, and glucose was reported (23), but more study is needed to determine how receptor interaction affects bacteriocin induction.

Here, we proved that carocin S1 has nucleotidase activity and can be induced by glucose. The activity of each domain of carocin S1 and the effect of the promoter region will need further study to determine the regulatory mechanism of glucose induction.

To our knowledge, this is the first time that an LMWB gene (the carocin S1 gene) from *E. carotovora* subsp. *carotovora* has been cloned and expressed. This gene can now be introduced into tobacco, Chinese cabbage, or other plant species by transgenic techniques to protect them against soft rot disease.

FIG. 4. Alignment of the amino acid sequences of carocin S1, pyocin S3A, and pyocin AP41. (A) Killer protein. (B) Immunity protein. The numbers refer to the positions of the amino acid residues in the sequence of each protein. The putative structural domains (I to IV) of carocin S1K are indicated.

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